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Lack of MD2 expression in human corneal epithelial cells is an underlying mechanism of lipopolysaccharide (LPS) unresponsiveness

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Abstract

In the present study we tested the responsiveness of human corneal epithelial cells (HCECs) and corneal fibroblasts to lipopolysaccharide (LPS), a TLR4 ligand. Purified *P 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 significantly more 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, RT-PCR analysis revealed that HUCL and primary HCECs, in contrast to THK cells, do not express MD-2. Thus, our results demonstrate that the LPS unresponsiveness of HCECs might be due to deficient expression of MD2, an essential component for LPS-TLR4 signaling.

Keywords

corneal epithelial cells; keratocytes; toll-like receptors; proinflammatory; cytokines; innate response

Introduction

The Gram-negative bacterium *P. aeruginosa* is an opportunistic bacterial pathogen and a leading cause of bacterial keratitis with increased incidence among contact lens users with

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extended wearing. [1,2] If left untreated, infections could lead to perforation of the cornea resulting in permanent loss of vision and potential loss of the eye. [3,4] *P. aeruginosa* elaborates a multitude of factors including glycocalyx, lipopolysaccharide (LPS), endotoxin, and flagellin.[5,6] These factors, notably LPS, may induce the release of multiple proinflammatory cytokines and chemokines from resident corneal cells and this initial inflammatory response plays a key role in containment of the infection. [6,7] LPS, a major component of the outer membrane of *P. aeruginosa*, is a virulence factor that can cause inflammatory responses in tissues, the consequences of which can be severe and even lethal. [8] LPS activates monocytes and macrophages which produce several inflammatory cytokines including TNF-α, IL-1, IL-6, IL-8 and IL-12, leading to serious systemic disorders. [9,10] LPS also induces inflammatory responses in endothelial cells [11,12] and respiratory, gastric, and bladder epithelial cells.[13–16] Other epithelium, such as intestinal epithelial [17,18] and well-differentiated human airway epithelial cells [19], are hyporesponsive to LPS. In contrast others have reported that intestinal epithelial cells recognize and respond to the internalized LPS.[20,21] These studies also revealed that the inflammatory response induced by LPS in epithelial and other cell types is related to Toll-like receptor (TLR) 4.

The major advancement in understanding the molecular mechanism of TLR4 in transducing LPS-mediated signals was the discovery of myeloid differentiation (MD)-2.[22] MD-2 is a glycoprotein of approximately 17–25 kDa, co-expressed with TLR4 at the surface of various cell types, principally those of myeloid and endothelial lineages.[23,24] Shimazu *et al* found that when MD-2 was co-transfected into HEK293 cells, it bound to TLR4 and greatly enhanced the response of TLR4-transfected cells to LPS.[22] In another study, a forward genetic screen of CD14-transfected CHO cells revealed that MD-2 is essential for LPS responses and MD-2 can be supplied as a soluble receptor component to a TLR4-positive cell. [25] In addition, MD-2 has been suggested to play a role in the trafficking of TLR4 to the cell surface, at least in murine cells.[26] Many mucosal epithelial cells such as those in the intestine [27] and in the airway[19,28] have a limited response to LPS, and low or absent expression of MD-2 was suggested as the underlying mechanism for these cells.

TLRs recognize repetitive patterns, thus they are termed pattern-recognizing receptors (PRRs). These patterns are present on diverse microbes including Gram-positive and Gram-negative bacteria and viruses and are key components of innate immunity. [29,30] An early study revealed that HCECs expressed TLR4 and its co-receptor CD14 and responded to LPS challenge to produce proinflammatory cytokines within 24 h. [31] Recently, Ueta et al 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 beta-defensin-2. [32] 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. [33] In contrast to the other TLRs, TLR4 is unique as its downstream signaling can occur via two independent pathways [34]. The first pathway depends on the MyD88 signal adaptor protein which is critical for the production of several proinflammatory cytokines, e.g., IL-6 and TNF-α, and in the recognition of Gram-negative bacteria. [35–37] In contrast, the MyD88-independent pathway,

which depends on the Toll IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF) signal adaptor protein, is mainly involved in production of type I IFN. 38

