



# Silencing TLR4/MyD88/NF- $\kappa$ B Signaling Pathway Alleviated Inflammation of Corneal Epithelial Cells Infected by ISE

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**Abstract**— The regulatory role of toll-like receptor 4 (TLR4) in the inactivate staphylococcus epidermidis (ISE)-induced cornea inflammation is not well investigated. Here, TLR4 silence could decrease inflammatory cytokines in corneal epithelial cells treated with ISE. The mouse corneal epithelial cells were exposed to ISE for 24 h, either alone or with the NF- $\kappa$ B inhibitor, TLR4 lentivirus to bilaterally (knock-down or and overexpression). The expression of TLR4 in mouse corneal epithelial cells was investigated using western blot and qRT-PCR assay. The inflammatory cytokine levels were evaluated by qRT-PCR and ELISA, respectively. The relative impact factors of TLR4-mediated NF- $\kappa$ B signaling detected using western blot assay. Results show the expression levels of TLR4 and some inflammatory cytokines were significantly increased in corneal epithelial cells treated with ISE. TLR4 Silence markedly decreased ISE-induced production of IL12, TNF- $\alpha$ , CCL5, and CCL9 in corneal epithelial cells. Furthermore, the nuclear translocation of NF- $\kappa$ B p65 and myeloid differentiation protein 88 (MyD88) in the cells treated with ISE were further reduced by silencing TLR4. Inhibition of TLR4-mediated NF- $\kappa$ B signaling by using BAY11-7082 also alleviated ISE-induced inflammation. In the rescue experiment, transfected the stable TLR4 silenced corneal epithelial cells with TLR4 overexpression lentivirus, we found that TLR4 overexpression can restore the down-regulation of TLR4 and inflammatory cytokines (IL12, TNF-

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$\alpha$ , CCL9) caused by TLR4 knocked down. Therefore, ISE-induced cornea inflammation was due to the activation of the TLR4/MyD88/NF- $\kappa$ B signaling pathway, and dramatically stimulated IL12, TNF- $\alpha$ , CCL9 secretion. TLR4 silence presented mitigates damage in corneal epithelial cells treated with ISE.

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**KEY WORDS:** toll-like receptor 4; corneal epithelial cells; inactivate staphylococcus epidermidis; inflammation; NF- $\kappa$ B; inflammatory cytokines.

## INTRODUCTION

Infectious keratitis is still a common cause of blindness worldwide [1, 2]. It is estimated that each year has 5 million people in the world suffer from various types of infectious keratitis, and unfortunately, about 20% are blinded due to eye infections [3]. Pathogenic microorganisms which caused microbial keratitis have bacteria, fungi, viruses, or parasites, among them bacterial infection is a common, latently sight threatening cornea inflammation [4, 5]. Comprehensive national conditions, staphylococcus epidermidis accounted for the largest proportion [6, 7]. Thus, we should pay enough attention to it. As a developing country, the incidence of infectious keratitis is higher in China, especially in many rural areas due to poor living conditions and health care awareness [8]. There are the most blind people in China of the world, with about 5 million blind people, accounting for 18% of the world's blind population, and each year about 45 million people blind in China. Even worse, if the current trend continue to remain the same, the blind people in China is expected 4-fold increase [9]. Therefore, there is an urgent need to prevention of corneal infection. It is a long and arduous task to study the mechanism of immunological regulation and drug development.

The innate immune system recognizes the structural components of pathogens, known as pathogen-associated molecular patterns (PAMP), and the corresponding recognition receptors are called pattern recognition receptors (PRRs) [10–13]. TLR members are a family of transmembrane receptors that can recognize PAMP, and the subsequent initiation of signal transduction can lead to the release of inflammatory mediators, which plays an important role in natural immune defense [14–17], and finally activate the adaptive immune system. Therefore, it is believed that TLRs control the transformation from innate immunity to acquired immunity, and it is a pathogen recognition receptor that recently attracted much attention in recent years [18–20]. It has been observed that TLRs can interact with MyD88, thereby leading to activate of p38, c-JNK,

and NF- $\kappa$ B, which is indispensable for the production of cytokines such as IL-6, IL-8, and TNF- $\alpha$  [21–24].

TLR4 contains 879 amino acids and is located on the short arm of human chromosome 9. Poltorak *et al.* first discovered TLR4 in 1998 [25]. TLR4 has multiple functions in the organism and is mainly expressed in CD14<sup>+</sup> monocytes and macrophages, while it is also widely expressed in CD14<sup>-</sup> endothelial cells [26]. TLR4 can recognize exogenous or endogenous pathogen-associated molecular patterns *in vivo* [27]. TLR4 is an important TLR on the surface of lymphocytes [28]. And it has been a research hotspot in the research fields of oncology, microbiology, and immunology to study the role of TLRs. Studies have shown that TLR4 which expresses in a variety of ocular tissues and cells can identify the particular pathogen and induce inflammatory cytokines expression as well as inflammatory cell infiltration, thereby killing and eliminating pathogens [29]. Gram-negative bacteria transmit infection signals to cells through lipopolysaccharide (LPS), lipopolysaccharide binding protein LBP, lipopolysaccharide receptor CD14, and TLR4, and then mediated by various intracellular signaling pathways, triggering a series of immune responses [30, 31]. However, the regulatory role of TLR4 in corneal infections induced with gram-positive bacteria, such as ISE, remains largely unclear.

