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Innate Immune Response of Corneal Epithelial Cells to *Staphylococcus aureus* Infection: Role of Peptidoglycan in Stimulating Proinflammatory Cytokine Secretion

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Abstract

Purpose—This study sought to elucidate the innate immune responses of cultured human corneal epithelial cells (HCECs) to infection by the Gram-positive bacterium *Staphylococcus aureus* and to determine the underlying mechanisms.

Methods—HUCL, a telomerase-immortalized HCEC line, and primary cultures of HCECs were challenged with live or heat-killed *S. aureus*, its exoproducts, or cell wall components lipoteichoic acid (LTA) and peptidoglycan (PGN). IκB-α phosphorylation and degradation as well as phosphorylation of MAPKs, p38, and JNK-1/2, were assessed by Western blot analysis. The expression of interleukin (IL)-6, IL-8, TNF-α, and β-defensin-2 were determined using RT-PCR and secretion of IL-6, IL-8, TNF-α, and β-defensin were measured using enzyme-linked immunosorbent assay and immunoblot analysis of culture medium.

Results—Exposure of HUCL cells to live, but not heat-killed, *S. aureus* resulted in NF-κB activation in a time-dependent manner, as assessed by the increase in IκB-α phosphorylation and degradation. Live bacteria also activated the p38 and JNK pathways. The effects of live bacteria on HUCL cells may be attributable to bacterial exoproducts, since the conditioned medium of *S. aureus* also effectively stimulated these signaling pathways. PGN, but not LTA, activated the NF-κB and MAPK pathways in a dose- and time-dependent manner. Concomitant with activation of NF-κB and MAPKs, transcriptional expression of IL-6, IL-8, TNF-α, and β-defensin-2 were induced in cells challenged with bacterial exoproducts and PGN. Secretion of IL-6, IL-8, TNF-α, and β-defensin-2 were also significantly increased in HCECs in response to bacterial exoproducts and PGN challenge.

Conclusions—Corneal epithelial cells possess the ability to recognize the presence of Gram-positive bacteria and to initiate the innate immune responses by the expression and/or release of proinflammatory cytokines and β-defensin-2 in the cornea.

The Gram-positive bacterium *Staphylococcus aureus* is a leading cause of bacterial keratitis and accounts for approximately one quarter of confirmed cases. There has been a recent gradual increase in *S. aureus* keratitis in both the United States and China.[1,2] Staphylococcal keratitis often leads to localized inflammation associated with cellular injury and tissue destruction. Although it is necessary for containing the infection,[3,4] the host inflammatory response also contributes to corneal destruction, which may lead to irreversible corneal scarring.[5–7]

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Corneal epithelial cells, like other mucosal epithelial linings in the body,[8,9] constitute the first line of defense against microbial pathogens and should possess the ability to detect the presence of pathogenic bacteria.[3,10] Recent studies have shown that rather than being a passive barrier, the epithelium is an active participant in the host response to infection through the expression of proinflammatory genes and the secretion of inflammatory cytokines that recruit inflammatory cells in response to pathogenic bacteria and their products.[11–17] Our previous work showed that corneal epithelial cells are able to recognize *Pseudomonas aeruginosa* flagellin and initiated inflammatory responses of the cornea by release of the proinflammatory cytokines IL-6 and -8.[10] Like *P. aeruginosa*, *S. aureus* infection may also be a cause of ulcerative keratitis as a result of inflammation.[5,7] *S. aureus* produces a large variety of virulence factors[18] that include cell surface proteins (collagen-binding proteins and fibronectin-binding proteins) and secreted toxins (α-, β-, δ-, and γ-toxins), and their specific roles in corneal infection have been documented in the literature.[19–21] Cell wall components of Gram-positive bacteria, particularly peptidoglycan (PGN) and lipoteichoic acid (LTA), are also known virulence factors that cause host inflammation. PGN is an alternating β-linked *N*-acetylmuramyl and *N*-acetylglucosaminyl glycan whose residues are cross-linked by short peptides.[22,23] LTA is anchored in the membrane by glycolipids found in many Gram-positive bacteria[24] and has been shown to induce the ERK signaling pathway in the cornea.[25] Like lipopolysaccharide, a component of the outer cell membrane of Gram-negative bacteria, isolated PGN and LTA can elicit most of the clinical manifestations of Gram-positive bacterial infection.[26] These bacterial agents stimulate the excessive release of proinflammatory cytokines like TNF, IL-1, IL-6, and other inflammatory mediators from immune cells, including macrophages.[27–29] However, there are contradictory reports regarding epithelial responses to PGN and LTA. A recent study showed that human intestinal epithelial cells are broadly unresponsive to PGN and LTA.[30] Heyer et al.[31] reported that shed and/or secreted *S. aureus* components including PGN, but not LTA, stimulate lung epithelial cells to produce IL-8 and GM-CSF in vitro and in vivo. In contrast, using the human β-defensin-2 (hBD2) promoter, Wang et al.[32] showed that LTA stimulates hBD2 expression in human airway epithelial cells in an NF-κB/Toll-like receptor (TLR)– dependent pathway. It appears that the ability of epithelial cells to recognize PGN and LTA is tissue-specific and related to the expression of TLRs at the cell surface.[30,33–35] The role of epithelium in recognition of Gram-positive bacteria and in the innate immune response of the cornea to *S. aureus* infection has not been established.[30,35,36]

Recognition systems used by epithelial cells to respond to microbial exposure include the action of TLRs. These receptors are termed pattern-recognition receptors because they recognize repetitive patterns—that is, pathogen-associated molecular patterns (PAMPs), present on diverse microbes including Gram-positive and Gram-negative bacteria and viruses.[37,38] The interaction of a TLR with its PAMP results in the activation of multiple intracellular signaling events such as NF-κB activation and subsequent production of cytokines such as IL-6, IL-8, and TNF-α.[39–41] The best studied TLR is TLR4, which acts as an lipopolysaccharide (LPS) receptor.[42] In contrast, TLR2 is required for recognition of Gram-positive PAMPs including bacterial LTA and PGN.[43–46] Mutations in TLR2 are associated with severe mycobacterial, staphylococcal infection and lepromatous leprosy.[47–49] Further studies have shown that combinations of TLR molecules are necessary for recognition of certain PAMPs. For example, recognition of PGN requires combined expression of TLR2 and -6.[37,49,50]

Relatively little is known about the role of TLR2 signaling in the cornea. In this study, we investigated how human corneal epithelial cells (HCECs) react to *S. aureus* and its exoproducts. We demonstrated that HCECs express TLR2 and -6 mRNA and respond to live *S. aureus*, its exoproducts, and PGN, but not LTA, by triggering activation of MAPKs and NF-κB signaling pathways. HCECs also responded by subsequent expression and secretion of proinflammatory cytokines as well as antimicrobial peptide hBD2. Thus, our results highlight the potential role

of the epithelium in sensing the presence of Gram-positive bacteria and providing signals that activate the defense mechanism of the cornea.

