Lack of MD-2 expression in human corneal epithelial cells is an underlying mechanism of lipopolysaccharide (LPS) unresponsiveness

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In the present study we tested the responsiveness of human corneal epithelial cells (HCECs) and corneal fibroblasts to lipopolysaccharide (LPS), a Toll-like receptor (TLR) 4 ligand. Purified Pseudomonas aeruginosa LPS was used to stimulate telomerase-immortalized HCECs (HUCL) and stromal fibroblast (THK) cell lines. Exposure of cells to LPS induced a time-dependent activation of NF-κB in THK but not in HUCL cells, as assessed by an increase in IκB-α phosphorylation and degradation. Concomitant with NF-κB activation, LPS-treated THK cells, but not HUCL cells, produced a significantly larger number of cytokines than control untreated cells. A cell surface biotinylation assay revealed that HUCL cells express TLR4 intracellularly, whereas TLR5 is expressed on the cell surface. Furthermore, reverse transcriptase-PCR analysis revealed that HUCL and primary HCECs, in contrast to THK cells, do not express myeloid differentiation (MD)-2. Thus, our results demonstrate that the LPS unresponsiveness of HCECs might be due to deficient expression of MD-2, an essential component for LPS-TLR4 signaling.

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that HCECs expressed TLR4 and its co-receptor CD14 and responded to LPS challenge to produce proinflammatory cytokines within 24 h.\textsuperscript{31} Recently, Ueta et al.\textsuperscript{32} reported that the incubation of human corneal epithelial cells with LPS did not lead to the activation of NF-κB or the secretion of inflammation-associated molecules such as IL-6, IL-8 and human β-defensin 2. However, the application of LPS to abraded corneal epithelium in vivo resulted in the secretion of proinflammatory cytokines that mediate recruitment of neutrophils to the corneal stroma, thus inducing stromal edema and structural changes in corneal architecture in a MyD88-dependent manner.\textsuperscript{33} In contrast to the other TLRs, TLR4 is unique as its downstream signaling can occur through two independent pathways.\textsuperscript{34} The first pathway depends on the MyD88 signal adaptor protein, which is critical for the production of several proinflammatory cytokines, for example, IL-6 and tumor necrosis factor-α, and in the recognition of Gram-negative bacteria.\textsuperscript{35–37} In contrast, the MyD88-independent pathway, which depends on the Toll IL-1 receptor domain containing adaptor-inducing interferon-β (TRIF) signal adaptor protein, is mainly involved in the production of type 1 interferon.\textsuperscript{38}

To date, although corneal fibroblasts (keratocytes) have been implicated in playing a role in keratitis,\textsuperscript{4} the putative involvement of keratocytes in innate immune responses as part of corneal host defense has not been characterized. Thus, we hypothesize that in addition to epithelial cells, other resident corneal cells such as stromal keratocytes might be involved in recognizing and initiating an innate response for the cornea to LPS challenge. We tested this hypothesis \textit{in vitro}, using primary and immortalized human corneal epithelial and stromal keratocyte cell lines, by assessing LPS-mediated signal transduction and the production of proinflammatory cytokines/chemokines.

**RESULTS**

In epithelial cells, the transcription factor NF-κB plays a central role in regulating genes that govern the onset of mucosal inflammatory responses. The primary consequences of TLR activation are NF-κB activation and cytokine secretion in epithelial cells. To investigate LPS-triggered NF-κB activation, we first determined the dose response by treating HUCL cells with different concentration of LPS ranging from 1 ng to 10 μg·ml\(^{-1}\). We observed that LPS did not induce NF-κB as assessed by IκB-α phosphorylation in HUCL cells up to 10 μg·ml\(^{-1}\), whereas 1 μg·ml\(^{-1}\) LPS significantly induced NF-κB activation in THK cells (data not shown). A time-course study (Figure 1) showed that LPS (1 μg·ml\(^{-1}\)) in the keratinocyte basic medium had no apparent effect on the levels of phospho-IκB-α and IκB-α in HUCL cells for up to 8 h (Figure 1a). However, M9-conditioned medium derived from PAO1 culture (1:20 dilution) induced IκB-α phosphorylation and degradation in a time-dependent manner, suggesting that HCECs are responsive to PAO1 exoproducts (Figure 1a). In contrast, in THK cells LPS stimulated rapid IκB-α phosphorylation and IκB-α degradation that was maximal at 15 min post-stimulation (Figure 1b). Thus, LPS induced NF-κB activation in THK, but not in HUCL cells.