To date, although corneal fibroblasts (keratocytes) have been implicated in playing a role in keratitis 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 formulating an innate response for the cornea to LPS challenge. We tested this hypothesis 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 is 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. We observed that LPS did not induce NF-κB as assessed by IκB-α phosphorylation in HUCL cells up to 10 μg/ml, whereas 1 μg/ml LPS significantly induced NF-κB activation in THK cells (data not shown). A time-course study (Figure 1) showed that LPS (1 μg/ml) in KBM 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 RT-PCR. IL-8 mRNA was not detectable in untreated or 1 μg/ml LPS-challenged HUCL cells (Figure 2A). However, a PCR product of the expected size (347 bp) was observed in HUCL cells 30 min post-stimulation with *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.

The effect of LPS on IL-6 and IL-8 secretion by HUCL cells was assessed by ELISA. Consistent with a lack of NF-κB activation, there was no induced accumulation of IL-6 and IL-8 in HUCL cells treated with either 100 ng/ml or 1 μg/ml LPS (Figure 3A). These cells however, responded to a challenge of 250 ng/ml flagellin that served as a positive control by producing significantly more IL-8 (5.8 fold) and IL-6 (2.6 fold) as compared to 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 LBP 31,39 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. THK cells, on the other hand, produced significantly more IL-6 and IL-8 in response to the LPS challenge even without the addition of serum. 100 ng/ml LPS was as effective as 1 μg/ml 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 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. While 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. While 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). Little 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-κB signaling pathway and pro-inflammatory 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-κB 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. Human corneal stromal fibroblast cells, on the other hand, expressed TLR4 and MD-2 and responded to 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 PMN into the site of infection.

Bacterial endotoxins (LPS) are among the most potent inducers of innate immune responses and are a major virulence factor 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 showed that *P. aeruginosa* LPS induces less inflammation and lower overall host responses compared with that induced by enterobacterial (*E. coli*) LPS. [45] 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 TIR 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 pro-inflammatory cytokine and chemokine production. [18,41,42,47] 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 TNF-α. [31] However, a recent study by Ueta et al revealed that HCECs are unresponsive to LPS challenge. [32] 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. Since 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, andMD-2. Thus, the LPS-MD-2 complex plays a crucial role in the LPS response by activating epithelial cells in the inflammatory microenvironment. [19,52–54] 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 MD2 expression in intestinal epithelial cells was attributed to the hyporesponsiveness of these cells to LPS. 18 Although the role of MD2 in LPS internalization has not been reported, the lack of MD-2 expression may also be responsible for the failure of HCECs to internalize LPS in vitro and in vivo. 32,55 A more recent study also showed that human conjunctival epithelial cells are unresponsive to LPS due to the lack of MD2 expression 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 towards the constant exposure of bacteria and non-threatening amounts of bacterial endotoxins.

While 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 50 or on the basolateral side of simple epithelium57. Because of 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 58,59, recognize the pathogens in the epithelial layer and initiate corneal innate immune responses by producing antimicrobial molecules such as hBD2 60,61 and recruiting neutrophils via IL-8. Many pathogens may be eliminated upon initial contact by this mechanism 62. However, this initial defense may be overwhelmed by the pathogens, leading to the penetration of bacteria or released endotoxin into the stromal layer 55. In other mucosal epithelial cells (urinary, intestinal, and pulmonary), flagellin and TLR5 is 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. 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 thatHCECs 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. Since 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, i.e 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 novel, specific therapies that could promote innate defense and prevent some of the destructive consequences of ocular Gram-negative bacterial infections.

