Original Research

Influence of Pseudomonas autoinducer N-3-oxododecanoyl homoserine lactone on human corneal epithelial cells

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Impact statement

Pseudomonas aeruginosa is the most common causative agent in cases of bacterial keratitis associated with contact lens use. *Pseudomonas* autoinducer, *N*-3-oxododecanoyl homoserine lactone (3OC12HSL), exerts an important role in the regulation of virulence gene expression in *P. aeruginosa* infection. Little is known, however, about the specific effects of 3OC12HSL on human corneal epithelial cells (HCECs). Our study investigates the crosstalk between the quorum-sensing signaling molecule 3OC12HSL and host immunity in HCECs. Results of the study indicate that 3OC12HSL can be recognized by the host innate immune system, followed by the triggering of an immune inflammatory response which involves the activation of TLR2 and an increase in activated nuclear factor-κB (NF-κB)-dependent interleukin-8 (IL-8) expression. This article proved that 3OC12HSL is an important modulator of inflammatory signaling between *P. aeruginosa* and HCECs. These findings reveal that targeted interventions against 3OC12HSL may have the potential to control *P. aeruginosa* keratitis.

Abstract

The quorum-sensing (QS) signaling-dependent extracellular virulence factors of *Pseudomonas aeruginosa* can cause infections such as *P. aeruginosa* keratitis. *P. aeruginosa* communicates by secreting and sensing small chemical molecules called autoinducers in QS system. The key QS signal molecule, *N*-3-oxododecanoyl-homoserine lactone (3OC12HSL), can affect the behavior of host cells and initiate immune response. In this report we investigated the influence of 3OC12HSL on human corneal epithelial cells (HCECs) and the mechanisms of 3OC12HSL on activated toll-like receptor 2 (TLR2)-dependent interleukin-8 (IL-8) secretion in HCECs. Cells were cultured under different concentrations of 3OC12HSL. Cell viability was assessed using Crystal violet staining and the cell counting kit-8 assay. We demonstrated the administration of 3OC12HSL decreased HCEC viability and survival in a concentration- and time-dependent manner. At high concentrations, 3OC12HSL rapidly promoted a time-dependent increase in the expressions of TLR2 and TLR4. It was found that the nuclear translocation and expression of nuclear factor-κB (NF-κB) were also increased in response to 3OC12HSL treatment. The significantly elevated expressions of TLR2, TLR4, and NF-κB, encouraged us to further test their mechanisms that cause inflammatory response. Among the inflammatory factors examined (IL-6, IL-8, IL-10, and TNF-α), we found that IL-8 was significantly increased after treatment with 3OC12HSL and its expression was inhibited when TLR2 was specifically blocked or silenced. These results indicated that the QS signaling molecule 3OC12HSL could be recognized by the host innate immune system in HCECs. This recognition then triggered an immune inflammatory response involving the activation of TLR2 and an increase in expression of IL-8. This crosstalk between 3OC12HSL and host immunity in HCECs contributes to the development and progression of *P. aeruginosa* keratitis.

Keywords: 3-Oxododecanoyl-homoserine lactone, toll-like receptor 2, nuclear factor-κB, interleukin-8, human corneal epithelial cells

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Introduction

Pseudomonas aeruginosa, an opportunistic pathogen producing acute corneal infection, can lead to serious consequences which are difficult to treat.[1–3] Superficial corneal damage and surface infection are often associated with contact lens wear and *P. aeruginosa* is the leading cause of bacterial keratitis.[4,5] Healthy corneal epithelial cells, which possess an intact barrier function appear to be resistant to *P. aeruginosa* infection. However, a damaged corneal epithelial barrier provides an opportunity for *P. aeruginosa* entry into the cornea.

The development of *P. aeruginosa* infection involves effects of the bacteria itself as well as effects upon the host immune response. Usually, it is the host immune response that results in severe corneal damage, including globe perforation and blindness.[6] In such cases, immune defense mechanisms of the host may then be a significant cause for tissue damage through activation of immune cells and secretion of cytokines.

As a major pathogen of *P. aeruginosa* keratitis, *P. aeruginosa* communicates by secreting and sensing small chemical molecules called autoinducers in a process known as the quorum-sensing (QS) system.[7,8] This communication system can organize cellular behaviors and modulate the gene expression of virulence factors.[9,10] *P. aeruginosa* constantly secretes auto-inducer molecules called *N*-acyl-homoserine lactones (AHLs) and there are two unique AHLs in the QS system of *P. aeruginosa*, 3OC12HSL and C4HSL (*N*-butanoyl homoserine lactone). Both of them are small lipid-soluble and membrane-permeant molecules which act as autoinducer of the QS system.

A significant number of studies have concluded that the *P. aeruginosa* QS signal molecule 3OC12HSL, but not C4HSL, can interact with different eukaryotic cells and modulate immune response in many disease models.[11–13] For example, it has been reported that 3OC12HSL can alter the barrier function of colon cells, suppress lipopolysaccharide-stimulated interleukin-12 (IL-12) secretion in macrophages, and induce IL-8 expression in lung fibroblasts and respiratory epithelial cells.[14–16] However, it is unclear as to whether 3OC12HSL is also involved in the regulation of corneal immune responses induced by *P. aeruginosa* infection.

The host organism primarily recognizes pathogen-associated molecular patterns through its pattern recognition receptors (PRRs).[17,18] PRRs are key components of the immune receptors that recognize the infectious agents and trigger immune responses.[19] With regard to *P. aeruginosa*, toll-like receptors (TLRs) are essential PRRs that mediate its recognition.[20] It has been reported that 3OC12HSL mediated TLR changes in PBMCs and monocytes.[21] Whether corneal epithelial cells recognize 3OC12HSL through TLRs, and exactly which TLR may play a key role in the recognition of 3OC12HSL remain unknown.

