

The Role of Toll-Like Receptor 4 in Corneal Epithelial Wound Healing

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PURPOSE. We evaluated the role of Toll-like receptor 4 (TLR4) in corneal epithelial wound healing.

METHODS. The expression of TLR4 during in vivo corneal epithelial wound healing was examined by immunostaining in mice. The expression and activation of TLR4 was studied in primary or telomerase-immortalized human corneal epithelial cells (HCEC). Scratch assay was performed to evaluate in vitro wound closure using live time-lapse microscopy. Transwell migration assay and Ki67 immunostaining were done to evaluate migration and proliferation, respectively. Lipopolysaccharide (LPS) was used to activate TLR4, whereas CLI-095 was used for its inhibition. The expression of inflammatory cytokines was determined by RT-PCR and ELISA. The activation of p42/44 and p38 was determined by immunoblotting.

RESULTS. In the murine model, TLR4 immunostaining was noted prominently in the epithelium 8 hours after wounding. There was a 4-fold increase in the expression of TLR4 6 hours after in vitro scratch wounding ($P < 0.001$). Confocal microscopy confirmed the membrane localization of TLR4/MD2 complex. There was a significant increase in migration, proliferation, and wound closure in HCEC treated with LPS ($P < 0.05$), while there was significant decrease with TLR4 inhibition ($P < 0.05$). Addition of LPS to wounded HCEC resulted in a significant increase in the expression of IL-6, TNF- α , CXCL8/IL8, and CCL5/RANTES at the mRNA and protein levels. Likewise, LPS increased the activation of p42/44 and p38 in wounded HCEC.

CONCLUSIONS. These results suggest that epithelial wounding induces the expression of functional TLR4. Toll-like receptor 4 signaling appears to contribute to early corneal epithelial wound repair by enhancing migration and proliferation.

Keywords: corneal wound healing, corneal epithelium, innate immunity, Toll-like receptor 4, TLR4

The corneal epithelium protects the eye against pathogen invasion and has an essential role in preserving corneal clarity. Following injury to the corneal epithelium, the epithelial cells undergo a highly coordinated repair process, involving migration, proliferation, and differentiation, that is orchestrated in part by its interactions with the extracellular matrix, growth factors/cytokines, and the resident cells, including keratocytes and infiltrating leukocytes. The innate immune response has an essential role during wound healing not only to prevent infections, but also to restore structural and functional integrity to the damaged tissues.

The Toll-like receptor (TLR) family of pattern-recognition receptors is a part of the innate immune system and may be an important modulator of inflammation during the wound healing process. These receptors, which are expressed by professional immune cells as well as local resident cells, participate in a number of innate and adaptive immune responses that involve the activation of stress signaling pathways, such as nuclear factor kappa B (NF- κ B), mitogen-activated protein kinase (MAPK), and activator protein (AP)-1.¹⁻³ During wound healing, the activation of TLRs by their ligands leads to the initiation of signaling cascades, which result in the expression of proinflammatory cytokines, chemokines,

adhesion molecules, antimicrobial peptides, and proteolytic enzymes that all take part in a complex cross-talk during the wound healing process, especially during the inflammatory stage.³

There have been 13 TLRs identified in humans and mice, which respond to lipids, proteins, or nucleic acids derived from bacteria, viruses, or endogenous factors released at the site of injury.^{1,4,5} Toll-like receptor 4 has been studied extensively as the receptor for lipopolysaccharide (LPS); however, it also can interact with other endogenous ligands that modulate its response.^{6,7} Upon TLR4 activation in the cornea, CXC chemokines recruit neutrophils to the corneal stroma, where IL-6 and other proinflammatory cytokines stimulate neutrophil degranulation, causing localized tissue damage with loss of corneal transparency and visual impairment.⁸ Since epithelial cells are located at the interface of the host and the external environment, it is critical that their LPS responses are tightly regulated to prevent excessive inflammatory responses that can cause tissue damage.^{8,9} Therefore, regulatory mechanisms exist in the corneal epithelium to prevent unnecessary TLR4 activation. These include deficient expression of TLR4 cofactors, such as myeloid differentiation (MD)-2 and endosomal expression of TLR4 rather than epithelial cell membrane.⁸⁻¹¹

Previous studies on TLR4 signaling in the cornea have focused primarily on its role during infections.^{1,2} Until now, it remains obscure whether TLR4 activation contributes to epithelial repair directly; that is, independently of inflammatory cells and mediators. In the present study, we investigated changes in the expression of TLR4 and its downstream signaling molecules in response to injury *in vitro* and *in vivo*. The results suggested that injury to the corneal epithelium activates TLR4, which regulates inflammatory cytokine production in the injured corneal epithelium, and contributes to the epithelial repair by inducing cell migration and proliferation.

MATERIALS AND METHODS

Mouse Model of Corneal Epithelial Wound Healing

Corneal wounding experiments in mice were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Illinois at Chicago (Protocol Number, 11-183). Six-month-old C57BL/6J mice were anesthetized with intraperitoneal injection of ketamine and xylazine. After applying topical 0.5% proparacaine, a 1.5-mm area of the central epithelium was demarcated and removed by gentle scraping using a blunt corneal scraper as described previously.¹² The eyes were enucleated at 0, 2, 4, 6, 8, 12, 24, and 48 hours.

Corneal Epithelial Cell Culture

Human corneal epithelial cell (HCEC) cultures were initiated from cadaver corneas kindly provided by the Illinois (Chicago, IL, USA) and Midwest Eye Banks (Ann Arbor, MI, USA). The 1.5-mm limbal rings were treated with Dispase (2 mg/mL; Gibco, Grand Island, NY, USA) at 37°C for 2 hours to separate the epithelial sheets, then digested in 0.25% trypsin-EDTA for 5 to 10 minutes. Cells were washed and resuspended in keratinocyte serum-free medium (KSFM; Invitrogen, Grand Island, NY, USA) and plated in collagen-coated tissue culture plates. Cells from passage 2 to 3 were used for experiments. In addition to primary corneal epithelial cells, human corneal-limbal epithelial (HCLE) cell line (telomerase-immortalized human corneal epithelial cell line kindly provided by Ilene Gipson, PhD) was used for some of the experiments. The HCLE cells were grown in KSFM.