Here, we established a corneal epithelial cells model stimulated by ISE, then performed by silencing or overexpression TLR4 and function of myD88 inhibitors. Herein, we reported that ISE-induced cornea inflammation was due to the activation of the TLR4/MyD88/NF- $\kappa$ B signaling pathway, and subsequently dramatically promoting IL12, TNF- $\alpha$ , CCL9 and CCL5 secretion. These findings revealed a novel immunoregulatory mechanism of TLR4 in the cornea inflammation infected by ISE. This project systematically illustrates the system immunological mechanism of TLR4 in the process of staphylococcus cornea infection. Moreover, this study could provide reference and basic experimental data for the clinical prevention and treatment of infectious keratitis.

## METHODS

### Reagents

The NF- $\kappa$ B inhibitor, BAY11-7082 was purchased from Merck (Temecula, CA, USA). The anti-rabbit TLR4 polyclonal antibody (ab13556),  $\beta$ -actin (ab8227), and anti-mouse p65 (ab16502), MyD88 (ab2064) were purchased from Abcam (Cambridge, UK). The anti-mouse and anti-rabbit secondary HRP-conjugated antibody for western blot was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals used in this paper were analytical or reagent grade and were from commercial sources.

### Cell Isolation and Culture

The mouse corneal epithelial cells were isolated from corneal tissues of C57BL/6J mice as described previously [32, 33]. In brief, the mice were euthanized by cervical dislocation, then quickly removed eyeballs and washed twice with 1 $\times$ PBS supplemented with penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA). Next, the corneal tissues were separated on a cold stage under sterile conditions and digested with 0.25% trypsin (Gibco, Carlsbad, CA, USA)/collagenase type IV (0.1%; Sigma, St. Louis, MO, USA) at 37 °C for 30 min. After that, the procured cell suspension was filter with a 100-mesh wire sieve and then centrifuged at 1200 rpm for 3 min. Finally, cells were cultured in DMEM containing with 15% fetal cattle serum (FCS, Hyclone, Logan, UT, USA); 10 ng/ml epidermal growth factor, IV collagen, and fibronectin; 5  $\mu$ g/ml recombinant human insulin and human transferrin;  $1.8 \times 10^{-10}$  M adenine, 400  $\mu$ g/ml hydrocortisone,  $10^{-6}$  M isoproterenol,  $2 \times 10^{-9}$  M triiodo thyronine, 2 mmol/l glutamine, and 1% penicillin/streptomycin at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were cultured and identified and as a subject for further study. For induction of inflammation, corneal epithelial cells were exposed to ISE for 24 hours. To further examine the mechanism of TLR4 in ISE-induced cornea inflammation, corneal epithelial cells were pre-incubated with 10  $\mu$ M BAY11-7082 for 2 h before ISE treatment.

### Construction of Recombined Lentivirus Vector and Transfection

The CDS sequence of TLR4 was downloaded from GenBank (accession no. AF177767.1). In order to silence the expression of TLR4, we obtained three candidate shRNAs and one scrambled shRNA by predicted on the line software ([http://](http://biodev.extra.cea.fr/DSIR/DSIR.html)

[biodev.extra.cea.fr/DSIR/DSIR.html](http://biodev.extra.cea.fr/DSIR/DSIR.html)), and packaged recombinant lentivirus. And at the meantime, the CDS sequence of TLR4 was sub-cloned into the BamHI, XhoI, and NotI sites of pCDH1-CMV-BXN-EF1-Neo vector (Invivogen, San Diego, CA) to generate the pCDH1-TLR4 plasmid (recombinant expression vector of TLR4), and packaged recombinant lentivirus. The empty pCDH1-CMV-BXN-EF1-Neo vector was employed as the negative control. The primers applied to construct the TLR4 recombinant plasmid are shown in Table 1. Transfection of TLR4 overexpression construct or TLR4 shRNAs lentivirus as well as the negative control after ISE was performed. Transfection was confirmed by qRT-PCR and Western blot analysis.

### Total RNA Isolation and Quantitative Real-time PCR (qRT-PCR)

Total cellular RNA was isolated from corneal epithelial cells by using Trizol Reagent (Invitrogen, Carlsbad, CA) under product instruments. Total RNA purity was detected by using an OneDrop 1000 spectrophotometer (Wuyi technology, Nanjing, China) at the 260/280 nm ratio. Then, the RNA (1  $\mu$ g) was subsequently reverse transcribed into cDNA using a Reverse Transcription Kit (Takara, Kusatsu, Japan). To check the mRNA expression level of TLR4, qRT-PCR was performed triplicate with the SYBR Green Master Mixture (Takara, Kusatsu, Japan) on a Lightcycler 96 system (Roche, Basel, Switzerland). The thermal cycling conditions comprised 5 min at 95 °C, 45 cycles at 95 °C for 20 s, 56 °C for 15 s, and 72 °C for 20 s. The expression of genes was analyzed using the  $2^{-\Delta\Delta CT}$  method and normalized using GAPDH. The Primer sequences used for qRT-PCR are listed in Table 2.