Materials and Methods

Reagents and Antibodies

LTA from *S. aureus*, which was repurified by phenolic extraction to remove any residual endotoxin,[51,52] was kindly provided by Siegfried Morath (University of Konstanz, Konstanz, Germany). Peptidoglycan (PGN) was purchased from Fluka Biochemika/Sigma-Aldrich (Buchs, Switzerland). This product has been shown to contain less than 0.0025 ng/mg endotoxin and are insensitive to polymixin B (binding to and inhibiting LPS).[53,54]

Anti-phospho-p38 MAPK mAb, anti-p38 antibody, anti-phspho JNK, anti-JNK antibody, anti-phospho-IκB-α, and anti-IκB-α antibodies were purchased from Cell Signaling Technology (Beverly, MA). Recombinant human β-defensin-2 protein and polyclonal anti-hBD-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

S. aureus Strain and Preparation of Bacterial-Conditioned Medium

S. aureus (strain 8325-4; a gift of John J. Indolo, Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK) was maintained in tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO). Before experimentation, bacteria were inoculated in 5 mL of TSB and incubated at 37°C until they reached midlogarithmic phase (optic density at 600 nm ($[D_{600}] \leq 0.5$). Bacteria were centrifuged and resuspended in prewarmed PBS to the desired cell density for inoculation of corneal epithelium cell cultures at a multiplicity of infection (MOI) of ~50 bacteria per cell, which was shown to be the lowest dose that stimulates optimal activation of signaling pathways in HCECs. Heat-killed bacteria were prepared in the same manner, but after suspension in PBS, they were heated to 80°C for 10 minutes and were assessed for nonviability by plating on TSB agar plates.

To prepare bacterial-conditioned medium, a chemically defined medium for staphylococci was used, and staphylococci grew as rapidly and reached high density in this medium as in tryptic soy broth.[55] This medium is known to enhance the production of cell-wall–associated products.[56] It consisted of five solutions. Solution 1 contained 20.1 g L-aspartic acid, L-$Na_2HPO_4 \cdot 12H_2O$; 3 g KH_2PO_4; 150 mg each of glutamic acid, L-isoleucine, L-leucine, L-proline, L-threonine, and L-valine; 100 mg each of L-alanine, L-arginine, glycine, L-histidine, L-lysine, L-methionine, L-phenylalanine, L-serine, L-tryptophan, and L-tyrosine; and 50 mg L-cystine, dissolved in 700 mL distilled water with pH adjusted to 7.2. Solution 2 contained 0.1 mg biotin, 2 mg nicotinic acid, 2 mg D-pantothenic acid, 4 mg pyridoxal, 4 mg pyridoxamine dihydrochloride, 2 mg riboflavin, and 2 mg thiamine hydrochloride dissolved in 100 mL distilled water. Solution 3 contained 20 mg adenine sulfate and 20 mg guanine hydrochloride dissolved in 0.1 M HCl with volume adjusted to 50 mL with distilled water. Solution 4 contained 10 mg $CaCl_2 \cdot 6H_2O$, 5 mg $MnSO_4$, and 3 mg $(NH_4)_2\ SO_4 \cdot FeSO_4 \cdot 6H_2O$ dissolved in 10 mL of 0.1 M HCl. Solution 5 contained 10 g glucose and 500 mg $MgSO_4 \cdot 7H_2O$ dissolved in 100 mL distilled water. Solutions 1 to 4 were mixed, and the volume was adjusted to 900 mL with distilled water. The mixed solution and solution 5 were autoclaved separately, and the two solutions were mixed before use. This medium was inoculated with bacteria from an agar plate and incubated overnight at 37°C with shaking. Subsequently, 1 mL of this growth culture was added to 25 mL of fresh medium and incubated at 37°C with constant shaking (150 rpm) for 8 to 10 hours, and bacterial growth was monitored by taking aliquots and measuring OD_{600} at various time intervals. The culture was stopped at late-logarithmic phase ($OD_{600} \leq 1$). Bacteria were removed by centrifugation at 12,000*g* for 30 minutes, and the supernatant was filtered through a 0.2-μm filter. The resultant conditioned medium was

assessed for its cytotoxic effects on epithelial cells by serial dilutions, and 1:8 or higher dilutions were found to have no significant effects on cell morphology after 24 hours' incubation. Thus, 1:10 dilution was used to challenge HCECs and THP-1 cells.

Human Corneal Epithelial Cell Cultures and *S. aureus* Challenges

Human telomerase-immortalized corneal epithelial (HUCL) cells, kindly provided by James G. Rheinwald and Irene K. Gipson,[57,58] were maintained in defined keratinocyte–serum-free medium (SFM; Invitrogen-Life Technologies, Carlsbad, CA) in a humidified 5% CO_2 incubator at 37°C. Before treatment, cells were split into culture dishes precoated with fibronectin-collagen (FNC; 1:3 mixture) coating mix (Athena Environmental Service, Inc., Baltimore, MD) and cultured in antibiotic-free defined keratinocyte-SFM. After cells were attached, the medium was replaced with keratinocyte basic medium (KBM) BioWhittaker (Walkersville, MD), and the cultures were incubated overnight (growth factor starvation).

To verify the results obtained from HUCL cells, HCECs were isolated from human donor corneas obtained from the Georgia Eye Bank. The epithelial sheet was separated from underlying stroma after overnight dispase treatment. The dissected epithelial sheet was trypsinized and the epithelial cells collected by centrifugation (500*g*, 5 minutes). HCECs were cultured in keratinocyte growth medium (KBM supplemented with growth factors; BioWhittaker) in T25 flasks coated with FNC and used at passage 3.

The human promonocytic cell line, THP-1, used as positive control for LTA- and PGN-stimulated cellular responses, was maintained in RPMI 1640 medium with 10% fetal calf serum, essential amino acids, and antibiotics. For stimulation, ~10^6 cells were plated in 2-mL cultures in six-well plates and challenged with LTA or PGN.

At the time of treatment, culture medium was replaced with fresh KBM containing live or heat-killed bacteria and the bacterial cell wall components LTA or PGN. At the indicated times, cells were processed for RNA preparation and immunoblot analysis, and conditioned media were collected for cytokine and hBD-2 assays.

Assessment of NF-κB, JNK, and p-38 Activation by Western Blot Analysis

Cells challenged either with bacteria or cell wall components were 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 phenylmethylsulfonyl fluoride [PMSF]), and protein concentration was determined with the bicinchoninic acid (BCA) assay (Micro BCA; Pierce Biotechnology, Rockford, IL). Proteins (30 μg/well) were separated by SDS-PAGE in Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) and electroblotted onto nitrocellulose transfer membranes. After blocking for 2 hours in PBST (20 mM Tris-HCl, 150 mM NaCl, 0.5% Tween) containing 5% nonfat milk, the blots were probed overnight with phospho-specific JNK and p-38 antibodies, as described by the manufacturer (Cell Signaling Technology). NF-κB activation was determined in terms of inhibitory IκB-α phosphorylation and degradation by using anti- Iκ-α and anti-phospho-IκB-α antibodies. After they were washed three times in PBST, membranes were incubated with secondary HRP-conjugated anti-mouse or anti-rabbit IgG for 1 hour. After the membranes were again were washed with PBST four times, 10 minutes each, proteins were visualized with reagents from Pierce (Supersignal).

RT-PCR Analysis of IL-6, IL-8, TNF-α, and hBD-2 Expression

RNA was isolated with extraction reagent (TRIzol; Invitrogen), and 2 μg of total RNA was reverse-transcribed with a first-strand synthesis system for RT-PCR (SuperScript; Invitrogen). cDNA was amplified by PCR with specific primers for human IL-6, IL-8, TNF-α, and hBD-2

(Table 1). IL-8 was amplified 30 cycles with annealing temperature at 58°C, and others were amplified 30 cycles with annealing temperature at 60°C. The PCR products (5 μL) and internal control GAPDH were subjected to electrophoresis on 1% agarose gels containing ethidium bromide. Stained gels were captured by digital camera (EDAS 290 system; Eastman Kodak, Rochester, NY).