Immunofluorescence Staining and Microscopy

Mouse corneal sections or primary corneal epithelial cells grown on the chamber slides were stained according to previously described protocols.¹³ Briefly, cells grown on chamber slides were fixed in 10% paraformaldehyde for 20 minutes. After three 5-minute washes with PBS, the cells/tissues were incubated with 5% normal donkey/goat serum in PBS containing 1% BSA for 1 hour at room temperature to block nonspecific binding. The cells then were incubated overnight at 4°C with the corresponding primary antibodies at optimal dilutions in corresponding blocking solution. The primary antibodies included rabbit anti-Ki67 (Abcam, Cambridge, MA, USA), rat anti-mouse TLR4/MD2 (MTS510; BioLegend, San Diego, CA, USA), and mouse anti-human TLR4/MD2 (HTA125; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). After three 5-minute washes with PBS, cells were incubated with the corresponding FITC-conjugated secondary antibody for 1 hour at room temperature, then washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted. Negative controls were stained in a similar fashion

using an irrelevant antibody to exclude nonspecific staining. The slides were visualized and photographed using a Zeiss LSM 710 microscope (Carl Zeiss, Jena, Germany).

Western Blots

Western blots were performed as described previously.¹³ Briefly, cells cultured on 6-well dishes were rinsed twice with PBS and harvested in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease/phosphatase inhibitors for total protein extraction. After measuring protein concentration (BCA assay; Pierce, Thermo Scientific, Rockford, IL, USA), equal amounts of each sample were mixed with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), denatured by heating at 70°C for 10 minutes, and subjected to electrophoresis on 4% to 20% Tris-Glycine gels (Invitrogen). The proteins were transferred to polyvinylidene fluoride (PVDF) membranes by iBlot gel transfer (Invitrogen). The blots then were incubated in 1% casein in TBST for 1 hour or 1% BSA in tris-buffered saline with Tween (TBST) followed by an overnight incubation (4°C) with primary antibodies at the optimal concentration. The membranes were washed with TBS with 0.03% Tween 20 and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Detection was performed with ECL-Plus Western Blotting Detection System (Amersham, Buckinghamshire, UK). The following antibodies were used: rabbit anti-human TLR4 (H80, dilution 1:1000; Santa Cruz Biotechnology, Inc.), rabbit anti- β -Actin (1:10,000; Cell Signaling Technology, Danvers, MA, USA), and rabbit monoclonal IgG to p44/42 MAPK, phospho-p44/42 MAPK, p38 MAPK, and phospho-p38 MAPK (MAPK assay kit; Cell Signaling Technology). Detection was performed by ImageQuant LAS 1040 detection system and quantified using ImageQuant software (both from GE Healthcare, Piscataway, NJ, USA).

Reverse-Transcriptase (RT) Polymerase-Chain Reaction

The RNA isolation from primary corneal epithelial cell cultures was performed as described previously.¹⁴ The RT reaction was likewise completed as before, followed by PCR with intron spanning primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_001256799), TLR4 (NM_003266), IL-6 (NM_000600), TNF- α (NM_000594), and CCL5/RANTES (NM_002985). These primers were chosen to eliminate genomic DNA contamination.

Cytokine Analysis

Primary corneal epithelial cells were grown to confluence in 6-well plates. The cells were growth factor-starved and the calcium concentration of the media was increased to 1.8 mM overnight. Two linear scratches were made perpendicular to each other using a sterile 200- μ L pipette tip and the wells were washed three times with PBS. Six hours after the scratch, the wells were washed three times and then 100 ng/mL ultrapure LPS (InvivoGen, San Diego, CA, USA) was added to the cultures for 1 hour (ultrapure LPS was used to minimize activation of other TLRs, which can occur with nonpurified LPS). After 12 hours, the supernatant from each well was collected and frozen at -80°C until the time of analysis. For the analysis, the samples were thawed on ice and then analyzed for selected cytokines using Multi-Analyte ELISArray Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and the absorbance at 450 nm was read within 30 minutes of stopping the reaction using plate reader (GENios plate reader; Tecan, Salzburg, Austria).

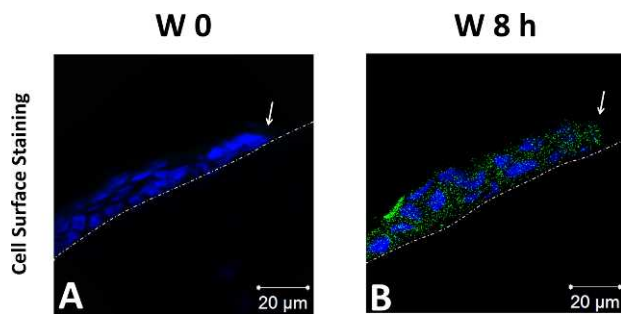


FIGURE 1. The expression of TLR4/MD2 complex was evaluated in the mouse corneal epithelium at 8 hours after a debridement wound. The expression of TLR4/MD2 complex is increased in the wounded epithelium at 8 hours (B) compared to 0 hours after wounding (A). Arrow indicates the wound edge in (A) and the leading edge in (B). Green, FITC; blue, DAPI.

In Vitro Live Migration Assay

The HCLE cells were grown to confluence on chamber slides. The cells were growth factor-starved and the calcium was increased to 1.8 mM overnight. A linear scratch was made using a sterile 200- μ L pipette tip and the wells were washed three times with PBS. Six hours after the scratch, 100 ng/mL ultrapure LPS was added to the cultures. To inhibit TLR4 signaling, the cells were pretreated with 5 μ M CLI-095 (InvivoGen) for 1 hour before and immediately after the scratch. The scratch area was captured serially using a spinning disc confocal microscope (Z1; Carl Zeiss), and photographed with an AxioCam camera (Carl Zeiss). The remaining wound area (RWA) was measured using ImageJ software (available in the public domain at www.nih.gov; National Institutes of Health [NIH], Bethesda, MD, USA).