### Western Blot Analysis

Total cellular proteins were harvested from corneal epithelial cells stimulated with ISE in the absence or presence of 10  $\mu$ M BAY11-7082 by using the RIPA lysis buffer. The BCA assay was adopted to determine the concentration of protein. A total of 20  $\mu$ g protein diluted in 5 $\times$ SDS loading buffer were boiled for 5 min and subjected to 12% SDS-PAGE gels. And the separated proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA) followed by blocking with 5% fat-free powdered milk in Tris-buffer saline and 0.1% Tween 20 (TBST) for 2 h at

**Table 1.** TLR4 shRNA Sequences

| shRNAs target | Sequences   |
|---------------|---|
| shRNA-mTLR4-1 | CCGGCCTGTAAGTTACCTGCATATTCTCGAGAATATGCA GGTAACCTACAGGTTTTTG |
| shRNA-mTLR4-2 | CCGGCCGTTGGTGTATCTTTGAATACTCGAGTATTCAAA GATACACCAACGGTTTTTG |
| shRNA-mTLR4-3 | CCGGCCATCATTATGAGTGCCAATCTCGAGATTGGCA C TCATAATGATGGCTTTTTG |

room temperature. Membranes were immunoblotted with primary antibodies against TLR4 (1:500), NF- $\kappa$ B p65 (1:500), MyD88 (1:500), or  $\beta$ -actin (1:500, internal control) overnight at 4 °C. Following washed thrice with TBST, the membranes were incubated with appropriate secondary HRP-conjugated antibodies (1:5000) for 2 h at room temperature. After washing, immunoreactive signals were detected using an ECL Kit, and scanned by chemiluminescence detection system (Tanon, Shanghai, China). Image J (NIH) was performed to analysis the bands. All experiments were conducted at least three times in triplicate.

### Cytokine Studies

To examine the secretion of cytokines, corneal epithelial cells were stimulated with ISE in the absence or presence of 10  $\mu$ M BAY11-7082 for 24 h. The cell-free supernatants were collected by centrifugation at 1000 rpm for 10 min. The secretion of IL1 (cat. No. ml027163, Inc. Biorbyt), IL2 (cat. No. KE10004, Inc. proteintech), IL12 (cat. No. KE10014, Inc. proteintech), INF- $\alpha$  (cat. No. ML1138, Inc. Abnova), TNF- $\alpha$  (cat. No. 29832165, Inc. eBioscience), CXCL1 (cat. No. KE10019, Inc. proteintech), CXCL2 (cat. No. KE10022, Inc.

proteintech), CCL5 (cat. No. ml024686, Inc. Biorbyt), and CCL9 (cat. No. EK1225, Inc. Boster) in cell-free supernatants of different groups was evaluated using the commercial corresponding ELISA Reagent kits according the manufacturer's instructions. Absorbance was measured at 450 nm using ELX-800 microplate reader (BioRad, Hercules, CA).

### Statistical Analysis

Values are reported as mean  $\pm$  standard deviation (SD). Each treatment experiment was performed at least three times in triplicate. Data were analyzed using GraphPad Prism 5. The results were analyzed using One-way ANOVA and *t* test. *P* < 0.05 was considered to be statistically significantly.