Materials and 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) was resuspended in 1 ml of endotoxin-free water containing 0.2% triethylamine (TEA) and extracted with water-saturated phenol as previously described 65. Purified LPS was re-suspended in keratinocyte basic medium (KBM, Biowittaker Inc., Walkersville, MD). One hundred percent recovery was assumed. This was referred to as "phenol re-extracted 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.66 The conditioned media was stored at 4°C and used at 5% concentration (i.e 5 % of saturated *P. aeruginosa* growth medium and 95% PBS). The flagellin of *P. aeruginosa* was purified as previously described 50.

Cell culture and stimulation

Human telomerase-immortalized corneal epithelial (HUCL) cells,67 kindly provided by Dr. Rheinwald and Dr. Gipson, were cultured in defined keratinocyte-SFM (Invitogen Life Technologies, Carlsbad, CA) at 37°C in a humidified incubator with 95% room air, 5%CO_2. HUCL cells were cultured in KBM overnight before experimentation (growth factor starvation). Human corneal stromal fibroblast cells (THK) were provided by Dr. James Jester 68 and were cultured in DMEM/F12 medium (Invitrogen-Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (Mediatech Inc., Herndon, VA) at 37°C in a humidified incubator with 95% room air, 5%CO_2. Before treatment, THK cells were washed with PBS two times and cultured in serum-free DMEM/F12 medium overnight for serum starvation. Both epithelial and stromal fibroblasts were treated with phenol re-extracted LPS in KBM or serum-free DMEM/F12 media for various times and then processed for Western blotting and cytokine measurement.

Primary HCECs were isolated from human donor corneas obtained from the Michigan Eye Bank. The epithelial sheet was separated from underlying stroma after overnight dispase treatment at 4°C. The dissected epithelial sheet was trypsinized, and cells were then

collected by centrifugation. Primary HCECs were grown in defined keratinocyte SFM in a humidified 5% CO_2 incubator at 37°C and then used at passage 3.

RT-PCR

RNA was isolated with extraction reagent (TRIzol; Invitrogen), and 2 μg of the total RNA was reverse-transcribed for cDNA synthesis using MLV-RT (SuperScript; Invitrogen). cDNA was amplified by PCR with specific primers for human IL-6 (sense: CTCCTTCTCCACAAGCGCCTTC, anti-sense: GCGCAGAATGAGATGAGTTGTC, product: 583bp), IL-8 (sense: GCAGTTTTGCCAAGGAGTGCT, anti-sense: GCATCTGGCAACCCTACAACA, product: 347bp), MD-2 (sense: TATTGGGTCTGCAACTCAT and anti-sense: CTCCCAGAAATAGCTTCAAC, product: 358 bp) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense: CACCACCAACTGCTTAGCAC and antisense CCCTGTTGCTGTAGCCAAAT, product: 515 bp). IL-6, IL-8 and MD-2 were amplified at 26, 32 and 28 cycles, respectively, with annealing temperature 58°C (45 sec) 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).

Cytokine ELISA

IL-6 and IL-8 secretion was determined by ELISA. HUCL cells or THK cells were plated at 4×10^5 cells/well in 12-well plates. After growth factor/serum starvation, cells were treated with 100 ng/ml or 1μg/ml 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). 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). Results were expressed as the mean pg of cytokine per mg cell lysate ± SE (n=3); P values were determined via ANOVA.

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). IκB-α phosphorylation and degradation were detected with rabbit anti-IκB-α and anti-phospho-IκB-α which were purchased from Cell Signaling Technology (Beverly, MA) and developed with Supersignal reagents from Pierce.

Cell Surface Biotinylation

Cells grown in 100 mm dishes were rinsed 6 times with HBSS (Mediatech, Inc., Herndon, VA), supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, and then incubated with freshly-prepared NHS-LC-Biotin (Pierce) diluted in the same solution (1mg/ml) for 5 minutes at

