Response of corneal epithelial cells to *Staphylococcus aureus*

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*Staphylococcus aureus* is a leading cause of invasive infection. It also infects wet mucosal tissues including the cornea and conjunctiva. Conflicting evidence exists on the expression of Toll-like receptors by human corneal epithelial cells. It was therefore of interest to determine how epithelial cells from this immune privileged tissue respond to *S. aureus*. Further, it was of interest to determine whether cytolytic toxins, with the potential to cause ion flux or potentially permit effector molecule movement across the target cell membrane, alter the response. Microarrays were used to globally assess the response of human corneal epithelial cells to *S. aureus*. A large increase in abundance of transcripts encoding the antimicrobial dendritic cell chemokine, CCL20, was observed. CCL20 release into the medium was detected, and this response was found to be largely TLR2 and NOD2 independent. Corneal epithelial cells also respond to *S. aureus* by increasing the intracellular abundance of mRNA for inflammatory mediators, transcription factors, and genes related to MAP kinase pathways, in ways similar to other cell types. The corneal epithelial cell response was surprisingly unaffected by toxin exposure. Toxin exposure did, however, induce a stress response. Although model toxigenic and non-toxigenic strains of *S. aureus* were employed in the present study, the results obtained were strikingly similar to those reported for stimulation of vaginal epithelial cells by clinical toxic shock toxin expressing isolates, demonstrating that the initial epithelial cellular responses to *S. aureus* are largely independent of strain as well as epithelial cell tissue source.

**Introduction**

*Staphylococcus aureus*, a commensal of the wet mucosa and skin,1-3 is a leading cause of invasive infection.4-6 It is also a leading cause of keratitis, a community acquired infection that occurs mainly in contact lens wearers and those with corneal injury.7,8 Hospital and community isolates both are increasingly methicillin resistant and resistant to third generation fluoroquinolones.9-11 Isolates of *S. aureus* are largely independent of strain as well as epithelial cell tissue source.9-11 Virulence 1:4, 223-235; July/August 2010; © 2010 Landes Bioscience

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have been shown to alter the biology of target cells by forming transient pores,25 and to constitute a translocation mechanism mediating the entry of bacterial effector molecules into eukaryotic cells26 (with the potential for added significance given the evidence cited above for intracellular expression of toll-like receptors), it was of interest to examine how toxin production by S. aureus contributed to the epithelial cell response. Therefore, the global transcriptional responses of human corneal epithelial cells to model strains of S. aureus either expressing pore forming toxins, or globally defective in toxin expression, were assessed. It was of further interest to determine whether the main transcriptional changes observed were reflected by the expression of modulators of the immune system, and to assess whether the main responses could be recapitulated using specific ligands for toll-like receptors.

**Results**

Changes in transcript levels in corneal epithelial cells resulting from exposure to S. aureus. Exposure of confluent monolayers of HCEC27 to S. aureus was optimized to permit the maximum stimulation of epithelial cells without inducing measurable damage caused by pore-forming toxins, such as α-toxin (Fig. 1A). It was found that seeding the monolayer with an initial S. aureus MOI of 20 resulted in >90% HCEC viability after 6 h incubation (Fig. 1A), and between 80% and 90% epithelial cell survival after 8 h. Rate of toxin production during by S. aureus strain RN6390 or control strain ALC135 during this coculture was also assessed (Fig. 1B). Toxin expression by RN6390, as evinced by hemolysis, was detectable after 4 h. Strain ALC135 (agr::tetM, sar::Tn917LTV1) did not produce measurable hemolytic activity under these conditions as expected.

Having established optimum conditions for epithelial cell exposure to S. aureus, the response of HCECs to exposure to agr/sar (RN6390) or agr/sar (ALC135) S. aureus was assessed by microarray. As illustrated in Figure 2, the abundance of about 650 species of mRNA was altered in HCECs exposed to either RN6390 or ALC135. Although some agr/sar-dependent differences were noted, the responses of epithelial cells to both S. aureus strains were strikingly similar. An especially large (~400 fold) increase in the abundance of mRNA encoding the antimicrobial dendritic cell chemokine CCL20,28 was noted. However, no significant differences were found in mRNA specifying human β-defensins, cathelicidin (LL37), liver-expressed antimicrobial protein (LEAP)1, and LEAP2, which are known to be expressed at a basal level by healthy human corneal epithelial cells.29 Other large (20–60 fold) increases in mRNA abundance were detected for proinflammatory factors as expected, including IL8, CSF2, CXCL1, IL24, IL6, IL1α, IL1β and TNFα (Table 1). Significant S. aureus-dependent increases in mRNA abundance were noted for several species involved in signal transduction, including SERPINB2, FOS, TNFAIP3, DUSP1 and INHBA (Table 1). Examination of S. aureus induced changes in gene expression in epithelial cells showed that some of the changes grouped by pathway related to inflammation, proliferation, and wound-healing (Table 2). A complete list of changes in gene expression is provided in Supplemental Table S1. Key microarray results were independently verified by real-time quantitative PCR (Table 3).
S. aureus stimulation could be recapitulated via TLR2 or TLR4 stimulation, HCECs were stimulated with Pam3Cys and LPS, respectively. To control for possible stimulation through NOD2, MDP was also tested. In shown in Figure 5A, LPS stimulation of HCEC resulted in a significant increase in CCL20 secretion, (CRY)AB, DNAJB4, and activator of heat shock 90 kDa ATPase homolog (AHA)1.