Determination of IL-6, IL-8, and TNF-α Secretion from HCECs

Secretion of IL-6, IL-8, and TNF-α was determined by ELISA. HCECs were plated 5×10^6 cells/well in six-well plates. After growth factor starvation, cells were treated with peptidoglycan or *S. aureus*–conditioned medium for 6 hours, and culture media were harvested for measurement of IL-6, IL-8, and TNF-α. The ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The amount of the cytokines in culture media was normalized with the total amount of cellular protein lysed with radioimmunoprecipitation assay (RIPA) buffer. Protein concentration of cell lysate was determined with a BCA assay (MicroBCA; Pierce Biotechnology). Results were expressed as mean nanograms of cytokine per milligram cell lysate. All values are expressed as the mean ± SD. Statistical analysis was performed using ANOVA, and each pair showed a significant difference in IL-6, IL-8 and TNF-α secretion in the treated HUCL cells as well as primary HCECs (P <0.001).

Immunoblot Analysis of β-Defensin-2 Secretion

Accumulation of hBD-2 in the culture media of HUCL and primary HCECs (5×10^6 cells/well) treated with PGN or *S. aureus*–conditioned medium was detected by immunoblot analysis. Briefly, culture media were collected 6 hours after treatment and centrifuged, and 100 μL was applied to a nitrocellulose membrane (0.2 μm; Bio-Rad, Hercules, CA) by vacuum, using a slot-blot apparatus (Bio-Rad). Recombinant human (r)hBD-2 peptide (Santa Cruz Biotechnology) at concentrations of 0, 10, and 50 ng/mL in 100 μL KBM was also applied to the membrane as a positive control and serving as the peptide standard. The membrane was fixed by incubating with 10% formalin for 2 hours at room temperature followed by blocking in Tris-buffered saline (TBS) containing 5% nonfat powdered milk for 1 hour at room temperature. The membrane was then incubated overnight at room temperature with rabbit anti-human hBD-2 diluted 1:1000 in TBS containing 5% nonfat powdered milk, 5% goat serum, 0.05% Tween-20, and 0.02% sodium azide. After washing, the membrane was incubated for 1 hour at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:2000 with 5% nonfat powdered milk. Immunoreactivity was visualized with reagents from Pierce Biotechnology (Supersignal).

Results

Expression of TLR-1, -2, -6, and -9 in HCECs

TLR2, with TLR1 or -6 as the coreceptor, is a major receptor for Gram-positive bacterial cell wall components such as PGN and LTA. To determine whether HCECs express these TLRs, we assayed for the presence of their transcripts by RT-PCR (Fig. 1). TLR1 and -2 were expressed in both primary HCECs and HUCL cells whereas TLR6 mRNA was barely detectable. TLR9, another bacteria-recognizing receptor, was also expressed in these cells. THP-1 cells, a human monocyte line used as a positive control, expressed all four TLRs.

S. aureus Stimulated NF-κB and MAPK Signaling Pathways in HCECs

To determine epithelial responses to *S. aureus*, HUCL cells were infected at MOIs of ~50 live bacteria per cell with *S. aureus* NCTC8325. Activation of NF-κB in HUCL cells in response to bacterial challenge was assessed by immunodetection of IκB-α phosphorylation and

degradation, whereas activation of MAPKs was detected with phosphospecific antibodies (Fig. 2). Live staphylococci induced the degradation of IκB-α in a time-dependent manner. Phosphorylated IκB-α was detected within 1 hour, reached a higher level at 4 hours, and remained at this level for up to 6 hours postinfection (PI) (Fig. 2A). Concomitant to the increase in IκB-α phosphorylation, IκB-α degradation was clearly detectable 4 and 6 hours PI. In addition to the NF-κB activation, *S. aureus* also stimulated the activation of MAPK pathways, as evidenced by the increase in JNK and p-38 phosphorylation detected at 4 and 6 hours PI. Heat-killed staphylococci, on the contrary, did not stimulate the activation (phosphorylation) of these signal pathways (Fig. 2B).

Epithelial Responses to Bacterial Exoproducts

To determine whether the observed effects of *S. aureus* on epithelial signaling pathways are due to the bacterial exoproducts, which contain both secretory factors and shed cell wall components, bacteria-conditioned medium was prepared by growing the bacteria overnight in a chemically defined medium. When a 1:10 dilution of conditioned medium in KBM—a concentration determined to exert little effect on HUCL cell morphology for 24 hours—was used to stimulate HCECs, a rapid increase in phospho-IκB-α and phospho-p-38 was observed within 10 minutes (Fig. 3A). An increase in phosphorylation of JNK was detected 20 minutes after challenge. Primary HCECs were also tested and found to be responsive to the challenge of *S. aureus*–conditioned medium in terms of NF-κB, p-38, and JNK activation (Fig. 3B).

Effect of Bacterial Cell Wall Components on NF-κB and MAPK Pathways in HUCL Cells

The expression of TLR2 as well as TLR1 and -6 in HCECs suggests these cells would be responsive to TLR2 ligands. To test this hypothesis, we measured the ability of the TLR2 ligands, PGN and LTA, to activate NF-κB in HUCL cells (Fig. 4). HUCL cells were responsive to PGN at levels as low as 5 μg/mL as measured by IκB-α phosphorylation. Treatment of HUCL cells with 20 μg/mL PGN resulted in the maximum phosphorylation of IκB-α (Fig. 4A). In contrast, LTA did not induce IκB-α phosphorylation, even at a concentration as high as 50 μg/mL (Fig. 4A). THP-1 cells, the control human promonocytic line, have a robust response to LTA and PGN, as 1 μg/mL LTA or PGN stimulated extensive IκB-α phosphorylation as well as IκB-α degradation (Fig. 4B).

Time course studies of HCEC response to PGN and LTA (25 μg/mL) were also performed (Figs. 5 and 6). HUCL cells (Fig. 5A) stimulated with 20 μg/mL PGN resulted in Iκ-α phosphorylation which was detectable at 20 minutes, and reached a peak at 40 minutes after stimulation, this peak was followed by a slow decline, although phospho-IκB-α was still apparent at 2 hours. Accompanying the increase in IκB-α phosphorylation, IκB-α degradation was observed 20 minutes after stimulation and was maximum at 60 minutes after stimulation (Fig. 5A). IκB-α phosphorylation and degradation were also observed in primary HCECs treated with PGN (Fig. 5B). PGN also stimulated the activation of p38 and JNK1/2 as assessed by tyrosine phosphorylation in a time course parallel to NF-κB activation in both HUCL cells (Fig. 5A) and in primary HCECs (Fig. 5B). No changes in IκB-α phosphorylation or p38 and JNK1/2 activation were detected up to 2 hours in HUCL (Fig. 6A) and 1 hour in primary HCECs (Fig. 6B) treated with 25 μg/mL LTA. Taken together, these results indicate that the corneal epithelium is responsive to PGN but not to LTA in activation of TLR-mediated intracellular signaling pathways.

Effect of *S. aureus*–Conditioned Medium and PGN on the Expression or Secretion of Proinflammatory Cytokines and β-Defensin-2

To assess the biological relevance of induced signaling pathways, we determined the effect of *S. aureus*–conditioned medium and PGN on the expression and secretion of proinflammatory cytokines. IL-6, IL-8, TNF-α, and hBD-2 mRNA expression were determined by RT-PCR

(Fig. 7). The transcripts of all three proinflammatory cytokines, IL-6, IL-8, and TNF-α, were not detectable in nontreated cells (lane marked C). Both PGN (Fig. 7A) and *S. aureus*–conditioned medium (Fig. 7B) induced the expression of TNF-α after 15 minutes and IL-6 and IL-8 within 45 minutes after stimulation. Levels of mRNA for all cytokines remained elevated during the course of the study (6 hours). hBD-2 expression was induced 2 hours after stimulation by both PGN and *S. aureus*–conditioned medium. The levels of GAPDH (control for RT-PCR) remained largely unchanged in control and challenged cells during the course of the study.