To assess the biological relevance of induced NF-κB activation, we measured the effect of LPS on proinflammatory cytokine expression and production (secretion) in HCECs and keratocytes. The effect of LPS on IL-8 mRNA expression was determined by reverse transcriptase-PCR. IL-8 mRNA was not detectable in untreated or 1 μg·ml\(^{-1}\) LPS-challenged HUCL cells (Figure 2a). However, a PCR product of the expected size (347 bp) was observed in HUCL cells 30 min after stimulation with \textit{P. aeruginosa} conditioned medium; the band intensity increased, peaked at 2 h and was still detectable at 6 h (Figure 2b). Similar to HUCL cells, IL-8 mRNA in unstimulated THK cells was barely detectable. However, incubation with LPS resulted in an increase in the expression of IL-8 mRNA in THK cells in a time-dependent manner.

![Figure 1](image1.png)

**Figure 1** LPS-stimulated IκB-α phosphorylation and degradation in THK, but not HUCL cells. HUCL (a) or THK (b) cells were stimulated with phenol-extracted LPS (1 μg·ml\(^{-1}\)) for the indicated times. As a positive control, HUCL cells were also treated with 5% supplemented \textit{P. aeruginosa}-conditioned medium (PA-CM) in KBM or KBM alone (Con). Total protein was extracted, and 20 μg was subjected to SDS-PAGE followed by phospho-IκB-α (p-IκB-α) and IκB-α immunoblotting using a chemiluminescence technique. The results are representative of two independent experiments. KBM, keratinocyte basic medium; LPS, lipopolysaccharide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

![Figure 2](image2.png)

**Figure 2** LPS induced IL-8 mRNA expression in THK, but not HUCL cells. (a) HUCL were incubated with 1 μg·ml\(^{-1}\) LPS for the indicated times; as a control, cells were also treated with 5% SMCM. (b) THK cells were treated with 1 μg·ml\(^{-1}\) LPS for the indicated times. Total RNA was extracted, reverse transcribed and amplified using IL-8 primers with GAPDH as control. PCR products were separated and stained as described in Methods. Results are representative of three independent experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; IL, interleukin.
The effect of LPS on IL-6 and IL-8 secretion by HUCL cells was assessed by enzyme-linked immunosorbent assay (ELISA). Consistent with a lack of NF-kB activation, there was no induced accumulation of IL-6 and IL-8 in HUCL cells treated with either 100 ng ml⁻¹ or 1 μg ml⁻¹ LPS (Figure 3a). These cells, however, responded to a challenge of 250 ng ml⁻¹ flagellin that served as a positive control by producing significantly more IL-6 (5.8 fold) and IL-8 (2.6 fold) as compared with the control untreated cells (P<0.01). To examine whether CD14 or LPS binding protein (LBP) is responsible for the unresponsiveness of HUCL cells to LPS challenge, human serum that provides soluble CD14 and/or LBP1,39 was added to the culture. No significant increase in IL-6 or IL-8 production was observed in LPS-challenged HUCL cells with or without human serum. In contrast, THK cells produced significantly more IL-6 and IL-8 in response to the LPS challenge even without the addition of serum. In total, 100 ng ml⁻¹ LPS was as effective as 1 μg ml⁻¹ LPS in inducing the secretion of the cytokines (Figure 4). Taken together, LPS differentially stimulates resident corneal cells by inducing IL-6 and IL-8 production in THK, but not in HUCL cells.

Recently, Ueta et al.32 reported the intracellular localization of TLR4 in HCECs. To confirm TLR4 localization in HCECs, HUCL cells were biotinylated with plasma membrane-impermeable NHS-LC-biotin, followed by avidin precipitation of biotinylated proteins and western blotting with TLR antibodies. Although HUCL cells expressed abundant TLR4, there was no surface biotinylated TLR4 detected. Under identical conditions, TLR5 was found to be labeled at the cell surface (Figure 5a).