Impact of S. aureus toxins on corneal epithelial cell defense responses. Supernatants from the HCEC/S. aureus co-cultures were assessed for the production of mediators of inflammation identified in transcriptome analysis and known to be elicited by S. aureus in other contexts.17,23,30-33 The greatest secretion of inflammatory mediators into the medium was noted for IL6, IL8, IL17, and interferon (IFN)γ (Fig. 3A). For most inflammatory mediators, release was independent of agr/sar regulated toxin expression. However, the non-toxigenic S. aureus strain induced greater IL-17 release and lower IL-6 release than its toxigenic parent. IFNγ production by corneal epithelial cells has not, to our knowledge, been reported in response to bacterial stimulation. To insure this finding was not an artifact of the SV40 immortalization of the HCEC cell line, two other corneal epithelial cell lines, HCLE and primary HCE, were also tested. As shown in Figure 3B, IFNγ was induced in both cell types by both S. aureus strains. IFNγ secretion was more abundant from bacterially stimulated HCEC cells, but primary cells also secreted readily detectable quantities of IFNγ. Interestingly, no detectable differences in IFNγ transcript levels in HCECs were observed, however (Table S1), indicating that the release of this factor may have stemmed from preformed pools or is controlled at the posttranscriptional level. Pools of IFNγ are known to exist in polymorphonuclear neutrophils (PMNs).29

CCL20 is secreted by HCEC and primary HCE cultures upon exposure to S. aureus. The large increase in CCL20 transcripts in HCEC co-cultured with either toxigenic or non-toxigenic S. aureus suggests that production of this antimicrobial chemokine is a major response of the corneal epithelial cell to sensing S. aureus. An increase in CCL20 in the culture supernatants of HCECs co-cultured with either S. aureus strain was also detected (3.2 ± 0.5 fold from exposure to RN6390 and 2.2 ± 0.6 fold from exposure to ALC135, Fig. 4A). No CCL20 release was noted in response to S. aureus stimulation of HCECs co-culture with Gram positive bacterium, Lactococcus lactis (Fig. 4A). Inclusion of cytochalasin D did not affect these results, indicating that S. aureus stimulation of HCECs did not require internalization (data not shown).34 To determine whether this response was specific to the HCEC cell line, primary HCE were stimulated with either RN6390 or ALC135, and the release of CCL20 was followed over time. CCL20 released by primary HCE was similar to that of HCECs, occurred as early as 1 h post-exposure to S. aureus, and was maximal at the end of the 8 h experiment (Fig. 4B).

Effects of TLR2 and TLR4 agonists on CCL20 expression. To determine whether CCL20 release by HCECs in response to S. aureus stimulation could be recapitulated via TLR2 or TLR4 stimulation, HCECs were stimulated with Pam3Cys and LPS, respectively. To control for possible stimulation through NOD2, MDP was also tested. In shown in Figure 5A, LPS stimulation of HCEC resulted in a significant increase in CCL20 secretion,
Inflammatory mediators

| Gene     | Rel. abundancea | GenBank No. | Biological function         |
|----------|-----------------|-------------|-----------------------------|
| CCL20    | 355             | NM_004591  | inflammatory chemokine      |
| IL8      | 58              | NM_000584  | inflammatory chemokine      |
| CSF2     | 42              | NM_000758  | inflammatory cytokine       |
| CXCL1    | 27              | NM_001511  | inflammatory chemokine      |
| IL24     | 25              | NM_006850  | inflammatory cytokine       |
| IL6      | 19              | NM_000600  | inflammatory cytokine       |
| CXCL3    | 17              | NM_002090  | inflammatory chemokine      |
| CXCL2    | 16              | NM_002089  | inflammatory chemokine      |
| IL1α     | 12              | NM_000575  | inflammatory cytokine       |
| IL1β     | 10              | NM_000576  | inflammatory cytokine       |
| TNFα     | 10              | NM_000594  | inflammatory cytokine       |