Here we demonstrate that 3OC12HSL positively regulates the inflammatory response in human corneal epithelial cells (HCECs). 3OC12HSL can be recognized by the host innate immune system through the activation of TLR2, and the neutrophil chemotactic molecule IL-8 is enhanced. Further studies show that the role of protein kinase B (AKT) and transcription factors (nuclear factor-κB [NF-κB]) in the production of IL-8 with activation of 3OC12HSL in HCECs.

Materials and methods

Antibodies and regents

The following antibodies were used in this study: TLR2 (ab108998; Abcam, Cambridge, MA, USA; 1:1000 for WB), TLR4 (ab13867; Abcam, Cambridge, MA, USA; 1:1000 for WB), NF-κB p65 (8242s; Cell Signaling Technology, Danvers, MA, USA; 1:1000 for WB, 1:400 for IF), phospho-NF-κB p65 (3033s; Cell Signaling Technology, Danvers, MA, USA; 1:1000 for WB), phospho-Akt (4060 s; Cell Signaling Technology, Danvers, MA, USA; 1:2000 for WB), Akt (9272 s; Cell Signaling Technology, Danvers, MA, USA; 1:1000 for WB), β-actin (12620 s; Cell Signaling Technology, Danvers, MA, USA; 1:1000 for WB). *N*-3-oxododecanoyl-homoserine lactone was purchased from Sigma-Aldrich (St. Louis, MO, USA); TLR2 agonist (HKLM, 10^8 HKLM/mL), TLR4 agonist (CRX-527, 10 ng/mL), TLR5 agonist (FLA-PA, 1 μg/mL), TLR6 agonist (FSL-1, 100 ng/mL), and TLRs antagonists (PAb-hTLR2, PAb-hTLR4, PAb-hTLR5, PAb-hTLR6, 5 μg/mL) from InvivoGen (San Diego, CA, USA); Lipofectamine 2000 from ThermoFisher Scientific (Basingstoke, UK); and BAY 11-7821 from Merck-Millipore (Darmstadt, Germany).

Cell culture and 3OC12HSL treatment

Immortalized HCECs were kindly provided by Zan Pan (New York University, NY, USA). Cells were cultured in keratinocyte serum-free medium (Thermo Fisher Scientific, UK) supplemented with 10% fetal bovine serum (FBS) and antibiotics (10 U/mL penicillin G and 10 mg/mL streptomycin) at 37°C and 5% CO_2. HCECs were grown in 100-mm plates until confluent monolayers were formed. These HCECs were then treated with different concentrations of 3OC12HSL, ranging from 0 to 200 μmol/L for 0, 4, 8, 12, or 24 h. Cells or supernatants were collected for subsequent experiments.

Treatments of TLR agonists or blocking antibodies

HCECs were pretreated with TLR agonists or TLR antagonists for 2 h before the administration of 3OC12HSL (100 μmol/L). Controls were similarly cultured with DMSO or control IgG.

Cell viability assay

The cell counting kit-8 (CCK-8 kit; CCK8, MedChem Express) was used to assay cell viability as per the protocol provided. Briefly, 10 μL of the CCK-8 solution was added to each well of the plate. When added to cells, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)) is modified by the reducing environment of viable cells and turns orange (WST-8 formazan) in color. After incubation for 2 h, the absorbance was measured at 450 nm and the cell viability under test conditions was reported as a percentage relative to the negative control.

Crystal violet staining

The cell culture medium was removed and HCECs were fixed with 11% glutaraldehyde. After washing with deionized water, HCECs were dried in an oven at 37°C. The HCECs were then stained with 0.1% crystal violet and shaken for 30 min. After drying, HCECs were observed and photographed under a microscope.

Quantitative real-time reverse transcription polymerase chain reaction

RNAiso plus (TaKaRa, China) and Trizol (Invitrogen, USA) reagents were used to isolate total RNA from HCECs. Then the total RNA was rapidly quantified by spectrophotometry. In order to measure the relative concentrations of gene expression, total RNA was used for first-strand cDNA synthesis according to the protocol for a reverse transcription system. The cDNA was then used for quantitative real-time PCR in the reaction volume. PCR cycling conditions were as follows: an initial denaturation step of 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Results were analyzed with the use of comparative cycle threshold method and normalized by GAPDH. The primers used in this study were synthesized by Sangon Biotech Corporation (Shanghai, China) according to the sequences as follows: GAPDH (forward: 5′-GTCAAGGC TGAGAACGGGAA-3′, reverse: 5′-AAATGAGCCCCAGC CTTCTC-3′), TLR2 (forward: 5′-TCTCCCATTTCCGTCT TTTT-3′, reverse: 5′-GGTCTTGGTGTTCATTATCTTC-3′), TLR4 (forward: 5′-GAAGCTGGTGGCTGTGGA-3′, reverse: 5′-TGATGTAGAACCCGCAAG-3′), TLR5 (forward: 5′-TTGCTCAAACACCTGGACAC-3′, reverse: 5′-C TGCTCACAAGACAAACGAT-3′), TLR6 (forward: 5′-GT GCCATTACGAACTCTA-3′, reverse: 5′-CTTGTTGGGAA-TGCTGTT-3′), IL-6 (forward: 5′-ACTCACCTCTTCAGA ACGAATTG-3′, reverse: 5′-CCATCTTTGGAAGGTTCA GGTTG-3′), IL-8 (forward: 5′-TTTTGCCAAGGAGTGCT AAAGA-3′, reverse: 5′-AACCCTCTGCACCCAGTTTTC-3′), IL-10 (forward: 5′-TCAAGGCGCATGTGAACTCC-3′, reverse: 5′-GATGTCAAACTCACTCATGGCT-3′), and TNF-α (forward: 5′-CCTCTCTCTAATCAGCCCTCTG-3′, reverse: 5′-GAGGACCTGGGAGTAGATGAG-3′).