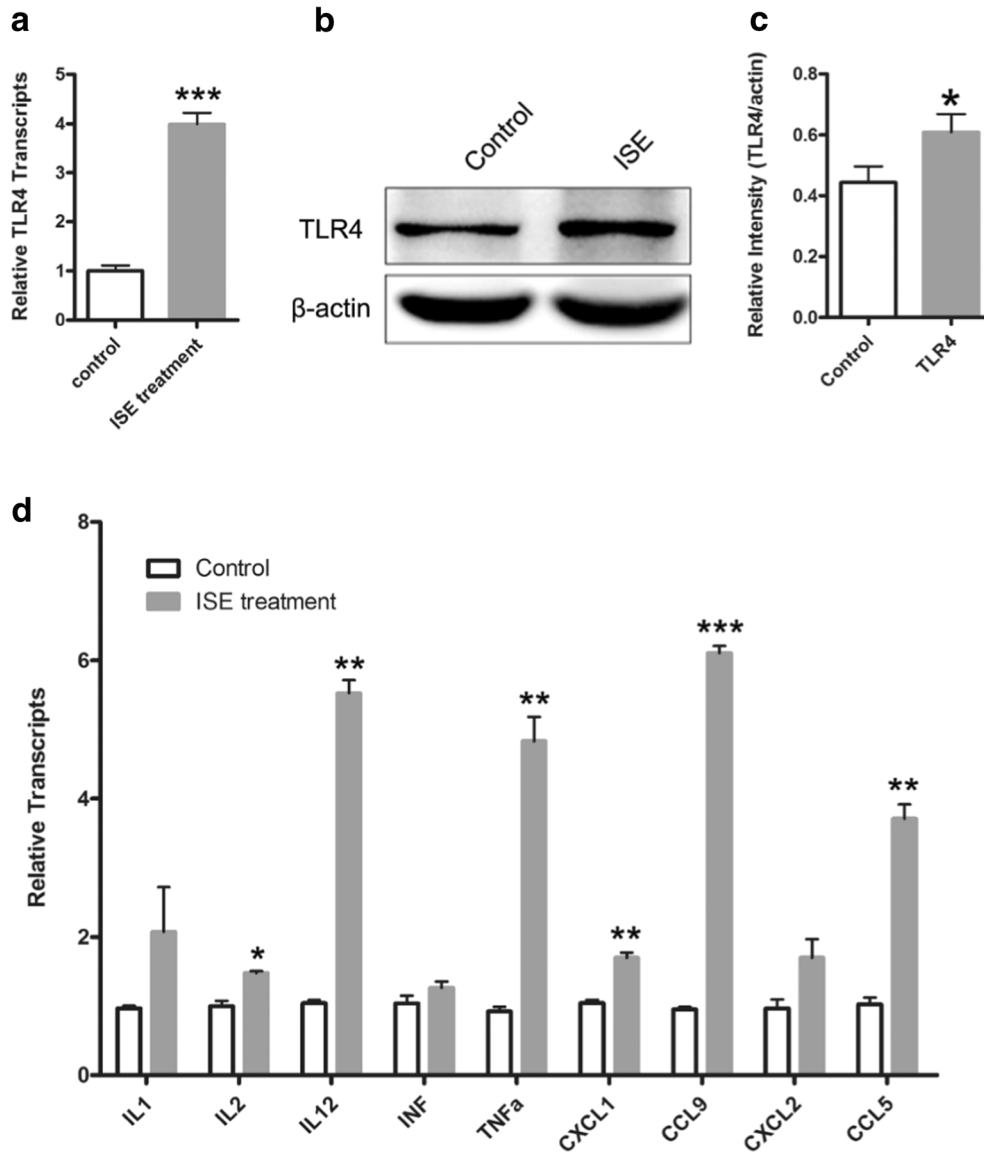
## RESULTS

### ISE-induced TLR4 and Related Inflammatory Cytokine Expression in Corneal Epithelial Cells

Initially, we evaluated the expression of TLR4 in corneal epithelial cells after exposed to ISE for 24 h, and then collected the total RNA and protein of the cells. The expression levels of TLR4 were detected by

**Table 2.** Primer Sequences Used for qRT-PCR

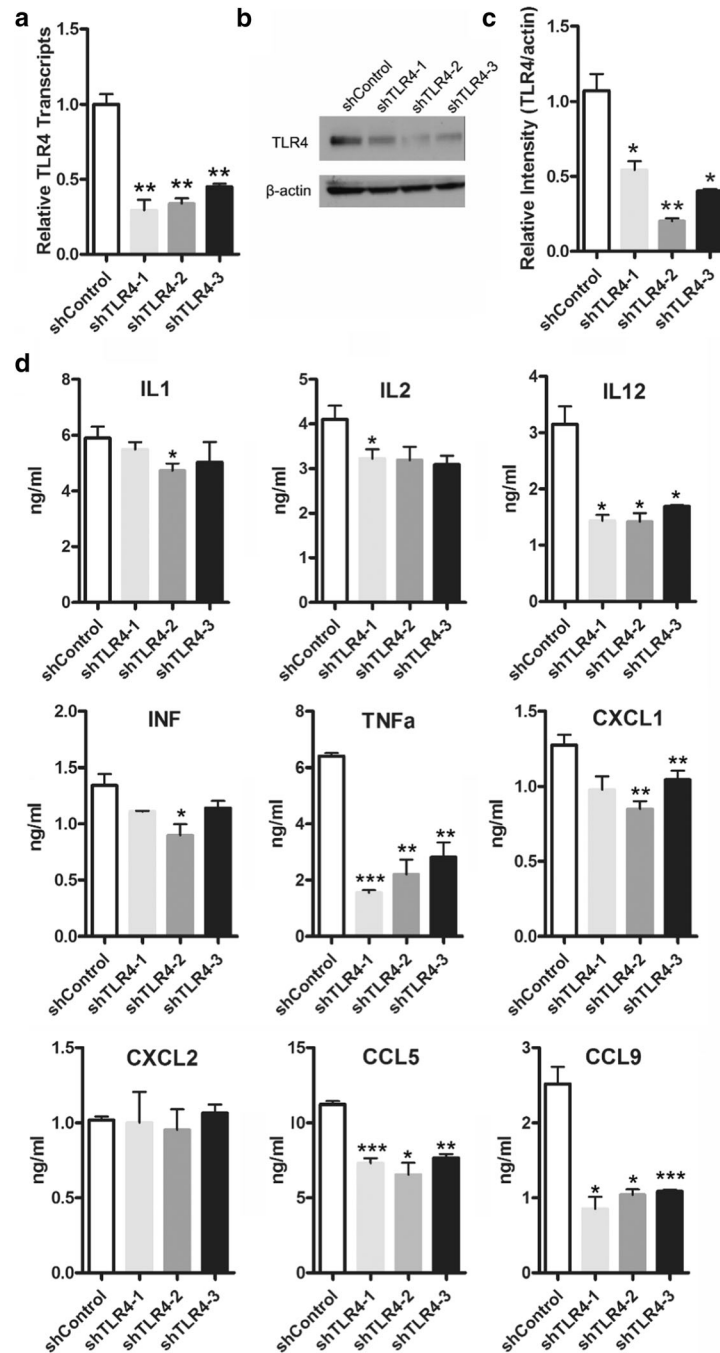
| Gene name     | Forward primer               | Reverse primer               |
|---------------|------------------------------|------------------------------|
| TLR4          | 5'-TCCCTGCATAGAGGTAGTTC-3'   | 5'-AGAGGTGGTGTAAAGCCATGC-3'  |
| IL1           | 5'-GCTTGAGTCGGCAAAGAAAT-3'   | 5'-GAGAGATGGTCAATGGCAGA-3'   |
| IL2           | 5'-CCAGGATGCTCACCTTCAAA-3'   | 5'-TGTTGTCAGAGCCCTTTAGTT-3'  |
| IL12          | 5'-CGTCTTTGATGATGACCCTGTG-3' | 5'-TCTGCTGATGGTTGTGATTCTG-3' |
| INF           | 5'-GACCTGTACACAGCTTCAGG-3'   | 5'-GAAACAGGTTGGAGGTGACA-3'   |
| TNF- $\alpha$ | 5'-ACTGAACCTCGGGGTGATCG-3'   | 5'-CCACTTGGTGGTTTGTGAGTG-3'  |
| CXCL1         | 5'-AAACCGAAGTCATAGCCACA-3'   | 5'-TTGGGGACACCTTTTAGCAT-3'   |
| CCL9          | 5'-CCGGGCATCATCTTATCAG-3'    | 5'-ATTGTTGTAGGTCCGTGGT-3'    |
| CXCL1         | 5'-3'CCAGACAGAAGTCATAGCCA    | 5'-CTTTGGTCTTCCGTTGAGG-3'    |
| CXCL5         | 5'-AATCTTGCACTCGTGTGTTGTC-3' | 5'-TAGGGGATTACTGAGTGGCA-3'   |
| GAPDH         | 5'-GGAGCGAGACCCCACTAAC-3'    | 5'-GGCGGAGATGATGACCCT-3'     |