room temperature. The reaction was quenched with 50 mM NH$_4$Cl, and cells were washed with PBS and lysed with a solution containing 1% Triton X-100, 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM EDTA, and 0.2% BSA supplemented with protease inhibitors. Cell extract supernatant was incubated with immobilized streptavidin agarose (Pierce) for 16 hours at 4°C to bind biotinylated proteins. Proteins bound to the agarose slurry were solubilized with Laemmli buffer and analyzed by SDS-PAGE and Western blotting with purified rabbit antibodies against TLR4 and TLR5 from Santa Cruz Biotechnology (Santa Cruz, CA).
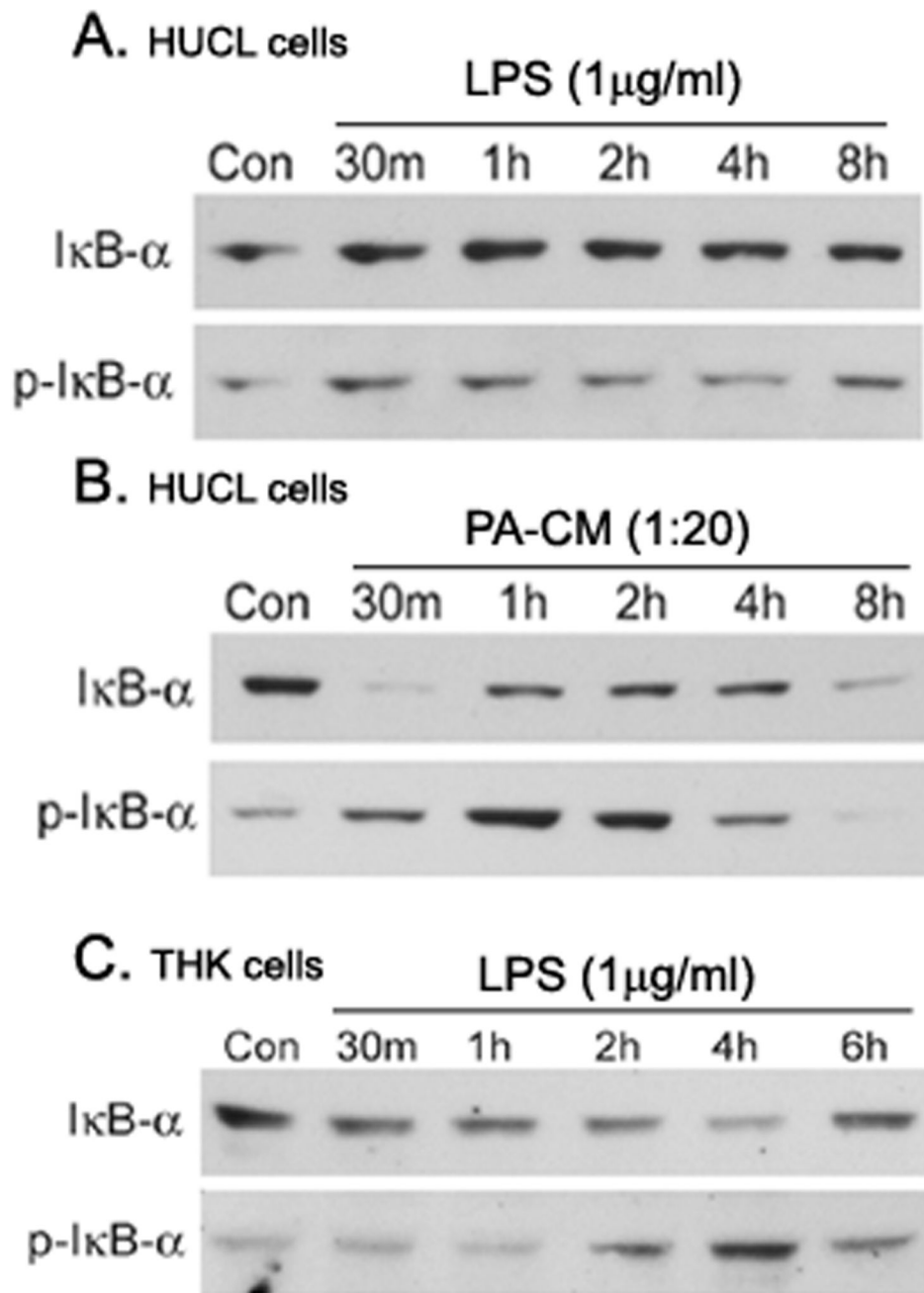
Acknowledgment