The effects of PGN and *S. aureus*–conditioned medium on IL-6, IL-8, and TNF-α secretion in both HUCL cells and primary HCECs were assessed by ELISA (Fig. 8). Significantly increased amounts of IL-6, IL-8, and TNF-α accumulated in the culture media of HUCL cells (Fig. 8A) as well as primary HCECs (Fig. 8B) cells treated with PGN or *S. aureus*–conditioned medium. PGN appeared to be more effective than *S. aureus*–conditioned medium in stimulating IL-6 and -8 and TNF-α secretion in HCECs.

The effects of PGN and *S. aureus*–conditioned medium on hBD-2 secretion in both HUCL cells and primary HCECs were assessed by slot-blot assay (Fig. 9) with synthetic hBD-2 peptide as the control (Fig. 9A). Although there was almost no hBD-2 detected in the medium of control, nontreated cells, treatment of both HUCL (Fig. 9B) and primary HCECs (Fig. 9C) with PGN and *S. aureus*–conditioned medium resulted in an increase in hBD-2 secretion. PGN appeared to be more effective in induction of hBD-2 secretion when compared with *S. aureus*–conditioned medium. More than 20 ng hBD-2 was secreted by 5×10^6 cells in a well of six-well plate.

Discussion

In this study, we demonstrated that *S. aureus* and its exoproducts stimulate multiple signaling pathways, including NF-κB, p38, and JNK and subsequent expression or secretion of proinflammatory cytokines as well as the antimicrobial peptide β-defensin-2 in HCECs. We also demonstrated that corneal epithelial cells express TLR2, -1, -6, and -9, the innate immunity receptors of Gram-positive bacteria, and respond to PGN, but not to LTA, by eliciting inflammatory responses. Thus, our data indicate that PGN from *S. aureus*, through its TLR2 receptor, may play a role in initiating an innate immune response in epithelium and subsequent inflammation in the cornea in response to infections such as Gram-positive bacterial keratitis.

A major cause of tissue damage during staphylococcal keratitis is the host inflammatory response to infection.[5] Because stromal infiltration is the hallmark of local inflammation,[59] it is not clear whether corneal epithelium, in addition to providing a biological barrier, plays any role in corneal response to staphylococcal infection. To invade the cornea, bacteria must first interact with and penetrate epithelial cells. The major findings of our study, using cultured epithelial cells as a model, are that corneal epithelial cells recognize and respond to *S. aureus* by expressing and secreting proinflammatory cytokines such as IL-6 and TNF-α, chemokines such as IL-8, and the antimicrobial peptide β-defensin-2. Although β-defensins may eliminate the invading bacteria through their direct antimicrobial action, the cytokines released by the epithelial cells may recruit inflammatory cells to the site of infection. Thus, the epithelial lining is an integral part of the innate immune response machinery and plays a role in the initiation of early cytokine and inflammatory responses to Gram-positive bacteria, such as *S. aureus*, in the cornea.[10]

S. aureus can potentially induce epithelial responses in several ways. First, bacteria may invade corneal epithelial cells as demonstrated by Jett and Gilmore.[60] The internalized bacteria may trigger an epithelial response by intracellular pattern recognizing receptors such as TLR9 or

nucleotide-binding oligomerization domain 1 (NOD1).[61] Second, secreted proteins such as α-toxin and elastase may have stimulative effects on epithelial cells. *Staphylococcus* α-toxin is released by bacteria as a 293-residue, single-chain polypeptide. On interaction with a target cell, monomers of α-toxin polymerize to form heptamers, which results in the formation of a transmembrane pore and causes membrane damage as well as an influx of extracellular calcium. This influx of calcium is a potent damage-related stimulus in many cells.[62] Both α-toxin and elastase were shown to be major virulence factors in *S. aureus* keratitis.[19,63,64] The ability of *S. aureus*–conditioned medium to stimulate a rapid HCEC response suggests a possible involvement of these factors. It is not clear whether these factors, in addition to their cytotoxic effects, can directly stimulate proinflammatory cytokine expression and production in epithelial cells. Third, the cell wall components of *S. aureus* such as PGN and LTA, either at the surface or as shed products, are recognized by epithelial cell surface receptors, the TLRs. This results in production of proinflammatory cytokines and innate defense molecules. Detection of TLR2, -1, and -6 in corneal epithelial cells and their rapid response to *S. aureus*–conditioned medium suggest that PGN and LTA may be PAMPs recognizable by corneal epithelial cells.

Cells of myeloid origin recognize and are activated by both PGN and LTA of Gram-positive bacteria in a TLR2-dependent manner.[37,49,50] However, intestinal epithelial cells that express low levels of TLR2 mRNA compared with THP-1 monocytes have been shown to be unresponsive to TLR2 ligands, including the *Staphylococcus*-derived PGN and LTA.[30] This broad unresponsiveness to TLR2 ligands provides an explanation as to why intestinal epithelial cells, although they are constantly exposed to a high density of Gram-positive bacteria, do not respond to and produce chronic proinflammatory cytokines in response to commensal Gram-positive bacteria in the gut. In contrast, airway epithelial cells respond to *S. aureus* and produce a variety of proinflammatory cytokines in a TLR-dependent manner in vitro and in vivo.[31,65] The epithelium-produced proinflammatory cytokines not only recruit polymorphonuclear leukocytes to the site of infection but also enhance their survival.[66] PGN, as a shed product, appears to be the molecule responsible for stimulating airway epithelial IL-8 expression.[31] Thus, the responses of corneal epithelial cells to *S. aureus* and its exoproducts that are documented herein suggest that corneal epithelial cells are similar to that of airway, but not intestinal epithelial cells, in innate immune responses to Gram-positive bacterial infection. Thus, we propose that TLR2 is the innate receptor for PGN and can function as a Gram-positive bacterial sensor in the cornea for recognizing the presence of bacteria or bacterial products and mediating corneal innate immune responses that result in the clearing of pathogen by corneal host mechanisms.

It was a surprise that LTA did not activate NF-κB signaling, although TLR-2 is expressed in the primary and HUCL cell lines used in this study. Recently, LTA was shown to be required in *S. aureus* nasal colonization.[67] It was also shown to induce cyclooxygenase-2 protein expression and mucin-2 production in human pulmonary epithelial cells.[68,69] In the latter case, the epithelial cell response to LTA, unlike that in macrophages, is dependent on transactivation of epidermal growth factor receptor and may not require TLRs. The recognition of PAMPs by epithelial cells is dependent not only on the expression but also on the distribution and function of TLRs, associated adapter proteins, and signaling kinases. A more complete analysis of epithelial TLR function in the cornea is necessary for understanding the mechanisms underlying epithelial cell responses to PGN, but not LTA.

In summary, our study suggests that corneal epithelial cells can recognize Gram-positive bacteria. These cells can also initiate innate immune responses that result in clearing of pathogens and, potentially, an excessive inflammatory response that results in corneal scarring and loss of vision. To the best of our knowledge, this study represents the first identification of an *S. aureus* surface ligand in the corneal epithelium and the first demonstration of its

importance in triggering inflammatory responses in the cornea. Understanding the molecular events of bacteria–epithelium interactions and the inflammatory consequences of TLR activation may permit the development of novel, specific therapies that can promote innate defense and prevent some of the destructive consequences of ocular Gram-positive infections.