Transwell Migration Assay

Six hours after wounding, HCLE cells were trypsinized, washed, and plated in 8.0- μ m pore size transwell inserts (Beckton Dickinson, Downers Grove, IL, USA) in serum-free Dulbecco's modified Eagle's medium (DMEM). The lower compartment was filled with DMEM, or DMEM plus either 100 ng/mL LPS or 5 μ M CLI-095 (after 1 hour pretreatment). After 12 hours, the cells on the upper side of the insert were removed by scraping and the cells that had migrated through were fixed on the lower side of the membrane with 10% formalin, then stained with crystal violet and quantified by counting the number of cells in 10 separate fields. The data were expressed as the number of migrated cells per micrograph field for each sample well.

Proliferation Assay

The effect of TLR4 activation on in vitro proliferation was assessed by immunofluorescence staining for the proliferation marker, Ki67. Primary human cells were plated in equal numbers in 4-well chamber slides and grown to confluence. The growth factors were eliminated from the media and calcium was increased to 1.8 mM. A scratch was made using a sterile 200- μ L pipette tip and the wells were washed three times with PBS. Six hours after the scratch, 100 ng/mL LPS was added to the cultures. To inhibit TLR4 signaling, the cells were pretreated with 5 μ M CLI-095 for 1 hour before the scratch was made. The cells were incubated for 14 hours in 37°C then fixed and stained for Ki67 as described above.

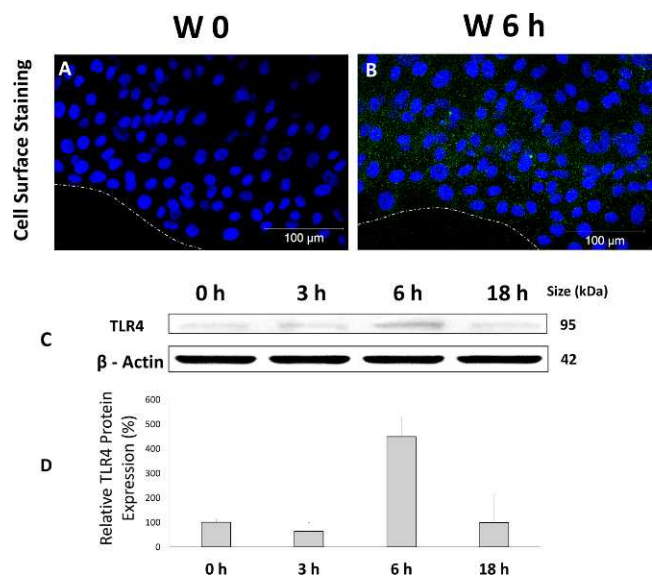


FIGURE 2. Primary HCEC were stained (without permeabilization) for TLR4/MD2 complex 6 hours after scratch wounding. Representative confocal images depicting the cells are distinctly positive for TLR4/MD2 complex staining (B) compared to 0 hour after wounding which is mostly negative (A). Green, FITC; blue, DAPI. Dotted lines: scratch wound edge. Primary HCEC were wounded and total lysates were collected in different time-points (C). Western blot analysis demonstrates TLR4 expression increased 6 hours after wounding compared to wound 0 (Chart [D]); $*P < 0.001$) and later returned to the baseline by 18 hours after wounding. Data shown are representative of three independent experiments. Data were normalized to β -actin as housekeeping protein. Error bars: standard deviation.

Statistical Analysis

Each experiment was replicated at least three times. Values were displayed as mean \pm SD. Comparisons between groups were analyzed by unpaired *t*-test (Microsoft Excel; Microsoft Corporation, Redmond, WA, USA). Results were considered statistically significant for $P < 0.05$.

RESULTS

Corneal Epithelial Injury Induces the Expression of TLR4

The expression of TLR4/MD2 complex was first examined in vivo in a murine corneal epithelial debridement model. The TLR4/MD2 complex was expressed prominently 8 hours after wounding (Fig. 1B) compared to 0 hours after wounding (Fig. 1A).

Likewise, after wounding in vitro, the expression of TLR4/MD2 complex was most prominent at 6 hours (Fig. 2B) compared to 0 hours, where only minimal staining was observed (Fig. 2A). The TLR4 expression was increased similarly in primary corneal epithelial cells after scratch wounding by Western blot. The peak expression of TLR4 was observed at 6 hours with return to baseline by 18 hours (Figs. 2C, 2D). Together, these results indicated that injury to the epithelium induces early expression of TLR4.

TLR4 After Wounding Is Responsive to its Ligand LPS

To test if TLR4 is functional after wounding, we challenged primary HCEC with a 1-hour exposure to 100 ng/mL ultrapure