**Fig. 1.** ISE-induced TLR4 and related inflammatory cytokines expression in corneal epithelial cells. The corneal epithelial cells were exposed to ISE for 24 h, and then collected the total RNA and protein of the cells. **a** TLR4 mRNA level was measured by qRT-PCR. **b** TLR4 protein level was determined by western blot. TLR4 was upregulated at both gene and protein levels. **c** The transcription level of inflammatory cytokines measured by qRT-PCR. Data are presented as mean ± SD (error bars). \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  vs Control.

qRT-PCR and western blot. As expected, the results indicated that the mRNA and protein levels of TLR4 were upregulated significantly in ISE treatment group compared with the control group (Fig. 1a–c). We next assessed the effects of ISE on the transcription level of inflammatory cytokines. As shown in Fig. 1d, the qRT-PCR assay declared that the inflammatory cytokines gene IL2, IL12, TNF-α, CXCL1, CCL9, and CCL5

levels of in corneal epithelial cells were increased significantly, and many studies have suggested that overstimulation of these cytokines can result in scar formation in the cornea and eventually bring to blindness [34]. However, no changes were discovered in the transcription level of IL1, INF, and CXCL2. The results indicated that corneal epithelial cells infected with ISE can activate the expression of TLR4 and



**Fig. 2.** TLR4 silencing reduced the secretion of related inflammatory cytokines in corneal epithelial cells. TLR4 shRNA knock-down vector was constructed, and packaged recombinant lentivirus. ELISA kits were used to detect the changes of various inflammatory factors in cells treated with ISE after TLR4 silencing. Three TLR4 shRNA lentivirus were packaged and transfected into corneal epithelial cells. **a** Extract RNA to detect TLR4 mRNA level by qRT-PCR; TLR4 mRNA levels were significantly down-regulated. **b** Protein was extracted, and the TLR4 protein level was down-regulated tested by western blot. **c** Statistical graph of figure **b**. **d** After TLR4 silencing, cell-free supernatants were collected for detected the relevant inflammatory factors by ELISA, in which IL12, TNF- $\alpha$ , CCL5, and CCL9 were significantly down-regulated. Data are presented as mean  $\pm$  SD (error bars). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs shControl.



subsequently promote the transcriptional level of multiple inflammatory cytokines.

### TLR4 Silence Reduced the Secretion of Related Inflammatory Cytokines in Corneal Epithelial Cells

To investigate the role of TLR4 in ISE-induced keratitis, the corneal epithelial cells were transfected with lentivirus to knock-down the expression of TLR4. qRT-PCR and western blot analysis displayed that the transcriptional and protein levels of TLR4 were markedly decreased (Fig. 2a–c). In addition, we analyzed the secretion of related inflammatory cytokines in cell-free supernatants. The ELISA assay demonstrated that TLR4 silence declined the secretion of inflammatory cytokines, especially inflammatory IL12, TNF- $\alpha$ , CCL5, and CCL9 secretion were dramatically reduced (Fig. 2d). Together, the results suggest that ISE induced the inflammatory response in corneal epithelial cells by activating the TLR4.