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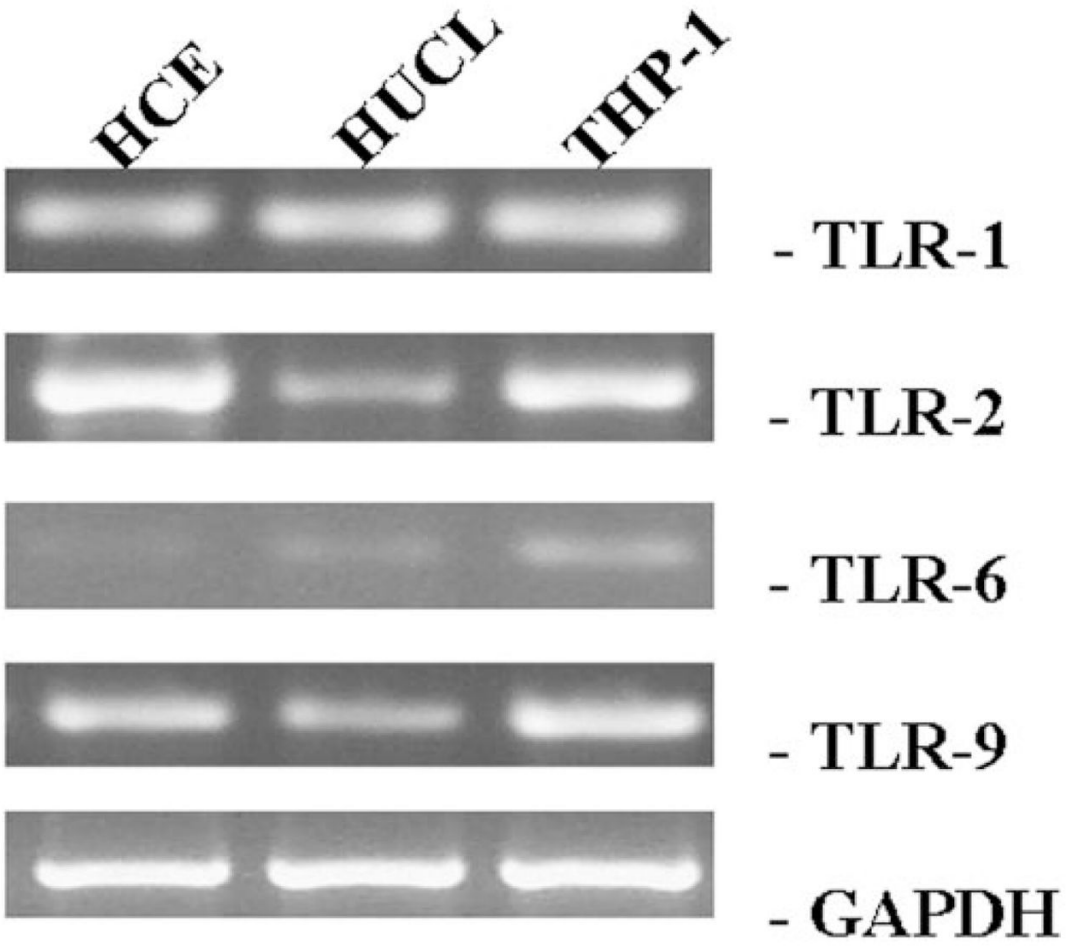

Figure 1.
Expression of TLR-1, -2, -6 and -9 in primary HCECs (HCE), HUCL, and THP-1 was analyzed by PCR after reverse transcription of mRNA. GAPDH was analyzed to verify similar cDNA loading. THP-1 was used as a positive control. Results shown are representative of three experiments with similar results.

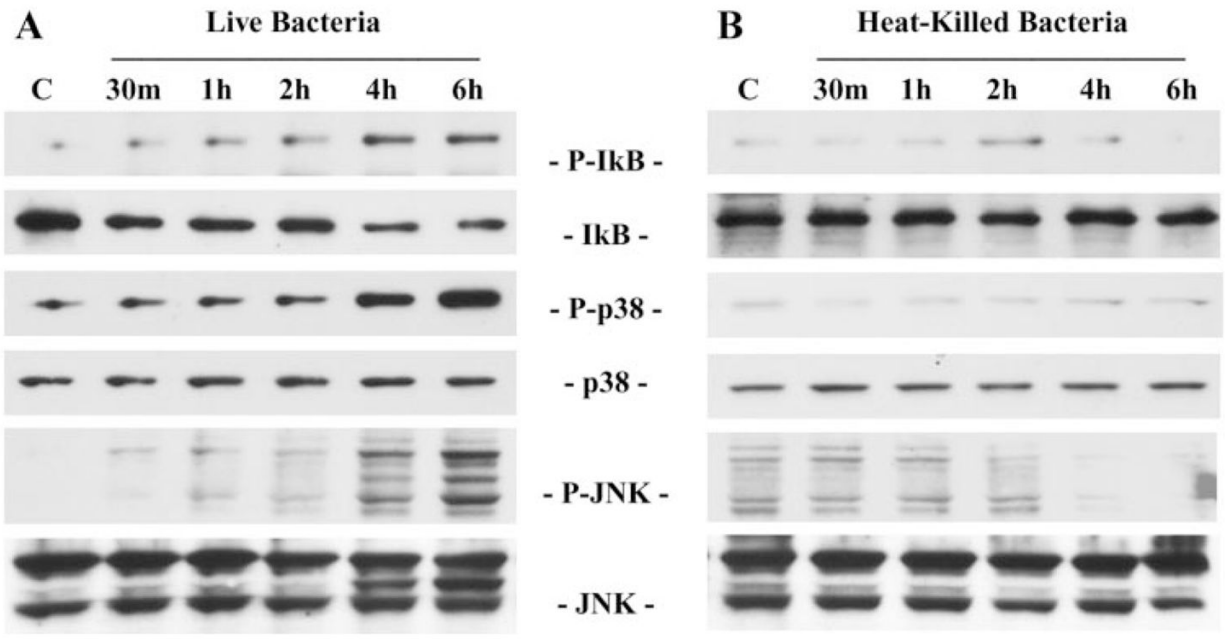

Figure 2.

HCECs were responsive to *S. aureus* exposure. HUCL cells were exposed to live *S. aureus* (**A**) or heat-killed bacteria (**B**) for the indicated times, and cells were lysed for Western blot analysis with antibodies against phospho-IκB-α (P-IκB-α), phospho-p38 (P-p38), phospho-JNK (P-JNK), and IκB-α (IκB). Antibodies against non-phosphorylated p38 and JNK were used to show that there were no changes in the protein levels and to normalize sample loading. HCECs challenged with live bacteria showed significant activation of NF-κB, p-38, and JNK signaling pathways in a time-dependent manner, whereas no such change was observed in cells treated with heat-inactivated bacteria. Results shown are representative of three independent experiments.