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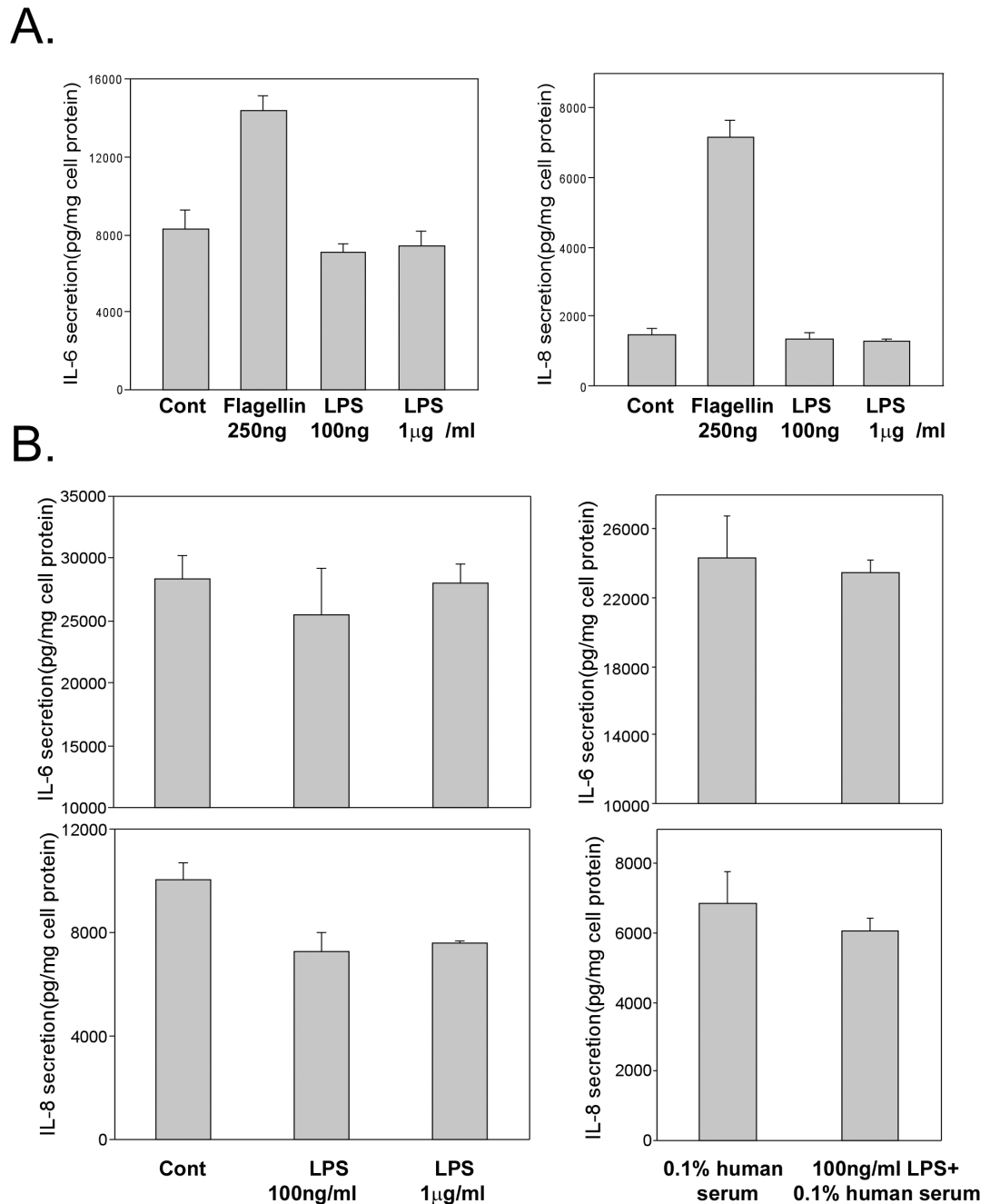
A. HUCL cells