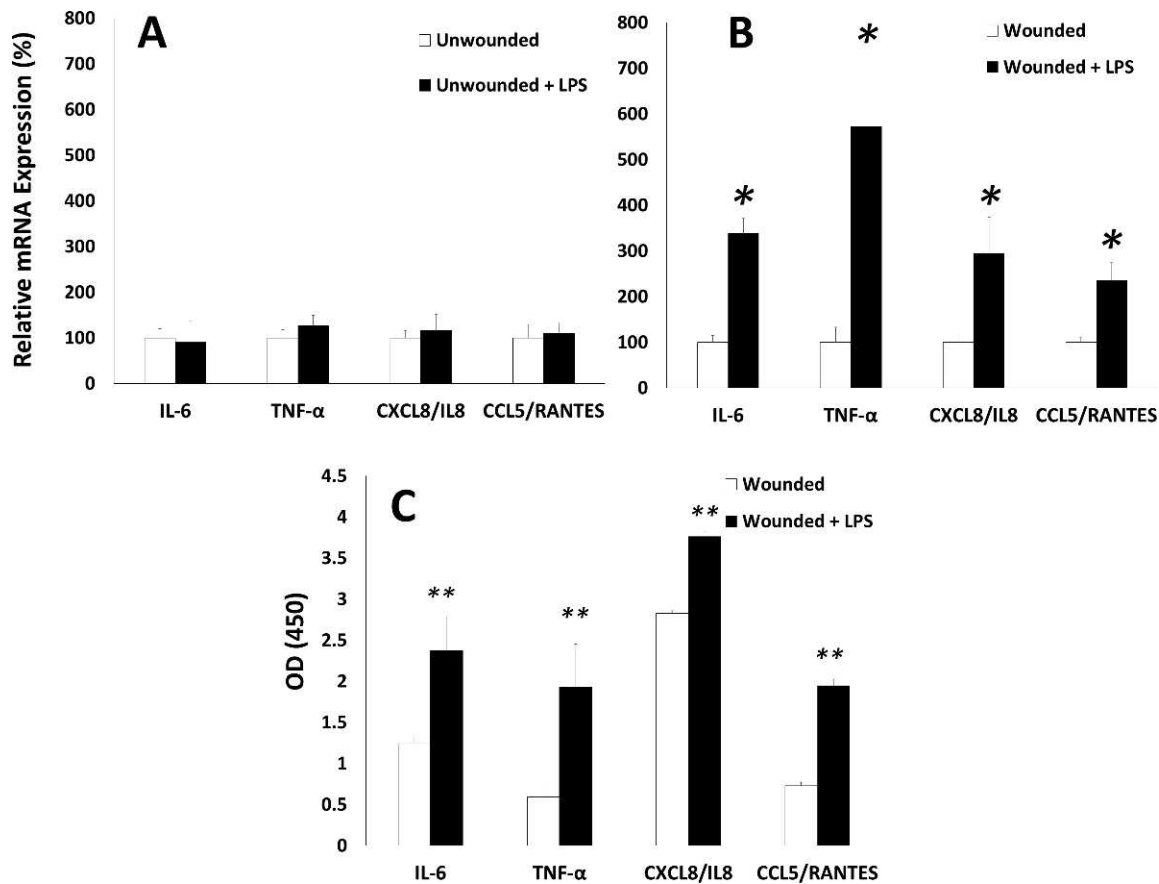


FIGURE 3. To evaluate if the TLR4 is responsive after wounding, primary HCEC were challenged with 100 ng/mL ultrapure LPS, and mRNA expression of IL-6, TNF- α , CXCL8/IL8, and CCL5/RANTES were evaluated in different time-points. There was no significant difference in cytokine expression between unwounded and unwounded + LPS (Chart [A]; $P > 0.05$). However, there was a significant increase in the cytokine expression in the cells that were challenged with ultrapure LPS at 6 hours after wounding compared to corresponding wounded cells not exposed to LPS (Chart [B]; $*P < 0.001$). Relative mRNA expression was calculated by normalization of each band signal intensity to GAPDH as a housekeeping gene. The level of each normalized sample was reported as its ratio relative to control. (C) Cytokine production by wounded primary corneal epithelial cell after being challenged with 100 ng/mL LPS. Cell culture supernatants from either wounded cells or wounded + LPS were analyzed for selected cytokine production. The x-axis represents individual cytokines tested. The y-axis represents average absorbance values at 450 nm ($**P < 0.01$). Data shown are representative of three independent experiments. OD, optical density. Error bars: standard deviation.

LPS. As shown by others,^{8-11,15-17} unwounded primary HCEC were unresponsive to ultrapure LPS with minimal induction of IL-6, TNF- α , CXCL8/IL8, and CCL5/RANTES compared to control (Fig. 3A; $P > 0.05$). In contrast, wounded epithelial cells demonstrated a significant increase in cytokine expression after being challenged with ultrapure LPS compared to control wounded cells not exposed to LPS ($P < 0.001$; Figs. 3B-D). These results indicated that the TLR4 expressed by epithelial cells after wounding is functional and responsive to LPS.

TLR4 Activation Accelerates Corneal Epithelial Wound Healing

The HCEC were exposed to either ultrapure LPS or CLI-095 and subjected to a scratch assay as well as transwell migration assay. The results indicated that 100 ng/mL ultrapure LPS accelerated scratch wound closure, while 5 μ m CLI-095 had an inhibitory effect (Fig. 4). Since wound closure in a scratch assay can be due to increased migration and/or proliferation, a transwell migration assay was used to evaluate migration more specifically. The results similarly indicated that LPS accelerated migration (Fig. 5A). On proliferation assay, primary HCEC also demonstrated a significant increase in the rate of proliferation

with LPS, while CLI-095 inhibited the rate of proliferation slightly, but significantly (Fig. 5B). Therefore, it was concluded that activation of TLR4 by LPS leads to an increase in migration and proliferation of wounded HCEC.

Activation of TLR4 After Wounding in Part Augments MAPK Signaling

The MAPK pathway is one of the important downstream signals that are activated by TLR4 signaling.¹ Previous studies have demonstrated the critical role of the MAPK pathway, particularly ERK and p38, in corneal epithelial wound healing.¹⁸⁻²² To evaluate specifically the effect of TLR4 activation on the MAPK pathway, primary HCEC were exposed to 100 ng/mL LPS after wounding and subjected to Western blotting. The results indicated that LPS increased the phosphorylation (activation) of ERK at 30 minutes after challenge ($P < 0.001$), which was reduced, but still above baseline, after 1 hour ($P < 0.05$; Fig. 6, Chart A). Likewise, the phosphorylation of p38 increased at 15 minutes after challenge ($P < 0.001$) with return to baseline after 1 hour (Fig. 6, Chart B). These results highlighted one potential mechanism by which TLR4 signaling may be modulating the epithelial wound healing response.