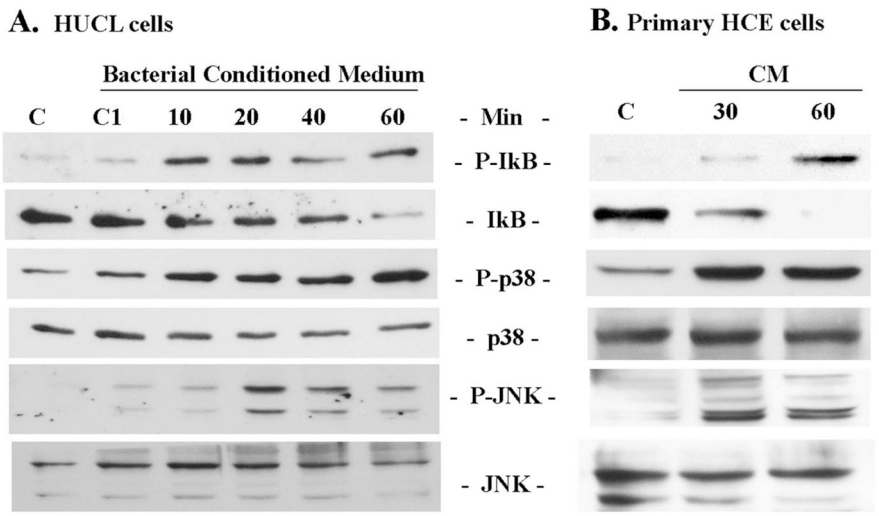

Figure 3.

S. aureus–conditioned medium induced activation of NF-*κ*B and MAPKs. HUCL cells (**A**) or primary HCECs (**B**) isolated from human corneas were incubated with 1:10 dilution of conditioned medium in KBM for the indicated times (in minutes) with KBM alone (C) and KBM + synthetic medium (C1) as controls. Cells were lysed and results displayed as described in Figure 2. *S. aureus*–conditioned medium induced rapid activation of NF-*κ*B, p-38, and JNK in HCECs. Results shown are representative of three independent experiments.

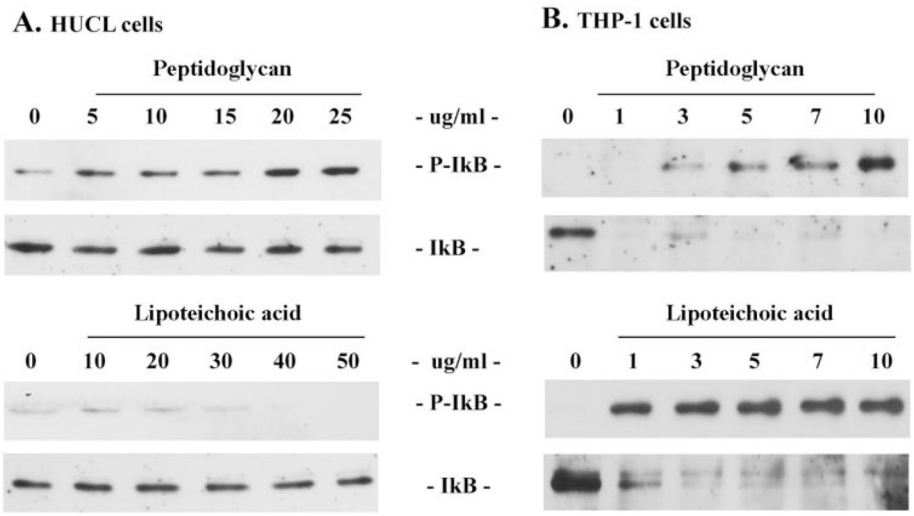

Figure 4.

Dose-dependent effect of PGN and LTA on NF-*κ*B activation. HUCL cells (**A**) or THP-1 (**B**) cells were stimulated with increasing concentrations of PGN or LTA for 1 hour and analyzed for I*κ*B-*α* phosphorylation (P-I*κ*B) and degradation (I*κ*B) by Western blot analysis. PGN, but not LTA, induced NF-*κ*B activation in HUCL cells in a concentration-dependent manner. Both TLA and PGN induced rapid and strong responses in the control THP-1 cells. Results shown are representative of four independent experiments.

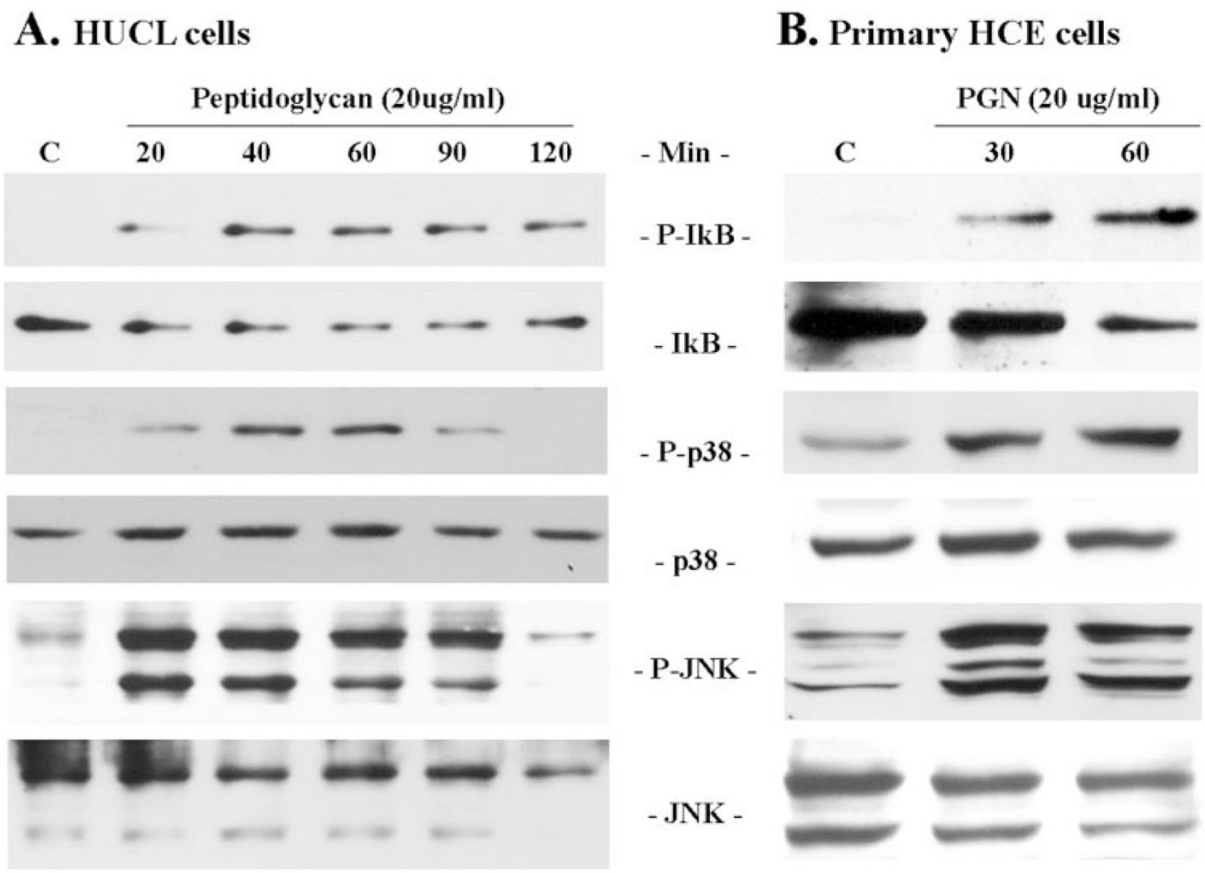

Figure 5.

PGN mediated activation of NF-κB and MAPKs. HUCL cells (**A**) and primary HCECs (**B**) were challenged for various time intervals with PGN (20 μg/mL). Cells were lysed for Western blot analysis using antibodies against phospho-IκB-α (P-IκB-α), phospho-p38 (P-p38), phospho-JNK (P-JNK), and IκB-α (IκB). Antibodies against p38 and JNK were used to show that there are no changes in protein levels and to normalize sample loading. PGN triggered NF-κB and MAPK activation in HUCL cells as well as in primary HCECs in a time-dependent manner. Results shown are representative of two independent experiments.