To understand the underlying mechanism of the hyporesponsiveness of HCECs to LPS, we assessed the expression of TLR4 co-receptors known to be required for LPS–TLR interaction. Although CD14 was expressed in both epithelial and stromal fibroblasts (data not shown), MD-2 was found to be abundantly expressed in THK cells as well as in THP-1 cells (a human monocytic leukemia cell line, sensitive to LPS), but not in HCECs, including both the HUCL cell line and primary HCECs (Figure 5b). Few or no RT-PCR amplicons of MD-2 were detected in either HUCL cells or primary HCECs.

**DISCUSSION**

In this study, we showed that *P. aeruginosa* LPS stimulates the activation of the NF-kB signaling pathway and proinflammatory cytokine and chemokine production in human corneal stromal fibroblast cells, but fails to activate an inflammatory response in HCECs. We demonstrated that HCECs express abundant TLR4 intracellularly. However, there was no detectable increase in LPS-induced NF-kB activation or production of proinflammatory cytokines in these cells exposed to LPS for up to 24 h. This finding is consistent with that reported by Ueta et al.32 We further demonstrated that the unresponsiveness of HCECs to LPS challenge is likely due to the lack of MD-2 expression in HCECs. In contrast, human corneal stromal fibroblast cells expressed TLR4 and MD-2 and responded to the LPS challenge by secreting proinflammatory cytokines. Thus, the cornea is able to recognize LPS once it has penetrated into the stroma, and mounts an innate immune response to the invading pathogens by producing proinflammatory cytokines that recruit polymorphonuclear leukocytes (PMN) into the site of infection.

Bacterial endotoxins (LPS) are among the most potent inducers of innate immune responses and are a major virulence factors of Gram-negative bacteria including *P. aeruginosa*. The structural analysis of LPS divides the molecule into a hydrophobic lipid A region, which replaces phospholipids in the outer membrane, a central core oligosaccharide region, and a repeating polysaccharide portion referred to as O antigen or O polysaccharide.40 The O antigen portion of the *P. aeruginosa* LPS is responsible for conferring serogroup specificity, which is defined by antibodies specific to the different variants of this antigen. Recent studies revealed that the inflammatory response to LPS in cells is mediated by its interaction with TLR4.41-42 Molecular interaction studies showed that TLR4 is essential for the recognition of the lipid A portion of bacterial LPS,43 and mediates both effective host resistance to infection as well as some of the pathology associated with LPS-induced shock.44 Therefore, it should be noted that the *Pseudomonas* LPS used in this study is unlikely to influence TLR4 signaling regardless of its serotype. A previous study by Pier et al.45 showed that *P. aeruginosa* LPS induces less inflammation and lower overall host responses compared with that induced by enterobacterial (*Escherichia coli*) LPS. However, more recent studies showed a high degree of variability in the *P. aeruginosa* lipid A structure that can be synthesized depending on the strain and growth conditions, and have also shown that TLR4-mediated responses are highly dependent on the level of acylation of lipid A.46

A maximally sensitive response to LPS requires the sequential participation of several extracellular and cell-surface LPS-binding proteins, including LBP, CD14, MD-2 and TLR4. Briefly, LBP binds to Gram-negative bacteria or aggregates of LPS, thus decreasing the binding energy of LPS monomers. The LPS molecule is shuttled to CD14, which transfers the LPS to MD-2. The binding of lipid A to MD-2 causes the rearrangement of TLR4, leading to the association of its intracellular Toll IL-1 receptor domains and the recruitment of adapter proteins. Therefore, MD-2 plays a pivotal role in LPS sensing, bridging the recognition of endotoxins initiated by LBP and CD14 in response to the activation of TLR4 to proinflammatory cytokine and chemokine production.18,41-42,44 The responsiveness of epithelial cells to LPS appears to be tissue-specific; respiratory, gastric and bladder epithelial cells have been shown to be activated by LPS, whereas intestinal and oral epithelial cells fail to recognize and respond to LPS.18,48,49 The reason for endotoxin hyporesponsiveness of intestinal epithelial cells is low levels of TLR4 and/or MD-2.18