LPS (1μg/ml)

Con   30m   1h   2h   4h   8h

IκB-α

p-IκB-α

B. HUCL cells

PA-CM (1:20)

Con   30m   1h   2h   4h   8h

IκB-α

p-IκB-α

C. THK cells

LPS (1μg/ml)

Con   30m   1h   2h   4h   6h

IκB-α

p-IκB-α

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) for the indicated times. As a positive control, HUCL cells were also treated with 5% supplemented *P. aeruginosa*-conditioned medium (PA-CM) in KBM or KBM alone (Con). Total protein was extracted, and 20 μg of protein were 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.

A. HUCL cells
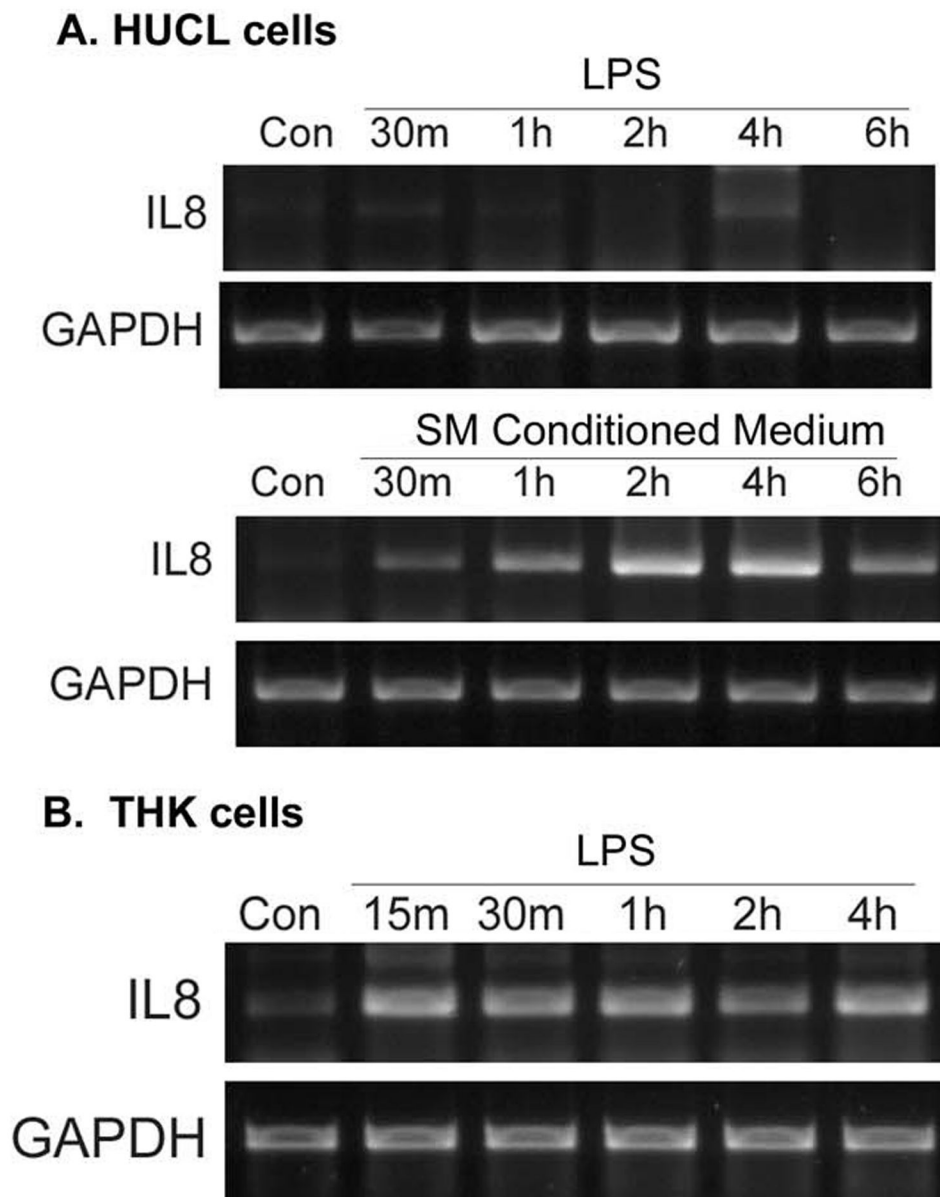
B. THK cells

Figure 2. LPS induced IL-8 mRNA expression in THK, but not HUCL cells
(**A**) HUCL were incubated with 1 μg/ml LPS for the indicated times; as a control, cells were also treated with 5% SMCM. (**B**) THK cells were treated with 1 μg/ml 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 Materials and Methods. Results are representative of three independent experiments.

A.

B.