Signal Transduction

| Gene     | Rel. abundancea | GenBank No. | Biological function       |
|----------|-----------------|-------------|---------------------------|
| SERPINB2 | 52              | NM_002575  | PLAT/PLAU regulator        |
| FOS      | 40              | NM_005252  | transcriptional regulator  |
| TNFAIP3  | 40              | NM_006290  | NFκB regulator            |
| DUSP1    | 34              | NM_004417  | MAPK regulator             |
| INHBA    | 31              | NM_002192  | TGFβ regulator             |
| GADD34   | 15              | NM_014330  | cell cycle regulator       |
| STC1     | 12              | NM_003155  | Ca/PO homeostasis          |

Downregulated molecules

| Gene     | Rel. abundancea | GenBank No. | Biological function       |
|----------|-----------------|-------------|---------------------------|
| ZAP      | -5              | NM_019589  | nucleoside kinase          |
| CCNF     | -5              | NM_001761  | cell cycle regulator       |
| ASPM     | -4              | NM_018136  | cell cycle regulator       |
| SC4MOL   | -4              | NM_006745  | cholesterol biosynthesis   |
| GJA5     | -4              | NM_005266  | gap junctions              |

*Relative abundance was calculated as the average normalized gene expression in a given *S. aureus*-infected group versus the untreated group, n = 3.

Table 1. Greatest changes in HCECs transcript abundance as the result of exposure to *S. aureus*

We found that human corneal epithelial cells respond to toxin-expressing, or non-toxigenic *S. aureus* in strikingly similar ways under the conditions tested, indicating that the initial epithelial cell responses are driven by toxin-independent mechanisms. By far the greatest change in the transcriptome was in the level of CCL20 expression. Furthermore, stimulation of CCL20 release appeared to be independent of TLR2 in primary HCEs and HCECs, whereas HCLE cells appeared to respond to the TLR2 agonist Pam3Cys. This cell line was the one observed by others to respond to TLR2 ligands derived from the *S. aureus* cell wall, and stimulation generated antimicrobial peptide production.

CCL20 displays both chemokine and anti-bacterial properties which has been shown to interact with CCR6, a characteristic shared with beta-defensins. Furthermore, this ligand-receptor pair acts as a chemoattractant of immature dendritic cells (DC). Originally it was thought that the cornea had no local immature DCs, but a report by Hamrah et al. identified bone marrow and monocyte derived DCs in the anterior normal stroma. Since these immature DCs exist, increased secretion of CCL20 may attract them to the site of infection for antigen processing. The rapid, large increase in CCL20 transcript abundance upon exposure of HCECs to *S. aureus* implicates this chemokine as an important early effector of the host response.

In intestinal epithelial cells, it has been shown that CCL20 can be induced by a variety of pathogens, including *Clostridium difficile*, and is not induced by non-pathogens such as *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Lactobacillus salivarius* and *Mycobacterium smegmatis*. Furthermore CCL20 expression is modulated by some commensals. Sibratic et al. showed that the presence of *B. infantis* could decrease the induction of CCL20 by pathogens and, in other work, *Lactobacillus rhamnosus* GG suppressed CCL20 expression from IECs exposed to flagellin or flagellated non-pathogenic *Escherichia coli*. In addition to bacteria and bacterial components, CCL20 can be induced by IL1β, TNFα or IFNγ which has been found in various epithelial cell lines, including our HCECs, responding to *S. aureus*. CCL20 production was not induced in our studies by non-pathogenic *L. lactis*, supporting the notion that there are epithelial sensing mechanisms that are pathogen-specific.

Discussion

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Table 2. Canonical pathways affected by *S. aureus*