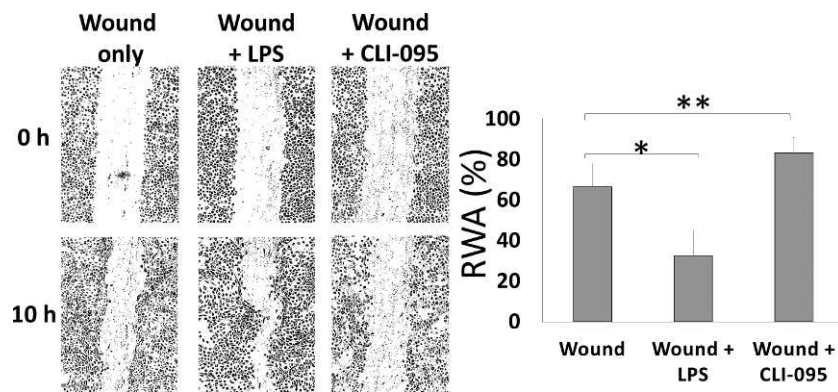


FIGURE 4. The effect of TLR4 exogenous ligand or its internal inhibitor on scratch assay wound closure rate was measured. Mechanically wounded HCLE cells were exposed to either 100 ng/mL ultrapure LPS or pretreated with 5 μ M CLI-095. Wound repair was assessed by measuring the remaining wound area (RWA) (* P < 0.01, ** P < 0.05). Data shown are representative of three independent experiments. Error bars: standard deviation.

DISCUSSION

Corneal epithelial cells provide an important physical barrier between the external and internal environments. Following an injury, the corneal epithelial defect must be resurfaced rapidly to avoid microbial infection and prevent further damage to the underlying stroma. Epithelial healing is achieved by migration of the epithelial sheet to cover the denuded surface and enhanced cell proliferation to reestablish epithelial stratification after resurfacing.¹⁹ The mechanisms underlying the complex and multistage wound-healing process are not yet completely understood. Innate immune signals, including TLRs, have an important role in tissue injury and repair. Importantly, the ligands for these receptors are many pathogen-associated molecular patterns (PAMPs), which are present in wounds because of the normal bacterial flora. Likewise, endogenous TLR ligands, the damage-associated molecular patterns (DAMPs), are present at the wound site as a result of tissue injury.²

The TLR4 receptor recognizes the gram-negative bacterial cell wall component LPS in association with cofactors, such as CD14, LBP, and MD-2.^{8,11,16,17} The combined activity of TLR4 accessory molecules facilitates recognition of LPS at picomolar concentrations.⁶ The TLR4 receptor also is an important mediator of immune responsiveness not just in response to

LPS, but also a variety of endogenous ligands, including calprotectins (S100 A8 and A9), heat shock proteins, high-mobility group box 1 (HMGB1), and extracellular matrix degradation products, including fibronectin, hyaluronic acid, and heparan sulfate.²³⁻²⁹ Expression of TLR4 was shown to increase in the early stages of murine skin wound healing, while TLR4 knockout mice had delayed wound healing, suggesting that TLR4 is an important regulator of wound healing and inflammation.³⁰ In our murine corneal epithelial injury model, increased epithelial expression of TLR4 was noted as early as 2 hours after wounding with gradual return to baseline by 24 hours.

Previous studies have shown that HCEC respond to TLR2,^{31,32} TLR3,^{33,34} and TLR5 ligands.³⁵ However, their response to TLR4 ligand normally is blunted. A similar response pattern has been shown in several mucosal epithelial cells (intestinal, airway, oral, and conjunctival), which continuously interact with commensal bacteria, whereby LPS responsiveness is minimized under homeostatic conditions.^{36,37} In particular, since the ocular surface bacterial flora includes gram-positive and -negative bacteria,^{9,38,39} it seems prudent for the corneal epithelial cells generally not to respond to such flora. However, upon injury, it is critical for the epithelium, as the first line of exposure to the outside

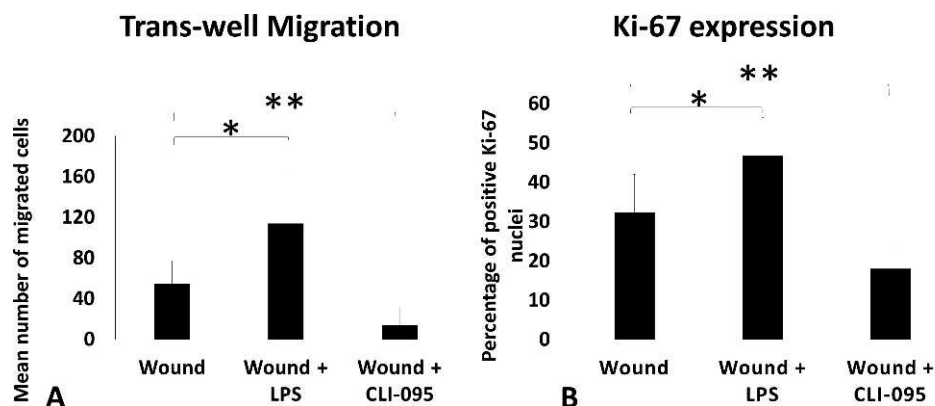


FIGURE 5. Wounded HCLE cells were trypsinized and plated in 8.0- μ m pore size transwells. Then, DMEM, LPS, or CLI-095 were added to the lower compartment. The data are expressed as mean number of migrated cells per micrograph field for each sample well. * P = 0.001, ** P < 0.001 (A). Primary HCEC were wounded and treated either with LPS or pretreated with CLI-095. At 14 hours after wounding, they were subjected to Ki67 proliferation marker staining and the percentage of Ki67-positive cell nuclei was quantified (* P < 0.001, ** P < 0.05) (B). Data shown are representative of three independent experiments. Error bars: standard deviation.