Western immunoblot analysis

HCECs were lysed with RIPA containing 1 mmol/L PMSF (100:1). Cell lysates were mixed with loading buffer (Beyotime, China) and boiled for 10 min. The cell lysates were assayed on SDS-PAGE gels (12%) and then transferred onto PVDF membranes (Millipore, Germany). After blocking with 5% non-fat milk solution, the membranes were incubated with primary antibodies, followed by relevant HRP-conjugated secondary antibodies. Colorimetric signals on the membrane were performed using Beyo ECL Plus reagents (Beyotime, China). The band image was visualized using a gel documentation system (UVP Biospectrum HR410, USA).

Construction of shRNA and lentiviral infection

The small hairpin RNA (shRNA) of TLR2 was acquired from the website (https://www.sigmaaldrich.com/) as follows: shTLR2 (forward: 5′-CCGGGCATCTGATAATGA CAGAGTTTTCAAGAGAAACTCTGTCATTATCAGATG CTTTTTTGGTACC-3′, reverse: 5′-AATTGGTACCA AAAAAGCATCTGATAATGACAGAGTTTCTCTTGAAA ACTCTGTCATTATCAGATGC-3′). After the plasmid of pLKO.1-puro (Sigma, CAT #SHC001) was digested with the endonucleases of AgeI and EcoRI, the shRNA fragment was inserted into the linearized plasmid. The shTLR2 was constructed, and pLKO.1-puro was used as shCtrl. Then the HEK-293T cell line was used to amplify the lentivirus. The shTLR2 (or shCtrl) plasmid was transfected into HEK-293T cell with the packaging plasmids of pMD2.G (Addgene, CAT# 12259) and psPAX2 (Addgene, CAT #12260) at a ratio of 5.7: 1.8: 4.5 using lipofectamine 2000. The medium was replaced with fresh 10% FBS culture medium after 8 h and the lentivirus was collected after 72 h. Then, HCECs were infected by lentivirus of shTLR2 or shCtrl. After one-week screening with puromycin (1 μg/mL; InvivoGen), the cells were harvested to conduct downstream experiments. Knockdown efficiency was analyzed by immunoblot.

Immunofluorescent staining

Immunofluorescent staining was used to visualize NF-κB proteins in the HCECs after administration of 3OC12HSL. HCECs (3×10^6 cells/well in 500 μL) were treated with 3OC12HSL for 1–4 h and then fixed in acetone at −20°C for 10 min. All nonspecific samples were blocked with 20% goat serum (Sigma-Aldrich) for 1 h. The NF-κB primary antibody was diluted 1:100 in 5% goat serum in PBS and then incubated for 1 h. After washing with PBS, samples were incubated with the secondary antibody for 1 h in the dark. Pictures were taken at 400× using an Eclipse E400 microscope equipped with a digital camera (DS-Qi1Mc; Nikon Instruments Inc, Melville, NY, USA).

Cytokine ELISA measurements

Culture supernatants exposed to different conditions were collected. Supernatants were centrifuged for 10 min at 2500*g* to remove cellular debris and stored at −80°C before analysis. Extracellular IL-6 and IL-8 cytokines were quantified with use of enzyme-linked immunosorbent assay kits (R&D, USA) according to the manufacturers' instructions.

Statistical analysis

GraphPad Prism 7.0 software was used for statistical analysis. The data were expressed as the mean ± SD of individual samples. Univariate analysis of variance (ANOVA) was used to perform comparisons among multiple groups with Bonferroni's test used for post-hoc comparisons. Values of $P < 0.05$ were required for results to be considered as statistically significant.

Results

3oc12hsl decreased HCECs viability

We first examined the effect of varying concentrations of 3OC12HSL (0 to 200 μmol/L for 12 h) on the survival of HCECs. Based on the results obtained with crystal violet staining and the CCK-8 assay, cell density and viability of HCECs decreased with increasing concentrations of 3OC12HSL (Figure 1(a) and (b)). To determine whether this reduction in HCECs survival to 3OC12HSL was time-dependent, HCECs were treated with 3OC12HSL at