| Gene       | Rel. Abundance | GenBank No. | Biological function               |
|------------|----------------|-------------|-----------------------------------|
| **MAPK signaling pathway (p = 5.3 x 10^-5)** |                |             |                                   |
| TNFα       | 10             | NM_000594   | inflammatory cytokine            |
| IL1α       | 12             | NM_000575   | inflammatory cytokine            |
| IL1β       | 10             | NM_000576   | inflammatory cytokine            |
| EGFR       | 4              | NM_201282   | growth factor receptor           |
| DUSP1      | 34             | NM_004417   | MAPK regulator                    |
| PTPRR      | 2              | NM_002849   | MAPK regulator                    |
| PPM1A      | 2              | NM_021003   | MAPKK regulator                  |
| MEK1       | 3              | NM_002755   | MAPKK regulator                  |
| TRIF       | 3              | NM_182919   | MAPKK regulator                  |
| FOSB       | 40             | NM_006732   | transcriptional regulator        |
| NUR77      | 10             | NM_002135   | transcriptional regulator        |
| JUN        | 9              | NM_002228   | transcriptional regulator        |
| NFκB1      | 3              | NM_003998   | transcriptional regulator        |
| JunD       | 2              | NM_005354   | transcriptional regulator        |
| **Cell cycle regulatory signaling (p = 0.002)** |                |             |                                   |
| CCNB       | -2             | NM_031966   | G1, M phase regulator            |
| PLK1       | -3             | NM_005030   | CCNB regulator                   |
| CDC25C     | -2             | NM_001790   | CCNB regulator                   |
| CDC20      | -2             | NM_001255   | M phase CCN regulator            |
| **(a) TGFβ-dependent** |                |             |                                   |
| INHBA      | 31             | NM_002192   | TGFβ regulator                   |
| BMP2       | 11             | NM_001200   | TGFβ-like factor                 |
| NOG        | 10             | NM_005450   | BMP2 regulator                   |
| FST        | 8              | NM_006350   | INHBA regulator                  |
| SMAD3      | 6              | NM_005902   | transcriptional regulator        |
| CDKN1C     | 3              | NM_000076   | G1 cyclin/Cdk regulator          |
| TGFβ2      | 2              | NM_003238   | secreted growth factor           |
| **(b) MAPK-dependent** |                |             |                                   |
| GADD34     | 15             | NM_014330   | S phase Cdk regulator            |
| GADD45A    | 8              | NM_001924   | S phase Cdk regulator            |
| CDKN1A     | 2              | NM_000389   | G1 cyclin/Cdk regulator          |
| **Fibrinolytic system (p = 0.0005)** |                |             |                                   |
| SERBIN82   | 52             | NM_002575   | PLAT/PLAU regulator              |
| SERPINE1   | 8              | NM_006062   | PLAT/PLAU regulator              |
| PLAT       | 5              | NM_000930   | plasminogen activator            |
| PLAU       | 5              | NM_002658   | plasminogen activator            |
| PLAUR      | 2              | NM_001005376 | PLAU receptor                     |
| **Sterol biosynthetic pathway (p = 0.0003)** |                |             |                                   |
| HMGCR      | -3             | NM_000859   | isopentenyl PP synthesis         |
| MVK        | -3             | NM_000431   | isopentenyl PP synthesis         |
| IDI1       | -2             | NM_004508   | isopentenyl PP synthesis         |
| SC5DL      | -3             | NM_001024956 | dehydrocholesterol synthesis   |
| SQLE       | -3             | NM_003129   | squalene epoxide synthesis       |

*Relative abundance was calculated as the average normalized gene expression in *S. aureus*-treated group versus the untreated group, n = 3.*

*p values were calculated using a hypergeometric test based on random, non-replacement sampling of a canonical pathway as defined by KEGG.*
Since TLR-based recognition of Gram positive bacteria is a main sensing mechanism of mammalian cells, we tested whether induction of CCL20 expression by HCECs and their primary cell counterparts is dependent on TLR2 or NOD2 as might be predicted. The observation that agonists of these receptors did not effect CCL20 expression by these cells would be consistent with the previous observation that little TLR2 occurs on the surface of the human cornea normally, suggesting that S. aureus is sensed by these cells via another pathway. It was previously noted that protein A, which is expressed by S. aureus early in log phase, is capable of stimulating TNFα and IL8 expression by corneal epithelial cells in a TLR2 independent fashion, supporting this prospect.

Irrespective of the role of TLR2, we observed increases in MAPK-related transcripts upon stimulation of human corneal epithelial cells by S. aureus. MAPK signaling proteins include both extracellular and intracellular mediators of inflammation and proliferation. One factor identified in our screen was epidermal growth factor receptor (EGFR), which can be activated by S. aureus α-toxin, which is known to interact with host cell membranes via two distinct mechanisms. At low concentrations (<50 nM), α-toxin binds with high affinity to an elusive receptor which is saturable. This process is thought to be important in binding to keratinocytes, platelets, leukocytes and to a lesser extent lymphocytes. At higher concentrations (>200 nM), α-toxin adsorbs nonspecifically to lipid bilayers which contributes to its hemolytic activity. Compared to streptolysin O, α-toxin forms relatively small pores (1–2 nm) at low concentrations, allowing only the transport of small ions, including potassium and sodium. This permeability has been shown to have important cellular consequences in fibroblasts, endothelial, and lymphocytes, such as ATP depletion and stimulation of mitogen activated protein kinase (MAPK) signaling pathways. It was also found that signaling could be blocked by extracellular ion chelators and high molecular weight dextrans; thus, it appears that osmosensing may constitute another tier of pathogen recognition.