In the cornea, an early study showed that HCECs express TLR4 and CD14, and respond to LPS stimulation through the production of IL-6, IL-1α, IL-8 and tumor necrosis factor-α.31 However, a recent study by Ueta et al.32 revealed that HCECs are unresponsive to LPS challenge. Our results are consistent with that reported by Ueta et al.32 We also confirmed intracellular localization of TLR4 in HCECs. The intracellular expression of TLR4 was suggested to be an underlying mechanism for the immunosilent environment at the ocular mucosal epithelium.32 Similarly, TLR4 was also found intracellularly in pulmonary and intestinal epithelial cells, but intracellular TLR4 in these cells is capable of recognizing internalized LPS. As we showed that HCECs respond to flagellin through TLR5,50 which shares the MyD88-dependent signaling pathways with TLR4,30 the unresponsiveness of HCECs to TLR4 ligand is not due to defects in intracellular signal transduction, but more likely due to the lack of molecules required for proper LPS–TLR4 interaction. CD14 and MD-2 are critical co-receptors for TLR4 signaling.51 MD-2 and LPS form complexes that induce TLR4-expressing epithelial cells to secrete IL-6, IL-8 and MD-2. Thus, the LPS–MD-2 complex plays a crucial role in the LPS response by activating epithelial cells in the inflammatory microenvironment.9,52-54 Our results have shown that there was little or no MD-2 mRNA detected in HCECs, in both HUCL cell lines as well as in primary cultured cells. In line with our study, the low level of MD-2 expression in intestinal epithelial cells was attributed to the hyporesponsiveness of these cells to LPS.30 Although the role of MD-2 in LPS internalization has not been reported, the lack of MD-2
Figure 3  LPS failed to induce IL-6 and IL-8 secretion in HUCL cells. (a) HUCL cells were stimulated with phenol-extracted LPS (1 μg ml⁻¹ and 100 ng ml⁻¹) or medium alone (control) for 8 h; as a positive control, cells were also treated with 250 ng ml⁻¹ purified flagellin for 8 h. (b) HUCL cells were treated with 1 μg ml⁻¹ phenol-extracted LPS with or without 0.1% human serum for 24 h. The effects of LPS and flagellin on IL-6 and IL-8 secretion were measured in cell culture supernatants by ELISA. Data are representative of triplicate experiments and are expressed as the mean ± s.d. Statistically significant differences in secreted IL-6 and IL-8 in LPS-treated cells were determined by ANOVA. ANOVA, analysis of variance; LPS, lipopolysaccharide; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.
expression may also be responsible for the failure of HCECs to internalize LPS \textit{in vivo}^{{32,55}}. A more recent study also showed that human conjunctival epithelial cells are unresponsive to LPS due to the lack of MD-2 expression.\textsuperscript{56} Thus, we conclude that the lack of MD-2 is a major factor which is responsible for the LPS signaling defect in HCECs, and for the tolerance of the cornea toward the constant exposure of bacteria and non-threatening amounts of bacterial endotoxins.

Although HCECs are unresponsive to LPS, the underlying stromal fibroblasts, keratocytes are rapidly activated by LPS. Unlike epithelia, stromal cells are not constantly exposed to bacteria or their products. When tissues such as the cornea are exposed to bacteria, the barrier function of epithelium would prevent bacteria from direct interaction with TLRs, which are usually located in the inner cell layers of stratified epithelium\textsuperscript{55} or on the basolateral side of simple epithelium.\textsuperscript{57} Owing to the barrier function and the unique localization of TLRs, the released endotoxin may also exhibit minimal effects on epithelial cells. However, once the barrier is breached and bacteria invade the epithelial layer, the pattern recognizing receptors, such as TLR5,\textsuperscript{58,59} recognize the pathogens in the epithelial layer and initiate corneal innate immune responses by producing antimicrobial molecules such as hBD\textsuperscript{2,60,61} and recruiting neutrophils through IL-8. Many pathogens may be eliminated upon initial contact by this mechanism.\textsuperscript{62} However, this initial defense may be overwhelmed by the pathogens, leading to the penetration of bacteria or released endotoxin into the stromal layer.\textsuperscript{55} In other mucosal epithelial cells (urinary, intestinal and pulmonary), flagellin and TLR5 are the major responsive unit, not LPS-TLR4. We believe that the unresponsiveness of epithelial cells is a way to avoid unwanted inflammation as these epithelial linings, unlike immune cells, are constantly exposed to LPS, though not to flagellin. This exception occurs because flagellin is a protein, which is usually not detected in the fluids that cover the epithelial lining in the body.