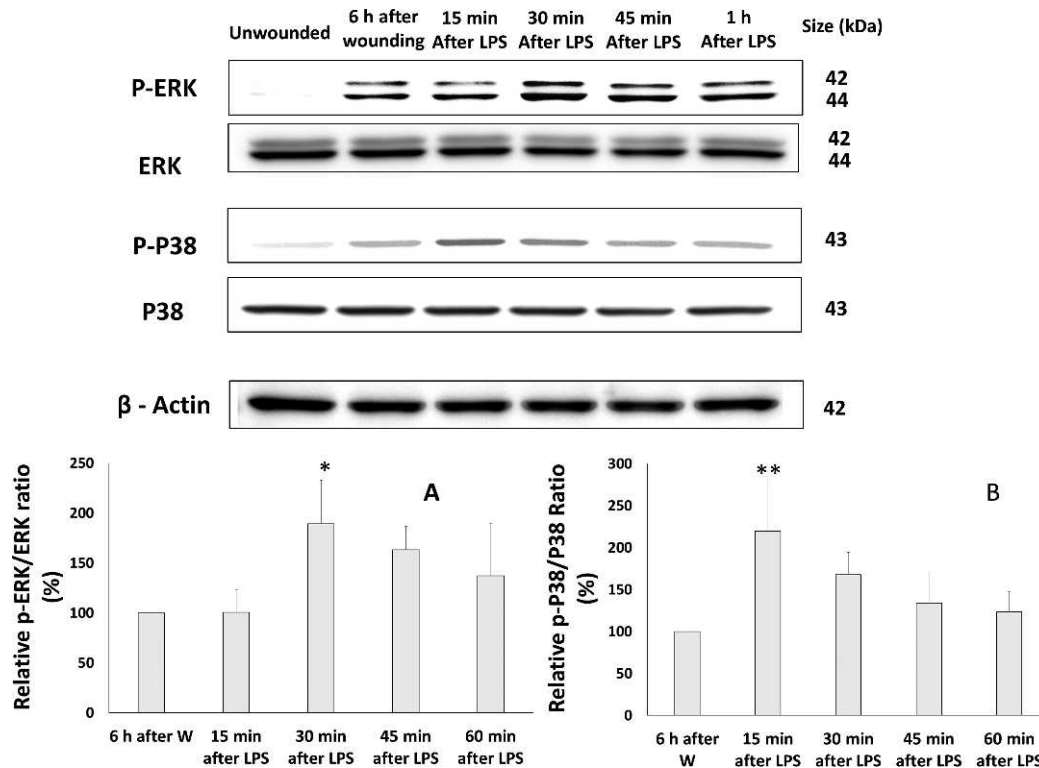


FIGURE 6. Primary HCEC were wounded and then challenged with LPS. The ratio of phospho-ERK/ERK increased 6 hours after wounding compared to unwounded cells. The ratio of phospho-ERK/ERK increased at 30 minutes after adding LPS and remained above baseline after 1 hour (Chart [A]; * $P < 0.01$). Likewise, phospho-P38/P38 increased after wounding compared to unwounded cells. After adding LPS to the 6-hour wounded cells, this ratio increased within 15 minutes (** $P < 0.001$) and returned to baseline after 1 hour (Chart [B]). Data shown are representative of three independent experiments. All values are normalized to co-stained β -actin signal intensity in each blot. *Error bars*: standard deviation.

environment, to become more responsive to danger signals and respond by enhancing the wound healing process.

This dampened TLR4 response in corneal epithelial cells has been shown to be due partly to the lack of expression of the MD-2^{8,11,17} and also to the intracellular localization of TLR4.^{9,10} It has been reported, however, that corneal epithelial cells can respond directly to LPS following infectious and inflammatory conditions in vivo, perhaps due to interactions with bone marrow-derived (e.g., macrophages, natural killer, and dendritic cells) or nonmyeloid cells in the vicinity.^{2,17} Likewise, Blais et al.¹⁶ have shown that human lacrimal glands express MD-2, indicating that tears may be a source of exogenous MD-2. In addition, MD-2 expression also can be induced by IFN- γ in conjunctival and corneal epithelial cells.^{1,40}

In this study, we evaluated the expression of TLR4/MD2 complex over the cell surface, which clearly showed the complex to be present after wounding in vivo and in vitro. Likewise, our cytokine studies confirmed that TLR4 is functional and responds to LPS. All TLRs (with the exception of TLR3) signal through the TIR domain to recruit the MyD88 common adaptor molecule, leading to NF- κ B translocation to the nucleus, and resulting in expression of proinflammatory cytokines and CXC chemokines that recruit neutrophils.^{1,41} In addition to the MyD88 pathway, TLR3 and TLR4 activate the TRIF pathway (non-MyD88 pathway), which induces IRF3 transcription, and production of type I interferons and CCL5/RANTES.^{1,2} Thus, TLR4 activates cells through two independent signaling pathways, leading to a diverse and amplified response.^{1,17} In our study, challenging wounded epithelial cells, which have upregulated TLR4, with ultrapure LPS resulted in an increase in IL-6, TNF- α , and CXCL8/IL8

(MyD88 pathway) and CCL5/RANTES (non-MyD88 pathway) cytokines suggesting that both pathways are likely activated upon stimulation in corneal epithelial cells. In addition to increased cytokine expression, we also demonstrated enhanced phosphorylation of the MAPKs ERK1/2 and p38 after TLR4 activation in wounded epithelial cells. The activation of MAPKs not only contributes to the inflammatory cytokine production, but also can directly enhance epithelial migration and proliferation as shown by others.^{18–22}

Clinically, it is well known that sterile corneal inflammation can occur in the absence of culturable bacteria, and can result in significant discomfort and visual impairment, most commonly in the setting of contact lens wear.² The etiology of these contact lens-induced inflammatory responses is thought to be bacterial products, particularly gram-negatives.² Based on our results, one might speculate that microtrauma may upregulate TLR4 responsiveness in corneal epithelial cells, which, in turn, may respond to dead bacteria or bacterial products associated with contact lens wear by releasing proinflammatory and chemotactic cytokines resulting in neutrophil recruitment to the corneal stroma—although, the role of other resident cells and other TLRs cannot be overlooked.² Previous animal studies have shown that LPS-induced keratitis only occurs if the corneal epithelium is abraded beforehand.^{42–44} This is thought to be largely because LPS can now reach the TLR4-responsive immune cells within the stroma. However, based on the findings of this study, it may also be due to increased TLR4 responsiveness in epithelial cells after wounding.