The response of human corneal epithelial cells to stimulation by model toxigenic or non-toxigenic S. aureus was also strikingly similar to that of human vaginal epithelial cells exposed to an.
S. aureus clinical isolate expressing toxic shock syndrome toxin-1 (TSST-1). In those experiments, the vaginal epithelial cells were also exposed to S. aureus for 6 h, but a larger inoculum was used (MOI of 100). In agreement with our data, they observed increased transcript abundance for CCL20, CSF2, CXCL3, CXCL1, IL8, CXCL2, TNFa, IL1α, IL6 and IL1β in remarkably similar magnitudes. Among the few differences, we did not observe an increase in the CSF3 transcript, which was reported by Peterson et al. to be 11-fold greater than the negative control. In that study a similar transcriptional response could be recapitulated with a culture fluid concentrate containing TSST-1. Our results indicate that most of the transcriptional responses are not specific to the toxigenic status of the S. aureus cell. RN6390 produces most S. aureus toxins, but it does not express TSST-1. We therefore cannot exclude that there may be a relationship between TSST-1 expression and CSF3.

The observations reported here and by Peterson et al. differ from results obtained with human airway epithelial cell cultures exposed to S. aureus strain 8325-4 (MOI = 50) for 3 h. The latter study did not show significant changes in transcript levels for most inflammation-related genes, except for IL1α, IL1β, IL6 and IL8. S. aureus strain 8325 is the parental strain of RN6390 and produces α-hemolysin, but not TSST-1. However, it is likely that the differences noted between this study and ours are more reflective of either (1) differences in the time of exposure or (2) differences in the nature of the responding epithelial cell, rather than strain background, since we found little toxin-dependent difference as late as 6 h after initiation of bacterial exposure, and because our results using model strains were very similar to those reported for a TSST-1 clinical isolate.

The response of corneal epithelial cells to S. aureus is similar in many ways to the response of HEp-2 cells to Streptococcus pneumoniae. Klenk et al. observed increased abundance of transcripts for CSF2, CXCL1, CXCL2, CXCL3, CCL20, IL6 and IL8, as well as transcription factors Jun and FosB. They also observed stimulation of release of IFNγ, corroborating for another Gram positive bacterial species the release of IFNγ by epithelial cells of the wet mucosa. However, S. pneumoniae stimulation of HEp-2 epithelial cells resulted in an increase in expression of the TLR2 transcript, a phenomenon not observed in the present study.

In conclusion, we find that epithelial cells from the immune privileged cornea respond to S. aureus in a manner that, as studied under the parameters used here, is mostly independent of toxin expression. Despite using well characterized isogenic mutants of a model S. aureus strain selected for its ready expression of cytolytic activity, the response of corneal epithelial cells to these strains was largely similar to the response of vaginal epithelial cells to S. aureus clinical isolates expressing TSST1, indicating that there is a response program that is common to epithelial cells of the wet mucosa, irrespective of other differences in regional immunity and immune privilege. Response to S. aureus invariably involved a large increase in abundance of transcripts encoding the antimicrobial dendritic cell chemokine, CCL20. Toxic expression by S. aureus was observed to induce a stress response, which may well have important consequences at later time points. We believe these studies highlight an underappreciated relationship between S. aureus detection by the epithelium, and its elicitation of a response by immune cells resident in the tissue involving the messenger, CCL20.

### Materials and Methods

**Bacterial strains.** S. aureus RN6390, an isogenic mutant of RN6390, ALC135, and MG1363 were used in this study, and routinely cultured in brain heart infusion (BHI) broth (BD San Jose, CA) broth at 37°C. For challenge experiments, overnight cultures were diluted 1:50 into 25 ml of pre-warmed BHI broth and grown at

| Table 4. Transcript in HCEC cultures influenced by S. aureus toxins |
|---------------------|---------------------|---------------------|
| Gene               | Rel. abundance     | GenBank No.         | Biological function     |
| Stress response     | RN6390/ALC135       |                     |                     |
| HSPA6              | 18                | NM_002155           | protein folding        |
| HSPA1A             | 7                 | NM_005345           | protein folding        |
| HSPA1B             | 6                 | NM_005346           | protein folding        |
| DNAJB1             | 7                 | NM_006145           | protein folding        |
| HSPD1              | 6                 | NM_002156           | protein folding        |
| CRYAB              | 3                 | NM_001885           | protein folding        |
| AHSA2              | 2                 | NM_152392           | activator of Hsp90     |
| DNAJB4             | 2                 | NM_007034           | protein folding        |
| Plasminogen activators |                     |                     |                     |
| PLAT               | 3                 | NM_000930           | plasminogen activator  |
| PRSS22             | 3                 | NM_022119           | activator of PLAT      |
| Membrane-associated |                  |                     |                     |
| SLC30A1            | 3                 | NM_021194           | Zn transporter         |
| SLC20A1            | 2                 | NM_005415           | Na/PO4 transporter     |
| CLDN4              | 2                 | NM_001305           | tight junctions        |
| Proliferative signaling |                  |                     |                     |
| RasD1              | 2                 | NM_016084           | NO-dependent signaling |
| FST                | 2                 | NM_006350           | TGFβ signaling         |
| IERS               | 2                 | NM_016545           | proliferation          |
| Negatively regulated |                 |                     |                     |
| SEMA4B             | -2                | NM_020210           | motility-related signaling |
| FGFRI              | -2                | NM_015633           | FGF signaling          |
| CCNF               | -2                | NM_001761           | cell cycle             |