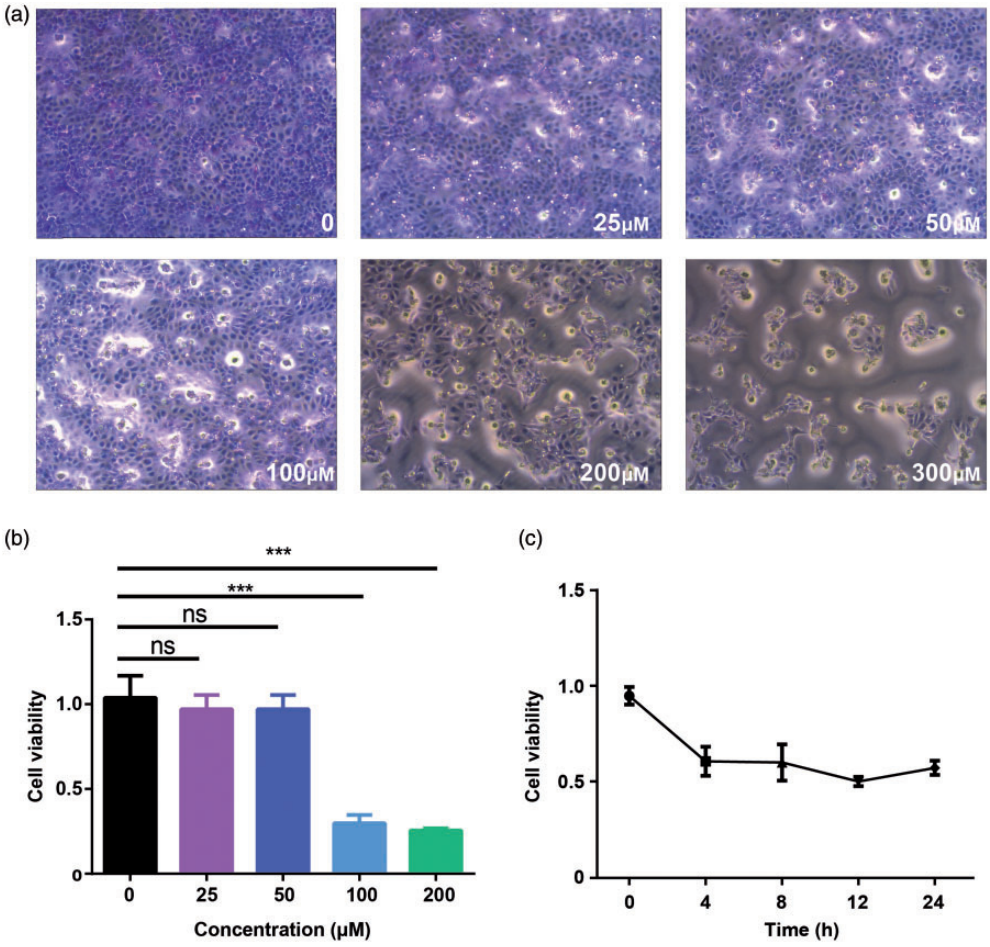

Figure 1. 3OC12HSL decreased HCECs viability. (a) The cell density of HCECs treated with varying concentrations of 3OC12HSL (0, 25, 50, 100, and 200 μmol/L for 12 h) by crystal violet staining. (b) HCEC viability in response to varying concentrations of 3OC12HSL as detected with use of the CCK-8 kit. (c) HCEC viability in response to varying durations of treatment with 100 μmol/L 3OC12HSL as detected with use of the CCK-8 kit. Data shown are the mean ± SEM; ***$P < 0.001$. Representative results are from at least three independent experiments. (A color version of this figure is available in the online journal.)

100 μmol/L for different times (0, 4, 8, 12, and 24 h) and assessed using the CCK-8 assay. We found that cell viability decreased as a function of exposure time (Figure 1(c)). The cell viability decreased sharply within 4 h, but the cell damage induced by 3OC12HSL was gradually attenuated by prolonged culture time after 4 h. Stimulation of HCECs for 12 h with an optimal dose of 100 μmol/L 3OC12HSL dramatically decreased the cell viability (50%). These results demonstrated that 3OC12HSL produced a dose- and time-dependent decrease in cell viability of HCECs.

3OC12HSL modulated TLR2 and 4 expressions in HCECs

In this experiment we determined whether 3OC12HSL mediated TLR (TLR2, 4, 5, and 6) expressions. HCECs treated with 3OC12HSL at various concentrations for 12 h showed significant increases in TLR2 and TLR4 mRNA expressions in a concentration-dependent manner as compared with the DMSO control, with maximal responses being observed at 100 μmol/L (Figure 2(a) and (b)). Next, the time-dependent effects of 100 μmol/L 3OC12HSL on the expressions of TLRs in HCECs were tested. We found that 3OC12HSL significantly increased TLR2 and TLR4 mRNA expressions after 4 and 8 h, respectively (Figure 2 (c) and (d)). With the stimulation of 3OC12HSL for 12 h, both TLR2 and TLR4 mRNA expressions were dramatically increased ($P < 0.001$). However, mRNA expressions of TLR5 and TLR6 were not significantly affected by any of the 3OC12HSL concentrations tested (Supplementary Figure S1(A) and (B)). These results show that 3OC12HSL modulated the cell density and viability of HCECs mainly through TLR2 and TLR4 but not TLR5 or TLR6. The significantly elevated mRNA expressions of TLR2 and TLR4 encouraged us to further assay their protein expressions with Western blot. Although not as dramatic, a similar induction in TLR2 and TLR4 protein expressions was found when treated with various concentrations of 3OC12HSL (Figure 2(e), (f), and (g)). Although the time-dependent effects of 3OC12HSL on expressions of TLR2 and TLR4 protein (Figure 2(h), (i), and (j)) were not quite in line with the previous trends of mRNA expressions, TLR2 and TLR4 protein expressions were dramatically increased with the stimulation of 3OC12HSL at 12 h ($P < 0.001$).