In our study, TLR4/MD2 complex expression returned to baseline by 24 hours after wounding in vivo. It has been shown that prolonged TLR4 expression and activation contributes to

the inflammation and impaired skin wound healing seen in patients with diabetes mellitus and chronic venous leg ulcers.^{45,46} Likewise, there is clear association between prolonged and unregulated TLR4 expression and activation in inflammatory bowel diseases.⁴⁷ Further studies are needed to determine whether misregulated expression of TLR4 is a contributing factor to inflammation and impaired wound healing in patients with ocular surface disease. Thus, modulating TLR4 signaling may provide a therapeutic approach for wound healing disorders of the epithelium.

In this study, we only evaluated one member of the TLR family. Previously, activation of TLR5 by bacterial flagellin, was found to induce epithelial migration and wound closure in an ex vivo model.⁴⁸ The role of other TLRs in corneal epithelial wound healing needs further investigation.

In conclusion, our study provides evidence that TLR4 activation can directly induce repair-related processes in corneal epithelial cells independently from the immune cells. Therefore, increased TLR4 expression after wounding acts like an alarm system, which upon activation accelerates wound closure and enhances innate immune responses. At this time, it is unclear to what extent TLR4 is activated by LPS released from the ocular flora versus endogenous ligands (DAMPs). Nonetheless, our results suggested that small amounts of bacteria on the ocular surface may be beneficial to wound repair as shown in other tissues.^{3,49,50} This further suggests that overly aggressive administration of antibiotics may have an inhibitory effect on wound healing by suppressing the normal flora. Further studies are needed to elucidate the role of the ocular surface microbiome and TLR signaling in normal and pathologic corneal epithelial wound healing.

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References

- Pearlman E, Johnson A, Adhikary G, et al. Toll-like receptors at the ocular surface. *Ocul Surf*. 2008;6:108-116.
- Pearlman E, Sun Y, Roy S, et al. Host defense at the ocular surface. *Int Rev Immunol*. 2013;32:4-18.
- Kostarnoy AV, Gancheva PG, Logunov DY, et al. Topical bacterial lipopolysaccharide application affects inflammatory response and promotes wound healing. *J Interferon Cytokine Res*. 2013;33:514-522.
- Barabino S, Chen Y, Chauhan S, Dana R. Ocular surface immunity: homeostatic mechanisms and their disruption in dry eye disease. *Prog Retin Eye Res*. 2012;31:271-285.
- Redfern RL, McDermott AM. Toll-like receptors in ocular surface disease. *Exp Eye Res*. 2010;90:679-687.
- Gioannini TL, Teghanemt A, Zhang D, et al. Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A*. 2004;101:4186-4191.
- Sun Y, Pearlman E. Inhibition of corneal inflammation by the TLR4 antagonist Eritoran tetrasodium (E5564). *Invest Ophthalmol Vis Sci*. 2009;50:1247-1254.
- Roy S, Sun Y, Pearlman E. Interferon-gamma-induced MD-2 protein expression and lipopolysaccharide (LPS) responsiveness in corneal epithelial cells is mediated by Janus tyrosine kinase-2 activation and direct binding of STAT1 protein to the MD-2 promoter. *J Biol Chem*. 2011;286:23753-23762.
- Ueta M, Nochi T, Jang MH, et al. Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. *J Immunol*. 2004;173:3337-3347.
- Lee HS, Hattori T, Park EY, Stevenson W, Chauhan SK, Dana R. Expression of toll-like receptor 4 contributes to corneal inflammation in experimental dry eye disease. *Invest Ophthalmol Vis Sci*. 2012;53:5632-5640.
- Zhang J, Kumar A, Wheeler M, Yu FS. Lack of MD-2 expression in human corneal epithelial cells is an underlying mechanism of lipopolysaccharide (LPS) unresponsiveness. *Immunol Cell Biol*. 2009;87:141-148.
- Movahedan A, Majdi M, Afsharkhamsheh N, et al. Notch inhibition during corneal epithelial wound healing promotes migration. *Invest Ophthalmol Vis Sci*. 2012;53:7476-7483.
- Movahedan A, Afsharkhamsheh N, Sagha HM, et al. Loss of Notch1 disrupts the barrier repair in the corneal epithelium. *PLoS One*. 2013;8:e69113.
- Djalilian AR, Namavari A, Ito A, et al. Down-regulation of Notch signaling during corneal epithelial proliferation. *Mol Vis*. 2008;14:1041-1049.
- Shaykhiev R, Behr J, Bals R. Microbial patterns signaling via Toll-like receptors 2 and 5 contribute to epithelial repair, growth and survival. *PLoS One*. 2008;3:e1393.
- Blais DR, Vascotto SG, Griffith M, Altosaar I. LBP and CD14 secreted in tears by the lacrimal glands modulate the LPS response of corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2005;46:4235-4244.
- Roy S, Karmakar M, Pearlman E. CD14 mediates Toll-like receptor 4 (TLR4) endocytosis and spleen tyrosine kinase (Syk) and interferon regulatory transcription factor 3 (IRF3) activation in epithelial cells and impairs neutrophil infiltration and *Pseudomonas aeruginosa* killing in vivo. *J Biol Chem*. 2014;289:1174-1182.
- Teranishi S, Kimura K, Nishida T. Role of formation of an ERK-FAK-paxillin complex in migration of human corneal epithelial cells during wound closure in vitro. *Invest Ophthalmol Vis Sci*. 2009;50:5646-5652.
- Saika S, Okada Y, Miyamoto T, et al. Role of p38 MAP kinase in regulation of cell migration and proliferation in healing corneal epithelium. *Invest Ophthalmol Vis Sci*. 2004;45:100-109.
- Nishimoto S, Nishida E. MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep*. 2006;7:782-786.
- Sharma GD, He J, Bazan HE. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. *J Biol Chem*. 2003;278:21989-21997.
- Imayasu M, Shimada S. Phosphorylation of MAP kinase in corneal epithelial cells during wound healing. *Curr Eye Res*. 2003;27:133-141.
- Yun TJ, Harning EK, Giza K, et al. EC144, a synthetic inhibitor of heat shock protein 90, blocks innate and adaptive immune responses in models of inflammation and autoimmunity. *J Immunol*. 2011;186:563-575.
- Wu H, Chen G, Wyburn KR, et al. TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest*. 2007;117:2847-2859.
- Vogl T, Tenbrock K, Ludwig S, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med*. 2007;13:1042-1049.