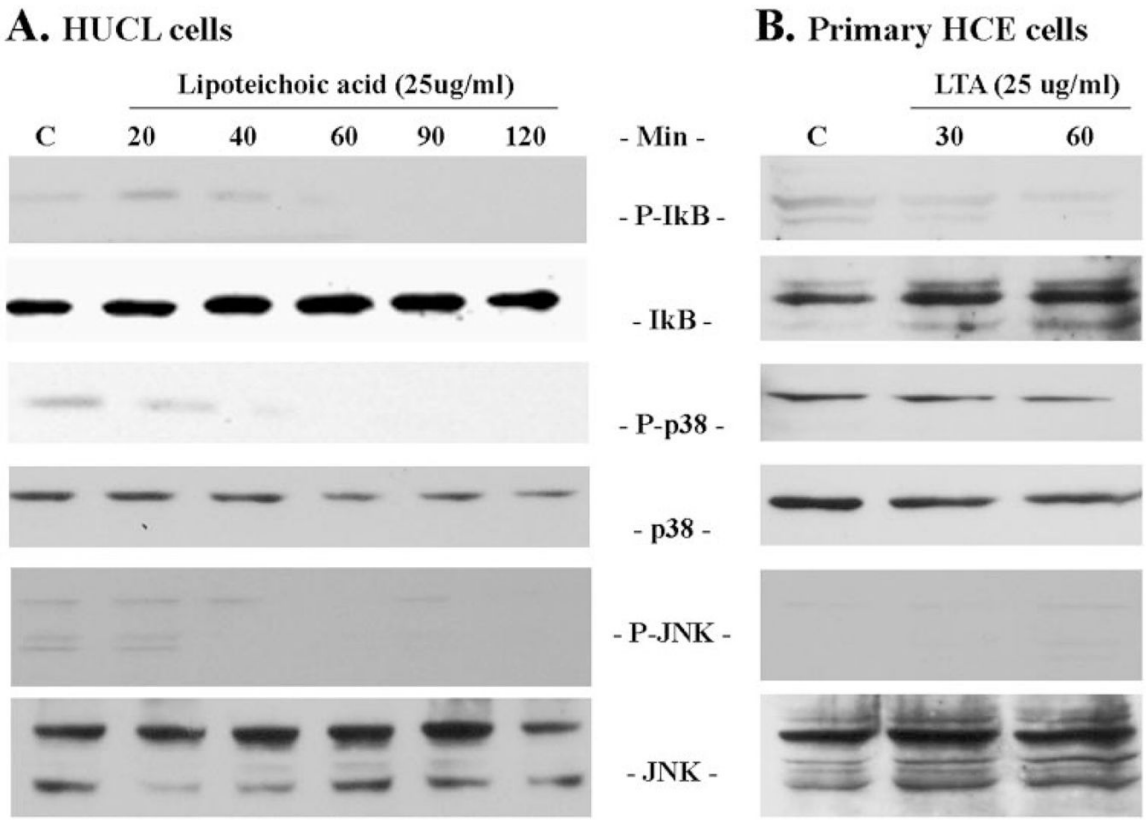

Figure 6.

LTA did not induce activation of NF-κB and MAPKs. HUCL cells (**A**) and primary HCECs (**B**) were stimulated with LTA (25 μg/mL) for various time intervals. Cells were lysed for Western blot analysis using antibodies against phospho-IκB-α (P-IκB-α), phospho-p38 (P-p38), phospho-JNK (P-JNK), and IκB-α (IκB). Antibodies against p38 and JNK were used to show that there are no changes in protein levels and to normalize sample loading. LTA did not trigger NF-κB and MAPK activation in HUCL cells or primary HCECs. Results shown are representative of three independent experiments.

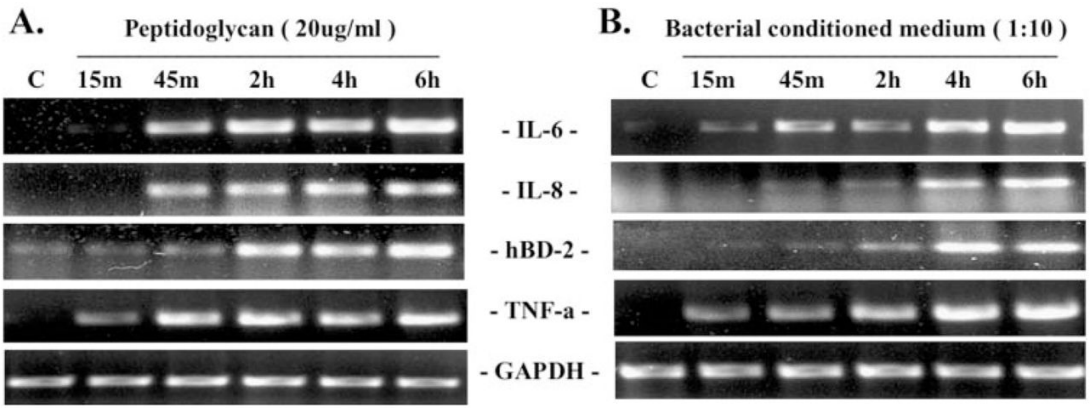

Figure 7.

PGN and *S. aureus*– conditioned medium induced IL-6, IL-8, hBD2, and TNF-α mRNA expression in HUCL cells. HUCL cells grown overnight in KBM were stimulated with 20 μg/mL PGN or 1:10 dilution of conditioned medium in KBM for the indicated times. Total RNA was extracted, reverse transcribed, and amplified using specific primers (Table 1), with GAPDH as the control. PCR products were separated by electrophoresis and stained. HUCL cells expressed the proinflammatory cytokines IL-6, IL-8, and TNF-α, as well as β-defensin-2, in response to PGN and *S. aureus*– conditioned medium challenge. Results shown are representative of three independent experiments.