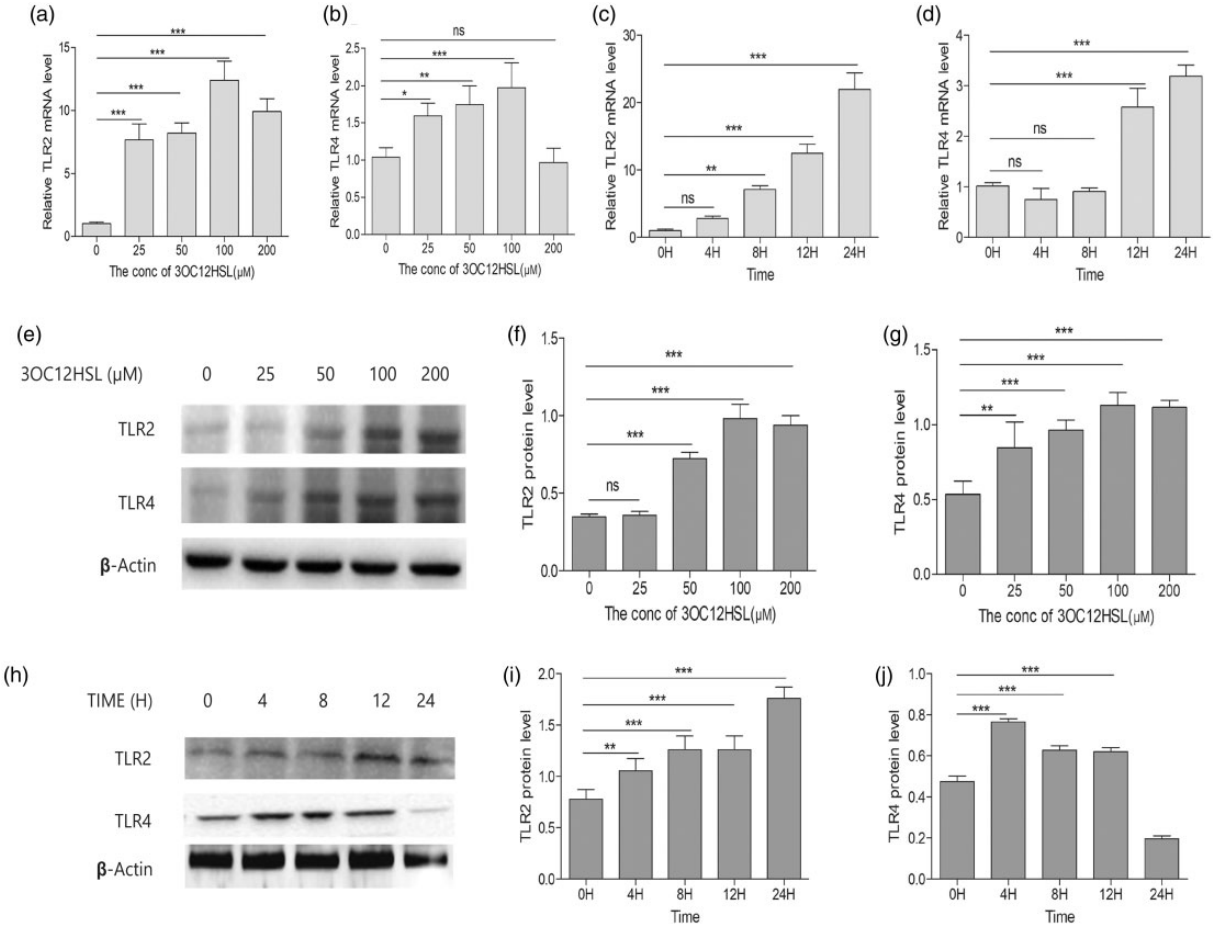

Figure 2. 3OC12HSL modulated TLR2 and 4 expressions in HCECs. (a and b) mRNA expressions of TLR2 and 4 in HCECs in response to a 12 h exposure of varying concentrations of 3OC12HSL (0 to 200 μmol/L). (c and d) mRNA expressions of TLR2 and 4 in HCEC treated with 100 μmol/L 3OC12HSL for 0, 4, 8, 12, or 24 h. (e) Protein expressions of TLR2 and 4 in HCECs in response to a 12 h exposure of varying concentrations of 3OC12HSL (0 to 200 μmol/L). (f and g) Quantification of TLR2 and 4 protein in HCECs in response to varying concentrations of 3OC12HSL. (h) Protein expressions of TLR2 and 4 in HCECs treated with 100 μmol/L 3OC12HSL for 0, 4, 8, 12, or 24 h. (I and j) Quantification of TLR2 and 4 protein in HCECs treated with 100 μmol/L 3OC12HSL for 0, 4, 8, 12, or 24 h. Data shown are the mean ± SEM; ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Representative results are from at least three independent experiments.

3OC12HSL induced NF-κB activation in HCECs

The NF-κB transcription factor family has been considered the central mediator of the inflammatory process and a key participant in TLR mediated signaling.[22] We examined the role of NF-κB in the activation of innate immunity induced by 3OC12HSL. Immunofluorescent staining of NF-κB p65 was performed in HCECs treated with 100 μmol/L 3OC12HSL for 0, 1, 2, or 4 h. Upon treatment with 3OC12HSL, NF-κB p65 translocated from the cytoplasm to the nucleus at 1 and 2 h (Figure 3(a)). Interestingly, this effect of 3OC12HSL on NF-κB p65 was reversible. Results from Western blot revealed that quantitative expression levels of nuclear NF-κB p65 were significantly elevated at 1 and 2 h, but returned to levels similar to that observed in controls at 4 h (Figure 3(b)). The most abundant form of NF-κB is a p50/p65 heterodimer, which associates with the NF-κB inhibitor α (IκBα). The phosphorylation level of p65 (p-p65) has been believed to be involved in the activation of innate immunity.[23] Therefore, we further explored the induction of p-p65 with stimulation of 3OC12HSL. The protein expression of phosphorylated NF-κB p65 was significantly increased after the cells were stimulated with 3OC12HSL for 1 h (Figure 3(c)). To better understand the activation of NF-κB in HCECs, we evaluated a potential role of Akt family kinases (also known as protein kinase B/PKB). When cells were stimulated with 3OC12HSL for 0, 1, 2, or 4 h, the active and phosphorylated forms of the Akt kinases were induced. Western blot showed that 3OC12HSL stimulation could activate p-Akt induction with increased expressions at 1 and 2 h (Figure 3(d)). These data showed that 3OC12HSL induced the transcription factor NF-kB activation in HCECs through the activation of an Akt kinase pathway.

3OC12HSL induced expressions of inflammatory cytokines in HCECs and a TLR2-dependent regulation of IL-8

An examination of 3OC12HSL on the production of inflammatory cytokines (IL-6, IL-8, IL-10, and TNF-α) in HCECs revealed that mRNA levels of IL-6 and IL-8 were significantly increased in response to 3OC12HSL, with maximal effects observed for IL-8 (Figure 4(a)). Addition of the NF-κB inhibitor BAY 11-7821, which blocked the activation of NF-κB, irreversibly inhibited the phosphorylation of