The presence of bacterial products such as flagellin and/or LPS in the stroma should act as a sign of stromal infection and a rapid response on the part of the resident cells is necessary for clearing the pathogens. The expression of functional TLR4, including its coreceptors CD14 and MD-2, and TLR5 (Yu et al., unpublished result) in keratocytes should allow the cells to rapidly recognize infection, and the release of proinflammatory cytokines and chemokines that recruit more neutrophils and monocytes to the infected stroma. It is interesting to note that corneal stromal fibroblasts stimulated with LPS secrete higher levels of cytokines than flagellin-stimulated HCECs when normalized with cellular proteins: over 2 times more IL-6 and approximately 15 times more IL-8 is secreted by THK cells. IL-8 is the major chemoattractant in the cornea for PMN infiltration.\textsuperscript{63,64} Thus, the increased level of IL-8 released from keratocytes may attract a large number of PMN to the stroma that clear the pathogen and may also cause severe local inflammation, leading to the development of keratitis.
In summary, the data presented in this study demonstrate that HCECs failed to respond to LPS. The unresponsiveness of HCECs is likely due to the lack of MD-2 expression, which is an important component of LPS-TLR4 signaling. As the cornea is constantly exposed to environmental stimuli including LPS, these findings suggest that the human corneal epithelium possesses a regulatory mechanism, similar to that observed in intestinal epithelial cells, for the inhibition of TLR4-mediated innate immunity. However, the breakdown of the epithelial barrier during infection or trauma leads to a direct interaction of bacterial products with submucosal cells, that is, stromal keratocytes. Therefore, these cells participate in innate immune responses by sensing and responding to bacterial products, such as LPS, that have penetrated into the subepithelial compartment of the cornea. Thus, targeting TLR4-mediated signaling pathways in keratocytes may permit the development of new, specific therapies that could promote innate defense and prevent some of the destructive consequences of ocular Gram-negative bacterial infections.

**METHODS**

**Purification and preparation of *P. aeruginosa* LPS and conditioned medium**

Commercially available LPS derived from *P. aeruginosa* (Catalog no. L9143, Sigma-Aldrich, St Louis, MO, USA) was resuspended in 1 ml of endotoxin-free water containing 0.2% triethylamine and extracted with water-saturated phenol as described earlier.63 Purified LPS was resuspended in keratinocyte basic medium (BioWhittaker Inc., Walkersville, MD, USA). Hundred percent recovery was assumed. This was referred to as ‘phenol reextracted LPS’.

*P. aeruginosa* (PAO1 strain) was cultured in supplemented M9 medium for 72 h and the culture media was centrifuged to remove bacteria and sterilized by filtration through a 0.2 μm filter.64 The conditioned media was stored at 4 °C and used at 5% concentration (that is, 5% of saturated *P. aeruginosa* growth medium and 95% PBS). The flagellin of *P. aeruginosa* was purified as described earlier.

**Cell culture and stimulation**

Human telomerase-immortalized corneal epithelial (HUCL) cells, kindly provided by Dr Rheinwald and Dr Gipson, were cultured in defined keratinocyte SFM (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37 °C in a humidified incubator with 95% room air and 5% CO2. HUCL cells were grown in defined keratinocyte SFM in a humidified 5% CO2 incubator at 37 °C (Invitrogen-Life Technologies) with 10% fetal bovine serum (Mediatech Inc., Herndon, VA, USA) at 37 °C in a humidified incubator with 95% room air and 5% CO2. Cells grown in 100 mm dishes were rinsed 6 times with HBSS (Mediatech Inc.), supplemented with 0.1 mM CaCl2 and 1 mM MgCl2, and then incubated with freshly prepared NHS-LC-Biotin (Pierce Biotechnology) diluted in the same solution (1 mg ml−1) for 5 min at room temperature. The reaction was quenched with 50 mM NH4Cl, and cells were washed with phosphate-buffered saline and lysed with a solution containing 1% Triton X-100, 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid and 0.2% bovine serum albumin supplemented with protease inhibitors. Cell extract supernatant was incubated with immobilized streptavidin agarose (Pierce Biotechnology) for 16 h at 4 °C to bind biotinylated proteins. Proteins bound to the agarose slurry were solubilized with Laemmli buffer and analyzed by SDS-polyacrylamide gel electrophoresis and western blotting with purified rabbit antibodies against TLR4 and TLR5 from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**RT-PCR**