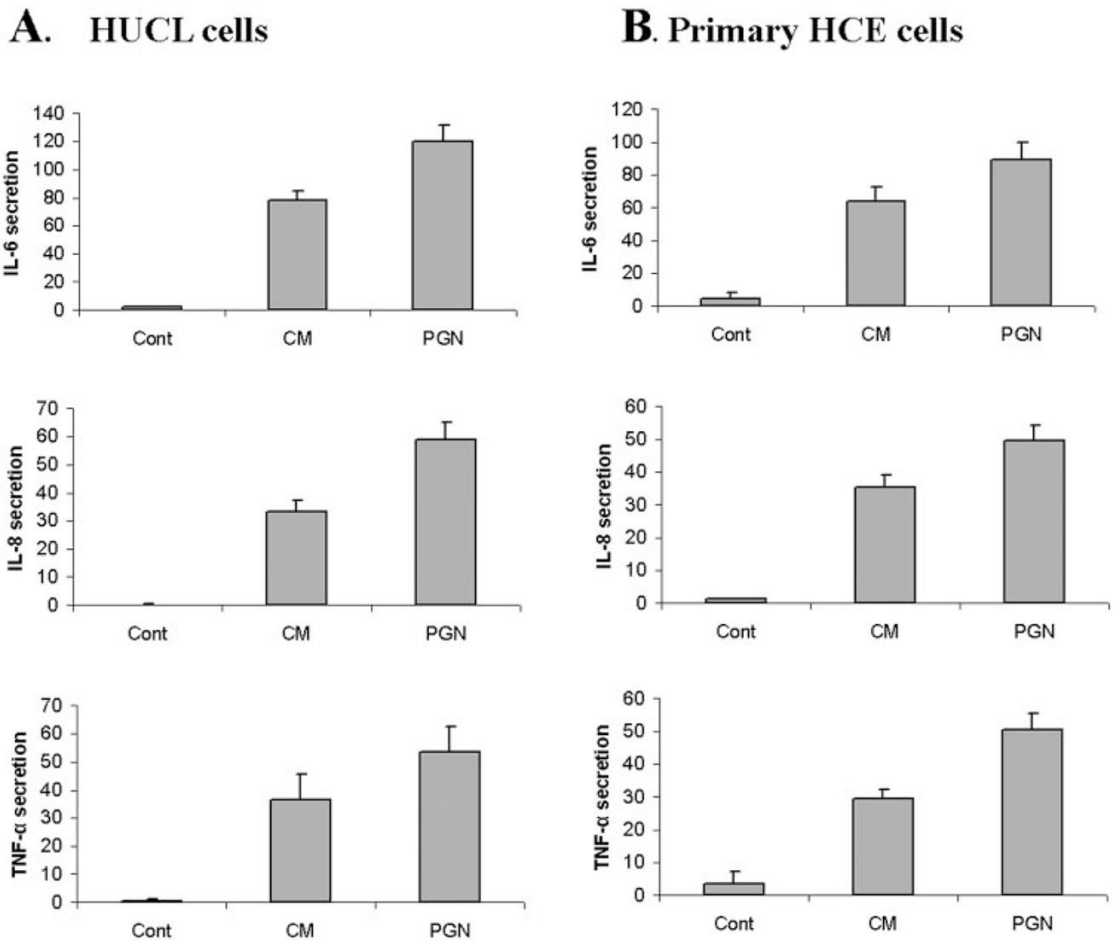

Figure 8.

IL-6, -8, and TNF-α secretion in HCECs in response to PGN and *S. aureus*–conditioned medium challenge. HUCL cells (A) and primary HCECs (B) grown overnight in KBM were treated with 20 μg/mL PGN or 1:10 dilution of *S. aureus*–conditioned medium for 6 hours, and the released IL-6, IL-8, and TNF-α in cell culture supernatants were measured by ELISA. The amount of cytokines was normalized with protein concentration of cell lysate (nanograms per milligram cell lysate). The data shown are representative of triplicate experiments.

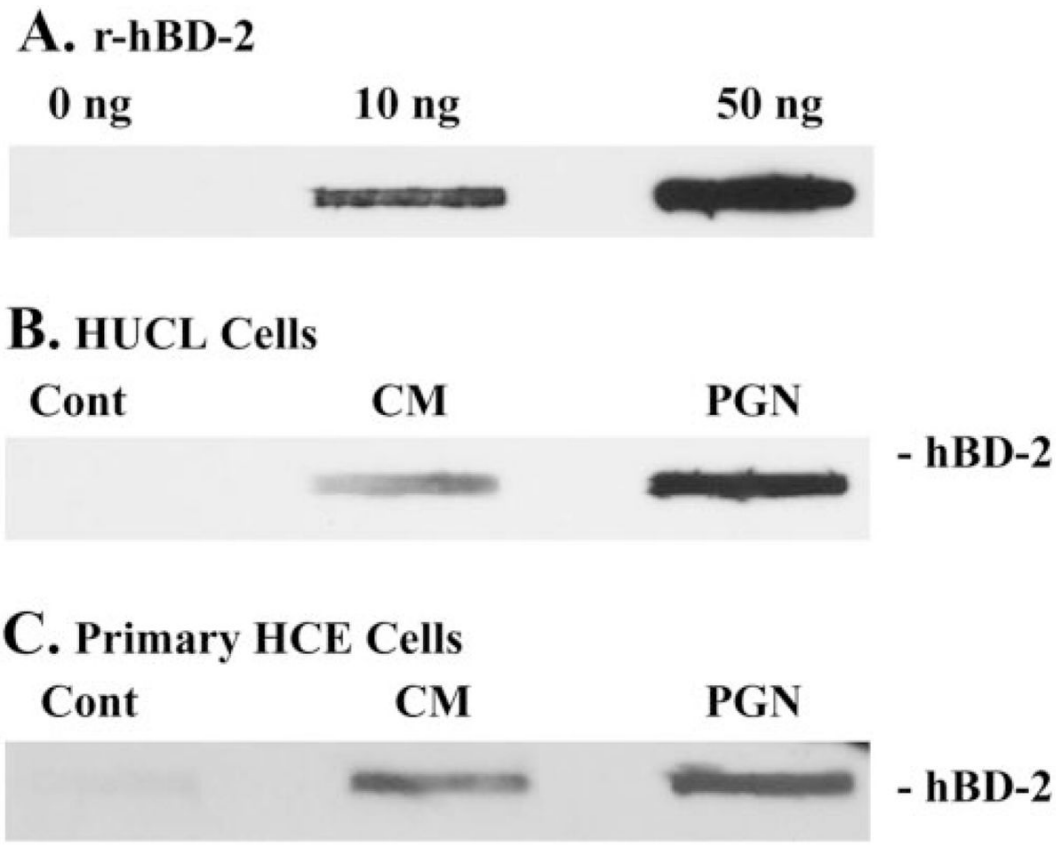

Figure 9.

PGN and *S. aureus*–conditioned medium induced hBD-2 secretion in HCECs. HUCL cells (**B**) or primary HCECs (**C**) were incubated with serum-free medium alone (Cont), media containing 20 μg/mL PGN (PGN), or a 1:10 dilution of *S. aureus*–conditioned medium (CM) for 6 hours. The secretion of hBD-2 peptide into the culture medium was detected by immunoblot analysis. To each slot-blot well, 100 μL culture medium was added and probed with anti-hBD-2 antibody. Recombinant human (r)hBD-2 peptide (10 ng/mL and 50 ng/mL) was included as positive control (**A**). Results are representative of triplicate experiments.

Table 1

Sequences and Product Sizes of PCR Primers

Gene	Primer	Sequence	Product Size (bp)
IL-6	Forward	**CTCCTTCTCCACAAGCGCCTTC**	583
	Reverse	**GCGCAGAATGAGATGAGTTGTC**	
IL-8	Forward	**GCAGTTTTGCCAAGGAGTGCTA**	376
	Reverse	**GCATCTGGCAACCCTACAACAAG**	
TNF-*α*	Forward	**GAAAGCATGATCCGGGACGTG**	510
	Reverse	**GATGGCAGAGAGGAGGTTGAC**	
hBD2	Forward	**CCAGCCATCAGCCATGAGGGT**	255
	Reverse	**GGAGCCCTTTCTGAATCCGCA**	
GAPDH	Forward	**CACCACCAACTGCTTAGCAC**	515
	Reverse	**CCCTGTTGCTGTAGCCAAAT**	
TLR-1	Forward	**GGCTGGCCTGATTCTTATAA**	335
	Reverse	**CTCTAGGTTTGGCAATAATT**	
TLR-2	Forward	**GTGGCCAGCAGGTTCAGGATG**	641
	Reverse	**AGGACTTTATCGCAGCTCTCAG**	
TLR-6	Forward	**TGTGACTGTGACCTCCCTCTGC**	366
	Reverse	**GTGATGGGCAAAATAGAGTTCG**	
TLR-9	Forward	**TCGCTGCTGGCTGTGGCTCTG**	604
	Reverse	**CGGTTATAGAAGTGGTGGTTG**	