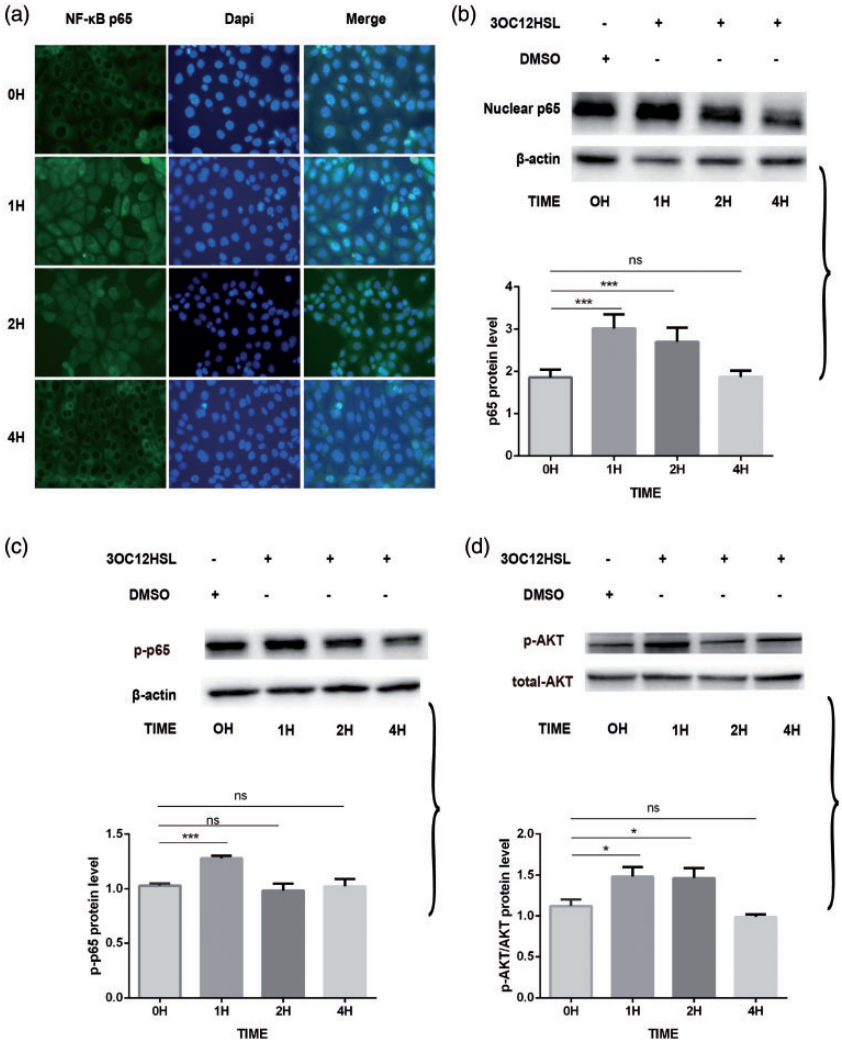

Figure 3. 3OC12HSL induced NF-κB activation in HCECs. (a) Nuclear translocation of NF-κB p65 in HCECs treated with 100 μmol/L 3OC12HSL for 0, 1, 2, or 4 h was observed with use of immunofluorescent staining. (b) Quantification of nuclear NF-κB p65 protein expression in HCECs treated with 100 μmol/L 3OC12HSL for 0, 1, 2, or 4 h was detected in a NF-κB p65 specific Western blot. (c) Quantification of phospho-NF-κB p65 protein expression in HCECs treated with 100 μmol/L 3OC12HSL for 0, 1, 2, or 4 h was detected in a p-p65 specific Western blot. (d) Western blot showed the protein levels of phosphorylation of Akt in HCECs treated with 100 μmol/L 3OC12HSL for 0, 1, 2, or 4 h and the quantification of p-Akt/Akt protein expression. Total cellular protein extracts were analyzed in p-Akt-specific Western blots. Then, the same blots were stripped and reanalyzed with antibodies against total Akt kinase. Data shown are the mean ± SEM; ns $P > 0.05$, *$P < 0.05$, ***$P < 0.001$. Representative results are from at least three independent experiments. (A color version of this figure is available in the online journal.)

NF-κB. When supernatants from NF-κB inhibitor pretreated cells were tested using specific ELISA assay kits, the induction of IL-8 protein was significantly reduced (Figure 4(b)), but there was no significant induction of IL-6 protein (Supplementary Figure S2). Therefore, IL-8 was selected for a more in-depth study of its expression in the supernatant as detected under different treatment conditions of 3OC12HSL. In response to different concentrations of 3OC12HSL (25, 50, 100, or 200 μmol/L for 12 h), significant increases in IL-8 protein levels were obtained in HCECs (Figure 4(c)). With regard to time-dependent effects, IL-8 protein levels were significantly elevated at 12 and 24 h following exposure to 100 μmol/L of 3OC12HSL (Figure 4(d)). The effect of TLRs on 3OC12HSL-induced IL-8 secretion was also investigated in HCECs pretreated with agonists or blocking antibodies of TLR 2, 4, 5, and 6 prior to 3OC12HSL stimulation. The TLR2 blocking antibody significantly decreased 3OC12HSL-induced IL-8 protein expression in supernatants of HCECs (Figure 4(e)). No obvious effects were obtained in response to treatments with TLR4 agonist or blocking antibody (Figure 4(f)). There were also no significant effects found when treated with TLR5 and TLR6 agonists or blocking antibodies (Supplementary Figure S3). To further evaluate the effects of TLR2 gene-silencing on the activation of NF-κB and secretion of inflammatory cytokine IL-8 in HCECs, cells were infected by lentivirus of shTLR2 or shCtrl (Figure 4(g)). The results showed that when TLR2 was effectively attenuated by TLR2 shRNA infection, the protein level of p-p65 in HCECs with 1 h of 100 μmol/L 3OC12HSL treatment decreased (Figure 4(h)), accompanied by a decreased protein level of cytokine IL-8 (Figure 4(i)). These results suggest that 3OC12HSL induced NF-κB activation and IL-8 expression through TLR2 signaling.