*Relative abundance was calculated as the average normalized gene expression in RN6390-treated group versus the ALC135-treated group, n = 3.
Tertiary passages of untransformed primary human corneal epithelial cells (HCEC-3, Cascade Biologics, hereafter referred to as primary HCE) were also used to compare the response of immortalized cells to untransformed cells. HCEs were cultivated in EpiLife media (Cascade Biologics, Portland, OR) according to manufacturer’s instructions. All epithelial cells were cultured at 37°C in 5% CO2 without antibiotics prior to bacterial stimulation.

**Bacterial challenge.** Single cell suspensions of HCEC and HCLE were seeded into 6-well (9 cm²/well) or 24-well (2 cm²/well) plates at 4.5 x 10⁴ epithelial cells/cm², and incubated at 37°C to a mid-logarithmic phase (OD₆₀₀ = 0.65). Bacteria were harvested by centrifugation (5,000 xg for 10 min), washed twice in phosphate buffered saline (PBS), and resuspended in tissue culture medium pre-equilibrated to 37°C. Bacteria were quantified by serial dilution in PBS containing 0.05% (v/v) Tween-20 (Sigma-Aldrich, St. Louis, MO), and plating onto BHI agar.

**Human corneal epithelial cells.** Three types of human corneal epithelium cells were used in this study. Microarray analyses were performed with SV40 transformed human corneal epithelial cells (HCECs) that were cultured in Defined Keratinocyte Serum Free Media (DK-SFM, Invitrogen, Carlsbad, CA). For comparison to previous studies, telomerase transformed human corneal-limbal cells (HCLE cells) were also included in analyses of cytokine expression. These cells were cultured in Keratinocyte Serum Free Media (K-SFM, Invitrogen) supplemented with 25 mg/ml bovine pituitary extract (Invitrogen), 0.2 ng/ml epidermal growth factor (Invitrogen), and 0.4 mM CaCl₂ (Sigma-Aldrich).

![Figure 3](image-url) **Figure 3.** Inflammatory mediators measured in HCEC culture supernatants following co-culture with *S. aureus* for 6 h at an MOI of 20. (A) Impact of *S. aureus* on HCEC cytokine and chemokine production. (B) Impact of *S. aureus* on IFNγ expression in primary HCE and HCLE cultures. Black bars represent media controls. Light and dark grey bars denote co-cultures with strain RN6390 or ALC135, respectively. These data represent the average ± SD of two independent experiments performed in triplicate. *Significantly different from the untreated group based on the Student t-test (p value <0.05). †Significant difference from RN6390-treated group (p value <0.05).
trypsin-EDTA (Sigma-Aldrich) using standard protocols. At confluency, the cell densities varied depending on the culture type, ranging from $1 \times 10^4$ cells/cm$^2$ (primary HCE) to $1 \times 10^5$ cells/cm$^2$ (HCEC). For challenges, mid-logarithmic phase bacterial cells were harvested and suspended in tissue culture media to a predetermined cell density to generate a desired multiplicity of infection (MOI). Epithelial cell supernatants were replaced with 2.5 ml of bacterial suspension (6-well plate) or 1 ml (24-well plate) and incubated at 37°C in 5% CO$_2$ for 1 to 8 h. Bacterial growth and toxin expression was monitored throughout each challenge.

To identify challenge conditions that maximize exposure of epithelial cells to *S. aureus* without compromising epithelial cell viability, confluent HCEC cultures (24-well plates, $2 \times 10^5$ cells/well) were infected with mid-log, toxigenic *S. aureus* RN6390, suspended in DK-SFM at MOIs from 0–200. At 2 h intervals, the medium was removed with gentle agitation, and bacteria in the medium were enumerated. Monolayers were gently washed twice with equal volumes of 37°C DK-SFM, and stained with 300 µl of 0.2% (w/v) trypan-blue, diluted in phosphate-buffered saline (PBS). Monolayers were visually assessed for dye exclusion using bright field microscopy at 200X magnification (Nikon Eclipse TE300) and photographed. Photographs of representative fields were analyzed for the proportion of staining (non-viable) and non-staining (viable) cells. Four independent experiments were performed in triplicate over MOI ranges 10–20, 30–50, 60–80 and >100. Based on the results shown in Figure 1, a standard challenge condition of 6 h co-culture with an initial MOI of 20 was chosen.