### ISE-induced Cornea Inflammation via the TLR4/MyD88/NF- $\kappa$ B Signaling Pathway

We next extended our study to illuminate the mechanisms by which ISE induces the inflammatory response in corneal epithelial cells. NF- $\kappa$ B was a potentially downstream molecule of activated TLR4 which was occurring through MyD88. NF- $\kappa$ B has been reported to be linked to various cell processes, such as pro-inflammation, cell proliferation, angiogenesis, and inhibition of apoptosis [35]. We confused whether NF- $\kappa$ B signaling pathway is involved in inflammatory response induced by ISE. Therefore, corneal epithelial cells were silenced the expression of TLR4 before stimulated with ISE. We noted that NF- $\kappa$ B p56 and MyD88 expression at corneal epithelial cells was lower in TLR4 silence group (shTLR4-1/2) than that in normal control group (Control) (Fig. 3a, c). To further test whether the ISE-induced inflammatory response was result from the activation of NF- $\kappa$ B signaling pathway. As depicted in Fig. 3b, d, the NF- $\kappa$ B p65 and MyD88 protein levels in ISE treatment group (ISE) were significantly increased compared with Control group ( $p < 0.01$ ). As expected, in the presence of an NF- $\kappa$ B inhibitor BAY11-7082 before treated with ISE dramatically abolished this effect. To further explore the effect of the activation of NF- $\kappa$ B signaling pathway on inflammatory cytokine secretion, we found that the increased IL12, TNF- $\alpha$ , and CCL9 levels in cells and cell-free supernatants stimulated with ISE were reversed in the presence of BAY11-7082 which detected by qPCR and ELISA, respectively. Our observation suggested that the activation of TLR4 triggered the

inflammatory response via MyD88/NF- $\kappa$ B signaling pathway in corneal epithelial cells treated with ISE.

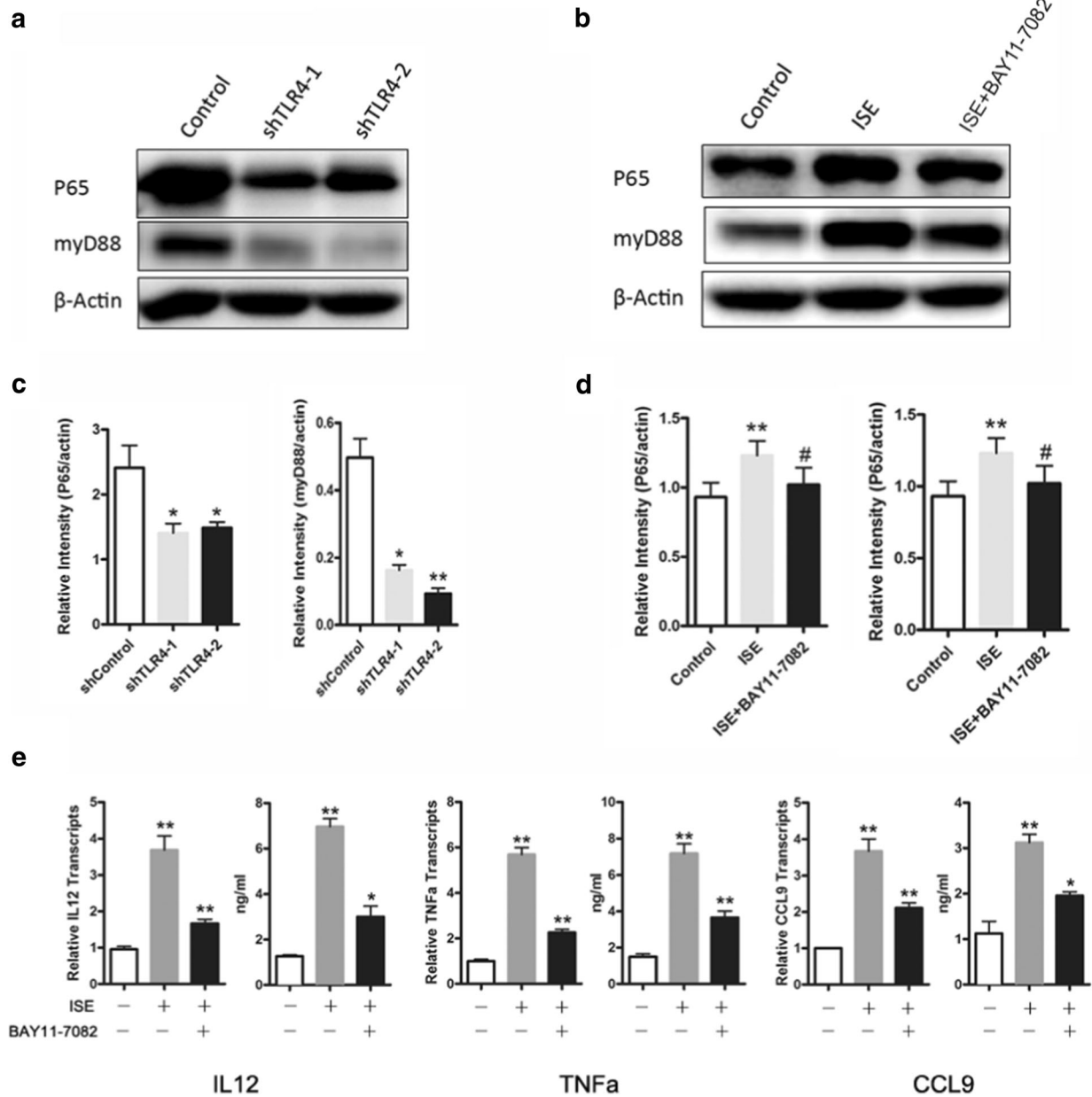
### TLR4 Overexpression Restored the Down-regulation of Inflammatory Cytokines Caused by TLR4 Silence