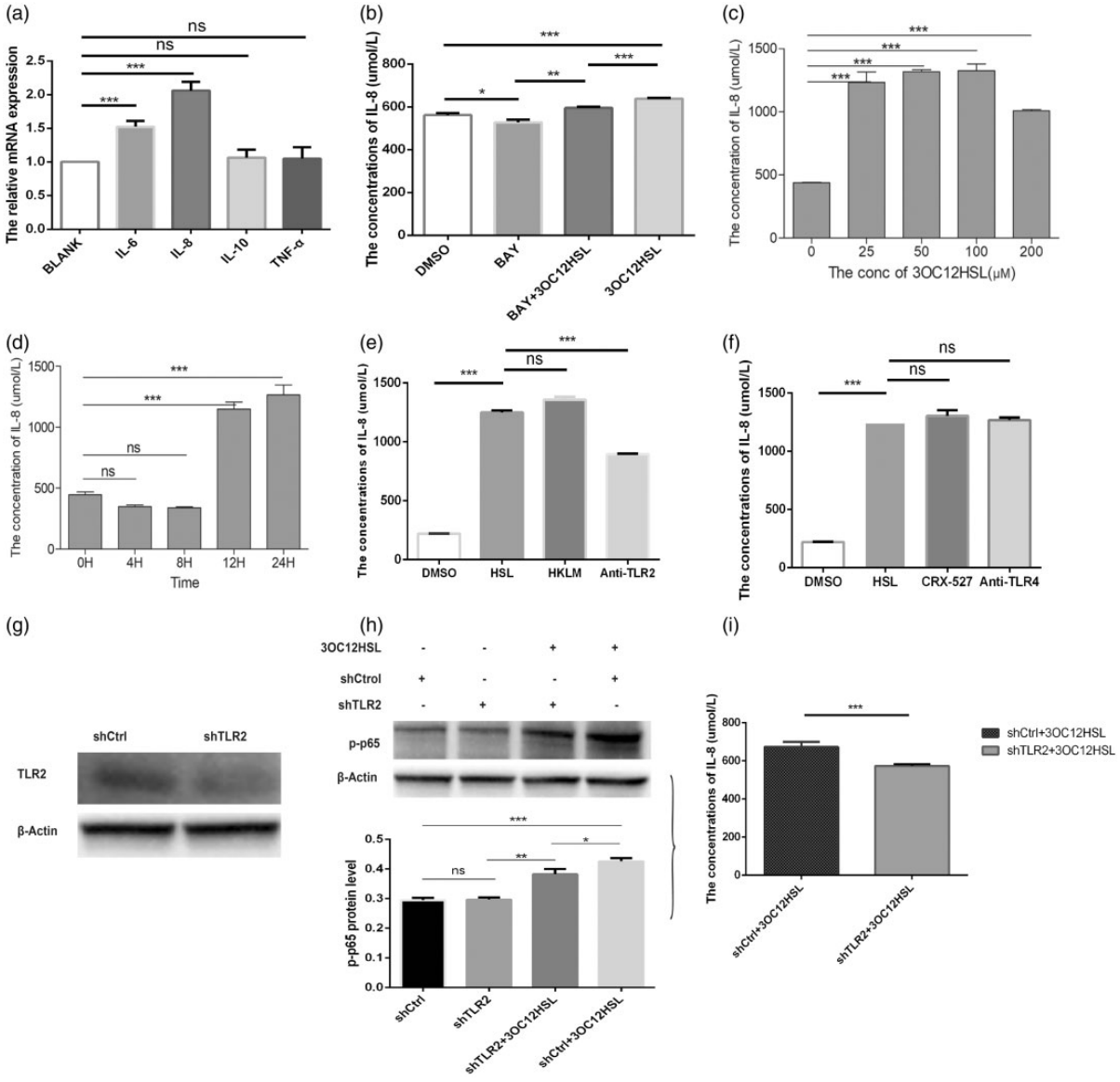

Figure 4. 3OC12HSL induced expressions of inflammatory cytokines in HCECs and a TLR2-dependent regulation of IL-8. (a) Effects of 3OC12HSL on IL-6, IL-8, IL-10, and TNF-α mRNA levels with use of RT-PCR. (b) Extracellular IL-8 detected by ELISA in specific NF-κB inhibitor (BAY 11-7821)-pretreated supernatants of HCECs after 24 h stimulation of 100 μmol/L 3OC12HSL. (c) Protein levels of IL-8 in supernatants of HCECs in response to a 12 h exposure of varying concentrations of 3OC12HSL (0 to 200 μmol/L). (d) Protein levels of IL-8 in supernatants of HCECs in response to treatment with 100 μmol/L 3OC12HSL for 0, 4, 8, 12, or 24 h. (e) IL-8 cytokine release was measured after 24 h stimulation of 100 μmol/L 3OC12HSL in HCECs pretreated with TLR2 agonist (HKLM) or TLR2 antagonist (PAb-hTLR2). (f) IL-8 cytokine release was measured after 24 h stimulation of 100 μmol/L 3OC12HSL in HCECs pretreated with TLR4 agonist (CRX-527) or TLR4 antagonist (PAb-hTLR4). (g) Immunoblot analysis of TLR2 expression in HCECs stably expressing shRNA against TLR2. (h) Effects of TLR2 gene-silencing on activation of NF-κB. Cells were infected with TLR2 shRNA or control shRNA and treated with 100 μmol/L 3OC12HSL for 1 h. (i) Effects of TLR2 gene-silencing on the secretion of IL-8 in HCECs. Cells were infected with TLR2 shRNA or control shRNA and treated with 100 μmol/L 3OC12HSL for 1 h. Data shown are the mean ± SEM; ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Representative results are from at least three independent experiments.

Discussion

The QS signaling-dependent extracellular virulence factors of *P. aeruginosa* initiate and maintain infection. They represent critical factors involved with *P. aeruginosa* keratitis through their capacity to invade and destroy corneal cells.[24] Local invasion is an important stage of *P. aeruginosa* corneal infection and, under intact/normal conditions, corneal epithelial cells appear to be resistant to *P. aeruginosa* infection. In this study, we found that 100 μmol/L 3OC12HSL significantly decreased HCEC viability, suggesting that high concentration of 3OC12HSL exerts a role in disrupting the integrity of corneal epithelial cells. However, the effective concentration of 3OC12HSL produced by *P. aeruginosa* on the ocular surface *in vivo* is still unclear, it may not be that high in the real case. It was reported that in the skin of mice model injected with 3OC12HSL, a concentration of 1 μ was sufficient to induce significant inflammation.[16,25] Since 3OC12HSL is a cell density-regulated molecule, the 3OC12HSL concentration changes based on the density of *P. aeruginosa* settled in the biofilm on the ocular surface.[14] *P. aeruginosa* has already established a prominent infection and cell death prior to

the production of significant amounts of 3OC12HSL. This ability for 3OC12HSL to induce the death of corneal epithelial cells may provide a means for entry of *P. aeruginosa* into the cornea and eventual corneal destruction.