**Toxin expression during challenge.** Supernatants from bacterial cultures were assessed for cytolytic toxin expression using hemolytic activity as a surrogate measure. Sterile filtered (0.2 µm) co-culture supernatants, sampled at 2 h intervals, were assessed for the ability to lyse 3% (v/v) packed human erythrocytes in prewarmed DK-SFM, following incubation at 37°C for 1 h. Unlysed erythrocytes were removed by brief centrifugation and hemoglobin release was measured at 450 nm using GENios Spectrophotometer (Tecan, Manndorf, Switzerland). Toxin expression was assessed in 3 independent experiments, each performed in triplicate.

**RNA isolation.** Following HCEC/*S. aureus* co-culture from an initial MOI of 20, culture medium was collected for enumeration of bacteria, and epithelial cell monolayers were washed 3X with PBS. HCEC were resuspended in RNAprotect® Cell Reagent (QIAGEN, Valencia, Calif.) for stabilization, according to manufacturer’s instructions, and stored at -70°C. RNA was extracted from thawed cells with an RNeasy Mini kit (QIAGEN, Valencia, CA), and was dissolved in DEPC-treated water, and quantified using a NanoDrop® ND-1000 Spectrophotometer V3.1.0 (NanoDrop Technologies, Wilmington, DE).

A control was also included for potential contamination of HCEC RNA by RNA derived from residual adherent or internalized *S. aureus*. Infected monolayers were washed three times with PBS and gently lysed with 1 ml trypsin/EDTA (0.25% w/v), Triton X-100 (0.025% w/v, Sigma-Aldrich). Lysates were serially diluted and associated bacteria plated onto BHI agar for enumeration. Based on triplicate measurements, the average burden of adherent and/or intracellular bacteria associated with harvested epithelial cells was estimated to be $1.2 \times 10^2$ CFU per epithelial cell, or $1.2 \times 10^9$ CFU/well in a 6-well plate. Using the RNA isolation protocol described above, the average RNA yield for $1.2 \times 10^9$ CFU of *S. aureus* was 300 ng, as compared to the average for $1 \times 10^6$ epithelial cells of 21.5 µg. Thus, contaminating *S. aureus* RNA comprised on average 1.3% of the total RNA prepared from epithelial cell co-cultures, and its presence was controlled for in microarray experiments.

**Microarray analysis of HCEC gene expression in response to *S. aureus*.** Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) were used to identify specific genes that are upregulated in response to *S. aureus* infection. Microarrays were performed in triplicate, and expression levels were analyzed using GeneSpring software. A fold change of ≥2 was considered significant.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Human corneal epithelial cells secret CCL20 in response to *S. aureus* exposure but not *L. lactis*. Supernatants from corneal epithelial and bacterial co-cultures were evaluated for CCL20 concentrations by ELISA. The results are reported as fold change relative to the uninfected control and represented as average values ± SD. (A) HCEC cultures treated with *S. aureus* or *L. lactis* for 6 h, based on five independent experiments performed in triplicate. (B) Primary HCEC cultures treated with *S. aureus* for 8 h that were sampled at regular time intervals. Data shown based on three independent experiments performed in triplicate.

*Significantly different from untreated group based on Student t-test (p value <0.05)."
strain RN6390 or non-toxigenic agr/sar- strain ALC135. RNA from triplicate confluent HCEC monolayers co-cultured with S. aureus (initial MOI = 20) in DK-SFM for 6 h were pooled, and RNA was purified from 3 independent pools of cells. RNA from the biological replicates (approximately 30 µg each) was labeled and hybridized to the microarrays by Genome Explorations (Memphis, TN). All samples were coded, and blind pairwise comparisons were made using the Affymetrix GeneChip Operating Software (GCOS). Comparisons were made between 4 groups: RNA derived from (a) RN6390/HCEC co-cultures, (b) ALC135/HCEC co-cultures, (c) HCEC cells cultured without bacteria, and (d) a control admixture of 98.7% cDNA from untreated HCEC and 1.3% RN6390 RNA.

Probe sets for which the GCOS Detection call was “present” in at least one group were screened for differences in signal intensity between groups, using ANOVA p-value ≤ 0.01 as a cutoff. Signal intensities were considered significantly different if the t-test p-value was ≤0.05 and differed by ≥2 fold in intensity between at least two groups. For each gene identified above, the reported fold-difference is based on the probe set demonstrating the lowest signal intensity. Transcripts, differing in relative abundance from the untreated group, were also screened for representation within canonical pathways defined in the Kyoto encyclopedia of genes and genomes (KEGG).72-74 Those pathways demonstrating an enrichment of transcripts were further tested using a hypergeometric test based on random, non-replacement sampling within pathway, and a p value ≤ 0.05 was considered significant.