Our previous research revealed ISE triggers the inflammatory response in corneal epithelial cells by activating the TLR4. In order to further solidify the role of TLR4 in cornea inflammation induced by ISE, we construct the pCDH1-TLR4 overexpression plasmid, and packaged recombinant lentivirus in the following rescue experiment. TLR4 was markedly up-regulated in RNA and protein levels in corneal epidermal cells transfected with TLR4 recombinant lentivirus (TLR4 overexpression, TLR4-OE) compared with the negative control group (Fig. 4a–c). As we know from Fig. 2d, TLR4 silence significantly reduced the secretion of IL12, TNF- $\alpha$ , CCL5, and CCL9 in ISE-treated cells. To confirm this effect was caused by TLR4 silence in this cells, TLR4 knock-down was rescued by TLR4-OE lentivirus and then examined the transcriptional levels of gene IL12, TNF- $\alpha$ , and CCL9. As shown in Fig. 4d, the down-regulation of IL12, TNF- $\alpha$ , and CCL9 in TLR4 silence groups (ISE+shTLR4-1/2) were reversed by TLR4 overexpression (ISE+shTLR4-1/2+TLR4-OE), which was verified by qRT-PCR. In other words, TLR4 overexpression restores the down-regulation of inflammatory cytokines caused by TLR4 silence in corneal epidermal cells stimulated with ISE. These outcomes suggest that activation of TLR4 triggered the inflammatory response via MyD88/NF- $\kappa$ B signaling pathway in corneal epithelial cells treated with ISE, and thus dramatically stimulated IL12, TNF- $\alpha$  and CCL9 secretion.

## DISCUSSION

Bacterial keratitis is corneal infection mainly caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and other pathogen [6], and which is the major cause of blindness in both developing countries and developed countries. The number of blindness and visual morbidity is projected to increase. Nevertheless, the overuse of antibiotics limits the effectiveness of antimicrobial therapy; it is urgently needed to develop additional therapeutic strategies in addition to antimicrobial therapy [7, 36].

TLRs play an important role in recognizing PAMP and are sentinels of innate immunity, which then leads to the development of adaptive immune responses. TLR4, one important member of TLRs, also plays a key role in the inflammatory response mediated by the NF- $\kappa$ B signaling pathways [37, 38]. According to reports, human