Another important factor that contributes to corneal damage during *P. aeruginosa* keratitis is the activation of the host defense system. TLRs are a well-characterized class of PPRs, which are key components of the immune receptors. The classification of TLRs is dependent on whether they are found anchored within the cell membrane or located intracellularly.[26] It has been reported that HCECs express TLR4 and TLR5, and respond to bacteria and bacterial products.[27–29] TLR2 and TLR6 have also shown an essential role in bacterial virulence and clearance in microbial keratitis.[30] TLRs that respond to lipid agonists include TLR4 and TLR2, which form a heterodimer with TLR1 or TLR6, respectively.[31] As the *P. aeruginosa* QS signaling molecule, 3OC12HSL is a kind of lipid agonist and is critical in modulating host immune responses.[32–34] In our current study, we have focused on investigating cell membrane-associated TLRs (TLR 2, 4, 5, and 6). We observed that 3OC12HSL increased the levels of TLR2 and TLR4, but not TLR5 or TLR6, as compared to that observed in the DMSO control. Since TLRs are key components of the immune receptors that recognize the infectious agents, the first step for immune responses activation in HCECs is the recognition of 3OC12HSL by TLRs. The increased expressions of TLRs could induce 3OC12HSL to be recognized more sufficiently, thereby initiating stronger cellular immune responses and inducing the increase of inflammatory chemokines to clear pathogenic agents. The increased TLRs were able to interfere with HCECs signaling pathways, modulating cellular death and the secretion of inflammatory factors with cell death. These findings strongly support a role for TLR2 and TLR4 in the recognition of microbial structures. They also suggest that there is a selective activation of TLRs in response to 3OC12HSL, with TLR2 and TLR4 being more susceptible to 3OC12HSL stimulation in HCECs.

The NF-κB pathway plays a vital role in inflammation and innate immunity, and its activation is tightly regulated. Nuclear translocation of NF-κB represents the most important means of regulating its activity. Specifically, when cells are activated, NF-κB is released and transferred to the nucleus where it mediates multi-gene transcription.[35,36] There exists considerable evidence linking 3OC12HSL with NF-κB. For example, in mouse embryonic fibroblasts, 3OC12HSL increased the activation of NF-κB in a PERK-dependent manner and COX-2 production induced by 3OC12HSL was shown to be regulated by the activation of NF-κB in primary normal human lung fibroblasts.[37,38] Moreover, 3OC12HSL-induced IL-8 production was regulated by the activation of a mitogen-activated protein kinase (MAPK) pathway that subsequently lead to interactions of NF-κB within respiratory epithelial cells.[39,40] Here, we also demonstrate that NF-κB activation could be regulated by 3OC12HSL in HCECs. Specifically, we found that NF-κB shifted from the cytoplasm to the nucleus in HCECs at 1–2 h after treatment with 3OC12HSL, followed by a return to conditions that approached controls starting at 4 h post-treatment. This intracellular translocation of NF-κB represented the change in the phosphorylation of NF-κB. The phosphorylated NF-κB in the nucleus was degraded at 4 h in HCECs, thus the transcription of multiple genes which played pivotal roles in immune, inflammatory, and cell death would not be inducted. Moreover, when stimulated with 3OC12HSL, the active and phosphorylated forms of the Akt kinases in HCECs were induced. Although we have not shown the involvement of MAPK kinases in 3OC12HSL activation of HCECs, we do know that 3OC12HSL induces the transcription factor NF-kB activation and nuclear translocation in HCECs, and these inductions may be realized through the activation of an Akt kinase pathway.

P. aeruginosa keratitis is characterized by the recruitment of inflammatory cells, in particular neutrophils. This recruitment of neutrophils is probably due to the production of the chemokine, IL-8.[41–43] While an increase in IL-8 production leads to a large infiltration of neutrophils, which is essential for the elimination of *P. aeruginosa*, the persistence of these cells may eventually contribute to corneal destruction. In this study, we showed that the 3OC12HSL was also a potential inducer of IL-8 production in HCECs, as 3OC12HSL treatment induced a significant production in IL-8 mRNA and protein. *P. aeruginosa* infection leads to extensive production of IL-8 followed by neutrophil migration into the cornea, which results in acute inflammation and tissue destruction. Such a mechanism may then represent an important source of corneal damage in *P. aeruginosa* keratitis. Accordingly, an understanding of the secretory mechanisms of IL-8 represents a critical component of this disease progression. In this study, addition of a specific NF-κB inhibitor or the TLR2 blocking antibody to cultures, 3OC12HSL stimulation of IL-8 was inhibited. TLR2 knockdown could reduce 3OC12HSL-induced NF-κB activation and IL-8 production. When collating these results obtained from our study, it appears that 3OC12HSL induced IL-8 secretion via the TLR2/NF-κB pathway in HCECs. However, we mainly investigated the influence of 3OC12HSL on HCECs *in vitro*, the investigation of 3OC12HSL on the ocular surface in keratitis patients or mice model may better reveal the molecular mechanisms involved in 3OC12HSL triggered immune responses. A further study related to 3OC12HSL *in vivo* or 3OC12HSL-controlled gene regulation during corneal infection is warranted.

In conclusion, *P. aeruginosa* QS signaling molecule 3OC12HSL can be recognized by the host immune system in HCECs, followed by the trigger of an immune inflammatory response which involves the activation of TLR2 and an increase in activated NF-κB-dependent IL-8 expression. These findings indicate the importance of 3OC12HSL as an inflammatory signal in innate immune responses and targeted interventions against 3OC12HSL may indicate the potential to control corneal *P. aeruginosa* infection.

AUTHORS' CONTRIBUTIONS

RH and KY wrote the main manuscript. RH, YZ, and JZ performed the experiments. YZ and XH provided advice on the

discussion. XJ designed the research and provided suggestions on the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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