Real-Time RT-PCR. RNA (2.5 µg) was treated with RNase-free DNase (1 U/µg) at 37°C for 30 min, according to manufacturer’s instructions (Promega, Madison, WI). cDNA was generated from randomly primed RNA using a High-Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA). cDNA (50 ng) was amplified using FAM™-labeled TaqMan® probes (Table 5) in Taqman® Gene Expression Master Mix (Applied Biosystems), and was detected on an ABI Prism 7900HT (Applied Biosystems). Commercially available GADPH primers (Applied Biosystem) were used to amplify an endogenous control from each sample. Differences in transcript levels were quantified by a comparing CT values (ΔΔCT), based on normalization of each amplicon to the endogenous control (GADPH).75 RT-PCR results are reported as fold-difference relative to control RNA from an untreated group, based on three independent experiments performed in triplicate.

Quantification of cytokine/chemokine release. Confluent monolayers of HCEC were co-cultured with bacteria under standard conditions. At 2 h intervals, supernatants were harvested, BSA (1 mg/ml) was added to stabilize CCL20 and other cytokines, and frozen at -20°C. Multiplex immunoassays were performed on thawed supernatants using a human cytokine panel (BioRad, Hercules, CA) and Luminex 100 detection system (Mirai Bio, Alameda, CA), according to manufacturer’s instructions. The human cytokine panel includes assays for interleukin (IL)1β, IL2, IL4, IL6, IL8, IL10, IL17, granulocyte macrophage-colony stimulating factor (CSF2), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα). IFNγ production was confirmed secondarily using the Proteoplex Human Cytokine
Array (Novagen, Darmstadt, Germany). CCL20 concentrations were directly measured using a colorimetric DuoSet ELISA development kit (R&D Systems, Minneapolis, MN) and GENios Spectrophotometer (Tecan, Manndorf, Switzerland) according to manufacturer specifications. Relative cytokine levels were compared using Student’s t-test with unequal variance in 2-tailed analyses, and were considered significantly different at p ≤ 0.05.

**Stimulation of corneal epithelial cells using pattern recognition receptors agonists.** To determine whether the induction of CCL20 was likely to have occurred via signaling through Toll-like receptors, pure pattern recognition receptor agonist preparations were used to stimulate the HCEC cells, HCLE cells, and primary human corneal epithelial cells. Each cell type was exposed to 10 μg/ml of TLR2 agonist Pam3Cys (Calbiochem, Gibbstown, NJ), 1 or 10 μg/ml of TLR4 agonist (MDP), a nucleotide-binding oligomerization domain containing protein (NOD2) agonist (Invivogen, San Diego, CA), for 12 h at 37°C under 5% CO2. After treatment, supernatants were filtered to remove cells and assayed for CCL20 as described above.

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**Note**

Supplementary materials can be found at:

www.landesbioscience.com/supplement/HeimerVIRU1-4-Sup.pdf

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**Table 5. List of Taqman gene expression assays used to measure relative transcript abundance by RT-PCR as described in materials and methods section**

| Gene   | Taqman Gene Exp. Assay | Primer pairs | Amplicon size (bp) |
|--------|------------------------|--------------|-------------------|
| CCL20  | Hs00171125_m1          | GAATCGAAGACGCAAGCACCATTGG | 81 |
|        |                       | GAAGCCTCAAAATATTTGGATGTA   |     |
| IL8    | Hs00174103_m1          | TGGTGAAAGGTGCAGTTTGGCAG   | 101 |
|        |                       | CAGTTCTTTAATAATTTGAGGGTG  |     |
| SERPINB2| Hs00234032_m1        | CAGATGGCCAAGGTCTCAGTTTA  | 120 |
|        |                       | ATCCAGGAATACCTCTTGGATC   |     |
| CSF2   | Hs00171266_m1          | TCCAACCCCGGAAACTCTCCTGCA  | 113 |
|        |                       | CTTGAGCTTGCTGCCAGCATCAAA |     |
| BDF4   | Hs00823638_m1          | TGCCCTTCCAGGTGGTTTGTGTA  | 86  |
|        |                       | AGGGCAAAAGACTGGATGACATAT |     |
| GAPDH  | Hs9999905_m1          | TTGGGGCCTCTGTACACGGGTCTG  | 122 |
|        |                       | GCCATGGGTGAAATCATATTGAAC |     |
| HSPA6  | Hs00275682_s1          | AAGATTCCCGGAGGACGGCCGACA  | 119 |
|        |                       | TCCAGCTTCTCCTCTGTATGCTAT  |     |
| HSPA1B | Hs01040501_sh          | CGGGATCCTCCTGCGCCGTTCACG  | 127 |
|        |                       | ACACCCCCACCCAGGAATGATTGTT  |     |
| DNAJB1 | Hs00356730_g1          | CCGGAGAAGAGGCTTAAAGGAGGT  | 103 |
|        |                       | CATGCCATGAGGGTCTCCATGGAAT |     |

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