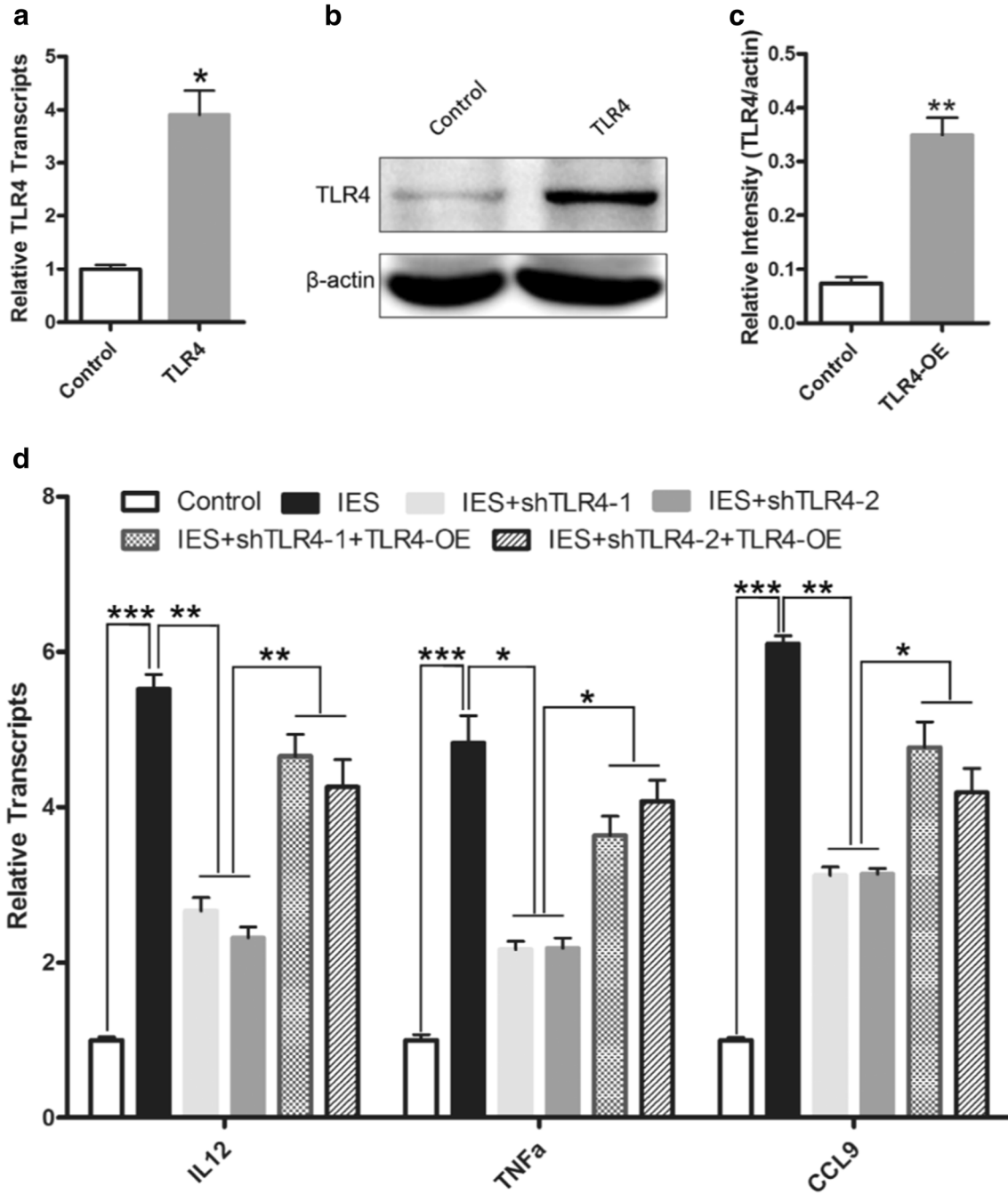


**Fig. 3.** ISE-induced cornea inflammation via the TLR4/MyD88/NF- $\kappa$ B signaling pathway. The expression of MyD88 and P65 in TLR4-mediated NF- $\kappa$ B signaling pathway. Mechanism of inflammation in corneal epithelial cells, **a** Cell proteins of the control group and TLR4 knock-down group (shTLR4-1/2) were collected after ISE treatment, respectively, and NF- $\kappa$ B signal was detected. **b** Cells were divided into untreated group (control), ISE treated group (ISE) and ISE+ BAY11-7082 (an NF- $\kappa$ B inhibitor, 10  $\mu$ M) treated group (ISE+ BAY11-7082), then the expression of p65 and MyD88 in each group was detected by western blot. **c** Statistical graph of figure **a**. **d** Statistical graph of figure **b**. **e** The cells were divided into control, ISE and ISE+ BAY11-7082 groups, and the expressions of IL12, TNF- $\alpha$ , and CCL9 were detected by qPCR and ELISA, respectively. Data are presented as mean  $\pm$  SD (error bars). \* $p$  < 0.05, \*\* $p$  < 0.01 vs Control, # $p$  < 0.05 vs ISE.

corneal epithelial cells stimulated with bacterially derived products such as lipopolysaccharides (LPS) can express TLR4 and secrete pro-inflammatory factors [39, 40].

MyD88 took part in inflammatory cytokine production during pathogen infection via the activation of NF- $\kappa$ B. TLR4 and MyD88 are upstream regulators of NF- $\kappa$ B





**Fig. 4.** TLR4 overexpression restored the down-regulation of inflammatory cytokines caused by TLR4 knockdown. Rescue experiment of TLR4 overexpression. Constructed the TLR4 overexpression plasmid, packaged lentivirus, and transfection was confirmed by qRT-PCR and Western blot analysis. **a** TLR4 mRNA level was measured by qRT-PCR. **b** TLR4 protein level was determined by western blot. **c** Statistical graph of figure **b**. TLR4 was upregulated at both RNA and protein levels. **d** Further verify whether TLR4 overexpression can restore the down-regulation of some inflammatory factors caused by TLR4 knockdown. TLR4 knockdown cells which treated with ISE were transfected with TLR4 overexpression plasmid lentivirus, then expression of related inflammatory factors was verified at mRNA levels. Data are presented as mean ± SD (error bars). \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs Control.

[41]. NF- $\kappa$ B is a nuclear transcription factor which regulates the expression of inflammation-related genes. NF- $\kappa$ B was isolated in an inactive form in the cytoplasm by binding to the I $\kappa$ B complex. After activation, NF- $\kappa$ B translocated to the nucleus, starting gene transcription in the nucleus, and producing various cytokines [42, 43]. In previous studies, gram-negative bacteria can transmit infection signals to cells and then mediated by various intracellular signaling pathways and trigger a series of immune responses in corneal epithelial cells [44]. However, the regulatory role of TLR4 in corneal infections induced with gram-positive bacteria, such as ISE, remains largely unclear.

In the present study, we sought to investigate the effects of TLR4 on the ISE-induced inflammation in corneal epithelial cells through a series of *in vitro* experiments. The present study showed that TLR4 as well as inflammation-related genes were upregulated dramatically in ISE-induced corneal epithelial cells. And the silence of TLR4 not only suppressed the secretion of inflammatory cytokines but also the protein levels of NF- $\kappa$ B p56 and MyD88 in ISE-induced corneal epithelial cells, in the other word, the underlying mechanism of ISE-induced inflammatory response in corneal epithelial cells may be the activation of TLR4/MyD88/NF- $\kappa$ B signaling pathway. To further explore the mechanism underlying ISE-induce the inflammatory response in corneal epithelial cells, we noted that NF- $\kappa$ B p56 and MyD88 expression was increased in ISE-induced corneal epithelial cells which was abolished by pre-administration of BAY11-7082. Moreover, in the final rescue experiment, TLR4 overexpression restored the down-regulation of inflammatory cytokines caused by TLR4 silence. Thus, the previous results showed that the activation of TLR4 triggered the inflammatory response via MyD88/NF- $\kappa$ B signaling pathway in corneal epithelial cells treated with ISE.

In conclusion, our study proposed a novel mechanism of ISE-induced inflammation in corneal epidermal cells, which was that the activation of TLR4 triggered the inflammation response via activating the MyD88/NF- $\kappa$ B signaling pathway. Moreover, our data may be promising for the therapeutic approach aiming to preventing ISE-induced inflammation of corneal by target silencing TLR4.

## FUNDING

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflicts of Interest.** We hereby declare that there is no conflict of interest between the authors of this manuscript.

**Ethical Approval.** All animal procedures were approved by the Lab Animal Ethical Committee of Nantong University and were performed according to the Standard Operating Procedures for Laboratory Animal Center of Nantong University.

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