RNA was isolated with extraction reagent (TRIzol; Invitrogen), and 2 μg of the total RNA was reverse-transcribed for cDNA synthesis using MIV-RT (SuperScript; Invitrogen). cDNA was amplified by PCR with specific primers for human IL-6 (sense: CTCCCTCTCTCCAGAAAGGCCTTC, anti-sense: GCGCA GAATGAGATGAGTTGTC, product: 583 bp), IL-8 (sense: GCAGTTTGGCC AAGGAGTGT, anti-sense: GCATCTGGCAACCTACAAA, product: 347 bp), MD-2 (sense: TATTTGGTCTGCACAT and anti-sense: CTCCCA GAATAGCTTCAAC, product: 358 bp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense: CACCAACACTGCTTACAGC, anti-sense: CCGCTGGTGGTGATGGCAAT, product: 515 bp). IL-6, IL-8 and MD-2 were amplified at 26, 28 and 28 cycles, respectively, with annealing temperature 58 °C (45 s) and extension temperature 72 °C (1 min). GAPDH was amplified 20 cycles under the same conditions. The PCR products (5 μl) were subjected to electrophoresis on 2% agarose gels containing ethidium bromide. Staining was captured by digital camera (Gel Logic 100 system; Eastman Kodak, Rochester, NY, USA).

**Cytokine ELISA**

IL-6 and IL-8 secretion was determined by ELISA. HUCL cells or THK cells were plated at 4 × 105 cells per well in 12-well plates. After growth factor/serum starvation, cells were treated with 100 ng ml−1 or 1 μg ml−1 phenol re-extracted LPS for the indicated time; the supernatants were harvested for the measurement of IL-6 and IL-8 using ELISA, which was performed according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). The amount of IL-6 and IL-8 in culture media was normalized with the total amount of cellular protein lysed with RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 100 mM sodium pyrophosphate, 3.5 mM sodium orthovanadate, proteinase inhibitor cocktails and 0.1 mM PMSF). The protein concentration of cell lysate was determined with a Micro BCA kit (Pierce Biotechnology, Rockford, IL, USA). Results were expressed as the mean picograms of cytokine per milligram cell lysate ± s.e. (n = 3); P-values were determined through analysis of variance.

**Western blot analysis**

LPS-treated HUCL and THK cells were lysed with RIPA buffer and protein concentration was determined with the Micro BCA kit (Pierce Biotechnology). IkB-α phosphorylation and degradation were detected with rabbit anti-IkB-α and anti- phospho-IkB-α, which were purchased from Cell Signaling Technology (Beverly, MA, USA) and developed with Supersignal reagents from Pierce Biotechnology.

**Cell surface biotinylation**

Cells grown in 100 mm dishes were rinsed 6 times with HBSS (Mediatech Inc.), supplemented with 0.1 mM CaCl2 and 1 mM MgCl2, and then incubated with freshly prepared NHS-LC-Biotin (Pierce Biotechnology) diluted in the same solution (1 mg ml−1) for 5 min at room temperature. The reaction was quenched with 50 mM NH4Cl, and cells were washed with phosphate-buffered saline and lysed with a solution containing 1% Triton X-100, 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid and 0.2% bovine serum albumin supplemented with protease inhibitors. Cell extract supernatant was incubated with immobilized streptavidin agarose (Pierce Biotechnology) for 16 h at 4 °C to bind biotinylated proteins. Proteins bound to the agarose slurry were solubilized with Laemmli buffer and analyzed by SDS-polyacrylamide gel electrophoresis and western blotting with purified rabbit antibodies against TLR4 and TLR5 from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

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**PROPRIETARY INTEREST**

None.
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