26. Uchida K. Redox-derived damage-associated molecular patterns: ligand function of lipid peroxidation adducts. *Redox Biol.* 2013;1:94-96.
27. Keyel PA. How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1. *Cytokine.* 2014;69:136-145.
28. Ellson CD, Dunmore R, Hogaboam CM, Sleeman MA, Murray LA. DAMPs and danger signals in IPF. *Am J Resp Cell Mol Biol.* 2014;51:163-168.
29. Sun Y, Lu Y, Engeland CG, Gordon SC, Sroussi HY. The anti-oxidative, anti-inflammatory, and protective effect of S100A8 in endotoxemic mice. *Mol Immunol.* 2013;53:443-449.
30. Chen L, Guo S, Ranzer MJ, Dipietro LA. Toll-like receptor 4 has an essential role in early skin wound healing. *J Invest Dermatol.* 2014;134:583.
31. Kumar A, Zhang J, Yu FS. Toll-like receptor 2-mediated expression of beta-defensin-2 in human corneal epithelial cells. *Microbes Infect.* 2006;8:380-389.
32. Adhikary G, Sun Y, Pearlman E. C-Jun NH2 terminal kinase (JNK) is an essential mediator of Toll-like receptor 2-induced corneal inflammation. *J Leukoc Biol.* 2008;83:991-997.
33. Johnson AC, Li X, Pearlman E. MyD88 functions as a negative regulator of TLR3/TRIF-induced corneal inflammation by inhibiting activation of c-Jun N-terminal kinase. *J Biol Chem.* 2008;283:3988-3996.
34. Ueta M, Hamuro J, Kiyono H, Kinoshita S. Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines. *Biochem Biophys Res Commun.* 2005;331:285-294.
35. Zhang J, Xu K, Ambati B, Yu FS. Toll-like receptor 5-mediated corneal epithelial inflammatory responses to *Pseudomonas aeruginosa* flagellin. *Invest Ophthalmol Vis Sci.* 2003;44:4247-4254.
36. Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med.* 2013;45:e66.
37. Beutler B. Endotoxin, toll-like receptor 4, and the afferent limb of innate immunity. *Curr Opin Microbiol.* 2000;3:23-28.
38. Hara J, Yasuda F, Higashitsutsumi M. Preoperative disinfection of the conjunctival sac in cataract surgery. *Ophthalmologica.* 1997;211(suppl 1):62-67.
39. Doyle A, Beigi B, Early A, Blake A, Eustace P, Hone R. Adherence of bacteria to intraocular lenses: a prospective study. *Br J Ophthalmol.* 1995;79:347-349.
40. Talreja J, Dileepan K, Puri S, et al. Human conjunctival epithelial cells lack lipopolysaccharide responsiveness due to deficient expression of MD2 but respond after interferon-gamma priming or soluble MD2 supplementation. *Inflammation.* 2005;29:170-181.
41. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* 2010;11:373-384.
42. Schultz CL, Morck DW, McKay SG, Olson ME, Buret A. Lipopolysaccharide induced acute red eye and corneal ulcers. *Exp Eye Res.* 1997;64:3-9.
43. Schultz CL, Buret AG, Olson ME, Ceri H, Read RR, Morck DW. Lipopolysaccharide entry in the damaged cornea and specific uptake by polymorphonuclear neutrophils. *Infect Immun.* 2000;68:1731-1734.
44. Khatri S, Lass JH, Heinzl FP, et al. Regulation of endotoxin-induced keratitis by PECAM-1, MIP-2, and toll-like receptor 4. *Invest Ophthalmol Vis Sci.* 2002;43:2278-2284.
45. Dasu MR, Jialal I. Amelioration in wound healing in diabetic toll-like receptor-4 knockout mice. *J Diabetes Complications.* 2013;27:417-421.
46. Pukstad BS, Ryan L, Flo TH, et al. Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers. *J Dermatol Sci.* 2010;59:115-122.
47. Fukata M, Abreu MT. TLR4 signalling in the intestine in health and disease. *Biochem Soc Trans.* 2007;35:1473-1478.
48. Gao N, Kumar A, Jyot J, Yu FS. Flagellin-induced corneal antimicrobial peptide production and wound repair involve a novel NF-kappaB-independent and EGFR-dependent pathway. *PLoS One.* 2010;5:e9351.
49. Kanno E, Kawakami K, Ritsu M, et al. Wound healing in skin promoted by inoculation with *Pseudomonas aeruginosa* PAO1: The critical role of tumor necrosis factor-alpha secreted from infiltrating neutrophils. *Wound Repair Regen.* 2011;19:608-621.
50. Edwards R, Harding KG. Bacteria and wound healing. *Curr Opin Infect Dis.* 2004;17:91-96.