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 and 100 ng/ml) or medium alone (control) for 8 h; as a positive control, cells were also treated with 250 ng/ml purified flagellin for 8h. (**B**) HUCL cells were treated with 1 μg/ml phenol-extracted LPS with or without 0.1% human serum for 24 h. The effects of LPS and flagellin on IL-6 and -8 secretion were measured in cell culture supernatants by ELISA. Data are representative of triplicate experiments and are expressed as the mean ± SD. Statistically significant

differences in secreted IL-6 and IL-8 in LPS-treated cells were determined by ANOVA with probabilities shown both for overall significance and pairwise comparison (*P<0.01).
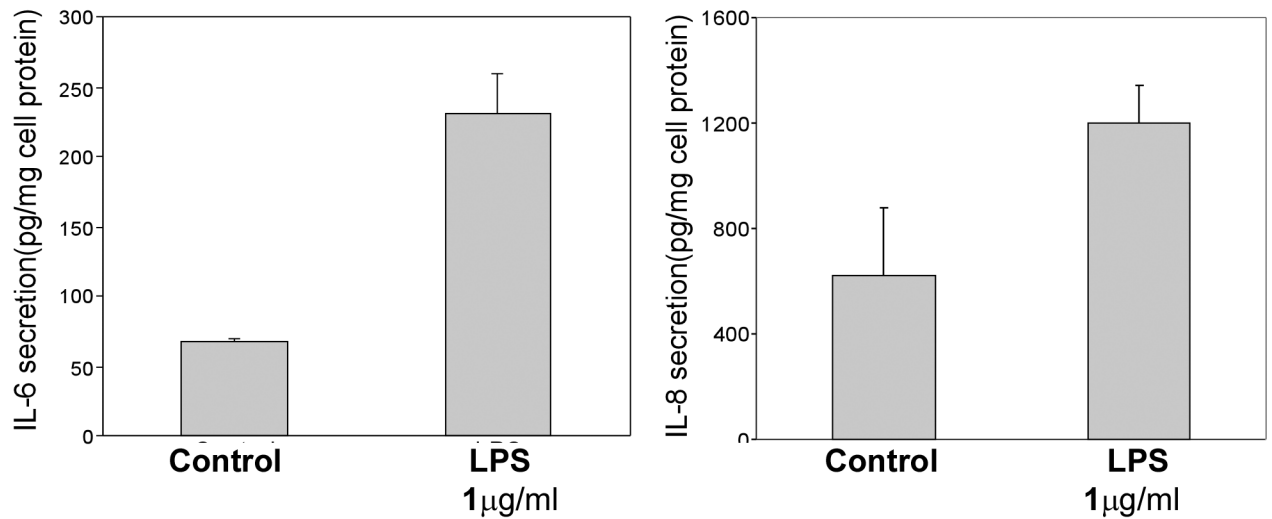
Figure 4. LPS stimulated IL-6 and IL-8 secretion in THK cells
THK cells were treated with phenol-extracted LPS (1 μg/ml) for 8 h, and with medium alone at the same time point as control. The effects of LPS on IL-6 and -8 secretion were measured in THK culture supernatants by ELISA. Data are representative of triplicate experiments and are expressed as the mean ± SD. Statistically significant differences in secreted IL-6 and IL-8 in LPS-treated cells were determined by ANOVA with pair-wise comparison (*P<0.01).

A.

B.
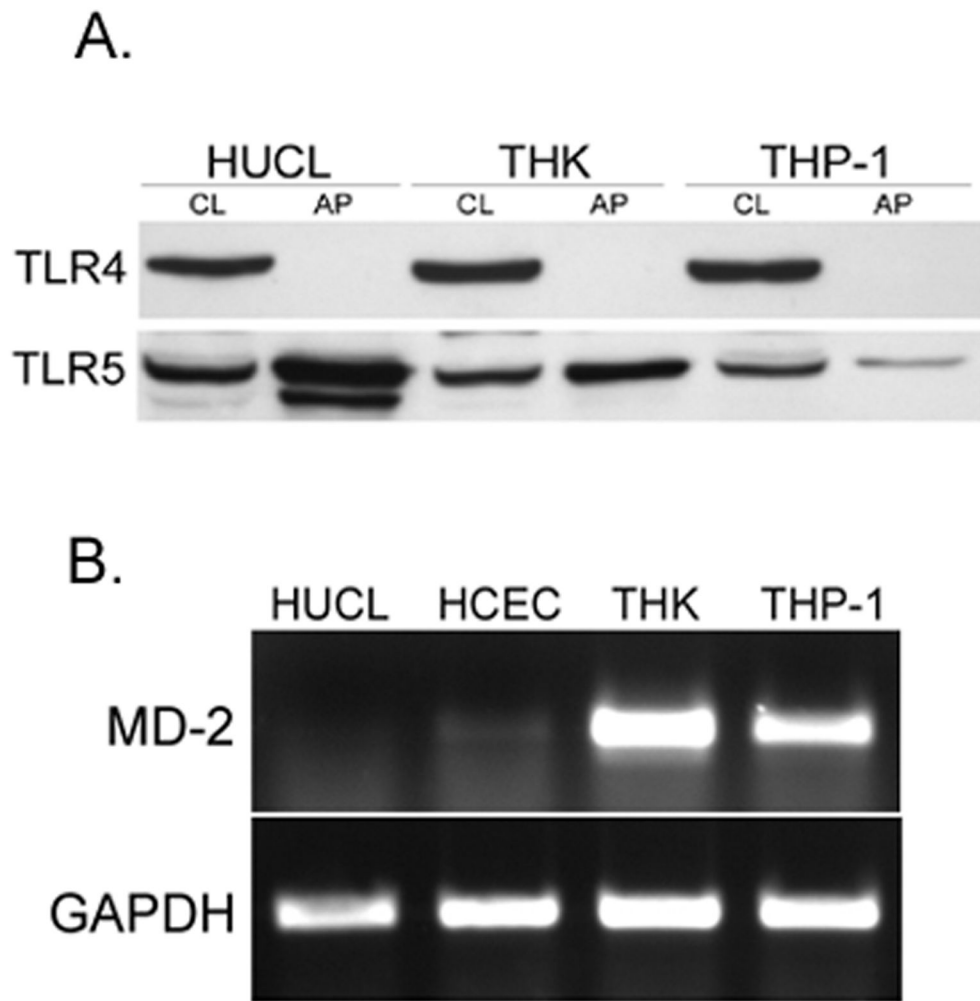
Figure 5. Lack of TLR4 cell surface expression and MD-2 expression in HCECs
(**A**) HUCL monolayers were labeled with NHS-LC-biotin. Biotinlyated proteins were either directly analyzed by Western blotting (C) or precipitated from the cell lysate by streptavidin-conjugated agarose and analyzed using Western blotting (M) with TLR4- or TLR5-specific antibodies. The figure is representative of three independent experiments. (**B**) HUCL, THK and THP-1 cells were extracted and the obtained RNA was reverse transcribed and amplified using human MD2 primers with GAPDH as control. PCR products were separated and stained. Results are representative of three independent experiments.