LBP and CD14 Secreted in Tears by the Lacrimal Glands Modulate the LPS Response of Corneal Epithelial Cells

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PURPOSE. Lipopolysaccharide (LPS) is one of the most powerful bacterial virulence factors in terms of proinflammatory properties and is likely to contribute to corneal bacterial keratitis. Better understanding of the spatial expression of the LPS receptor components at the tear–corneal interface might facilitate enhanced functions of the LPS receptor complex in ocular defense against Gram-negative infections.

METHODS. The expression of LPS-binding protein (LBP), CD14, toll-like receptor (TLR)-4, and MD-2 in human lacrimal glands, reflex tears, and corneal epithelia was examined by ELISA, RT-PCR, Western blot analysis, and immunofluorescence. The release of proinflammatory cytokines after the activation of primary and immortalized corneal epithelial cells with LPS and human tears was measured by ELISA.

RESULTS. LBP and CD14 proteins were detected in reflex human tears. Human lacrimal glands and corneal epithelia expressed LBP, CD14, TLR4, and MD-2 mRNAs and proteins. In the corneal epithelium, LBP was mainly expressed by superficial and basal epithelial cells, whereas CD14, TLR4, and MD-2 expression were limited to the wing and basal epithelial cells. In a dose-dependant manner, tear CD14 and LBP mediated the secretion of interleukin (IL)-6 and IL-8 by corneal epithelial cells when challenged with LPS.

CONCLUSIONS. Tear CD14 and LBP complemented the LPS receptor complex expressed by the corneal epithelia to trigger an immune response in the presence of LPS. The complementation of these tear and corneal immune proteins could play an important role in LPS recognition and signaling and, therefore, could modulate ocular innate immunity. (Invest Ophtalmol Vis Sci. 2005;46:4235–4244) DOI:10.1167/iovs.05-0543

Eyelids, tears, epithelium, and stroma help protect the outer eye against the environment. One mechanism of particular importance is tear fluid, because its continuous flow protects the eye by bathing the ocular surface and flushing foreign particles from the epithelium. In addition to their hydrodynamics, tears contain several immune molecules with bactericidal properties. Of these molecules, secretory IgA opsonizes bacteria and prevents their adherence to the epithelium.1 Another tear component, lactoferrin, limits bacterial growth and biofilm formation by sequestering iron and destabilizing bacterial membranes.2 These defense molecules use tears as a medium to help protect the entire ocular surface.3

Another barrier contributing to ocular protection is the intact corneal epithelium. Several epithelial features are essential in mediating this protection: tight cell junctions, cell polarity, and continuous epithelial turnover.4 Structural integrity is essential in preventing bacterial adherence and infiltration into deeper epithelial layers and subsequent infections.5 Rather than merely being a passive barrier, corneal epithelial cells actively participate in the ocular immune defense as they express innate immune receptors, such as CD14,6 TLR4,7 and TLR5,8 targeted against pathogenic products.

Microbial keratitis is responsible for up to 30% of the blindness in developing countries and can represent as much as 90% of keratitis cases in temperate climates.9 Bacterial keratitis is mostly associated with predisposing factors such as extended contact lens wear and LASIK surgery in industrialized society9,10 or trauma in tropical and subtropical communities,11 where Pseudomonas aeruginosa remains the primary Gram-negative causative agent.12 In recent years, a constant increase in keratitis incidence and subsequent treatment costs,10 combined with long-standing occupational insults, has demanded better understanding of the innate immune defense mechanisms present in the ocular environment to prevent infections. An important characteristic of innate immunity is the rapid recognition of a wide range of pathogens through highly conserved structural motifs present on many different microorganisms. The best-studied example of such an innate system is the recognition of lipopolysaccharide (LPS, or endotoxin), a highly conserved molecular pattern of Gram-negative bacteria. In the bloodstream, the sequential action of at least four extracellular and cell surface proteins—LPS-binding protein (LBP), CD14, TLR4, and MD-2, referred to as the LPS receptor complex—is required for maximum sensitivity of endotoxin signaling.12 LPS recognition is mainly initiated by LBP, a soluble serum lipid transfer protein synthesized by the liver, whose function is to extract LPS monomers from aggregated endotoxin structures for their subsequent delivery to membrane-bound or soluble (s)CD14.13,14 LPS is thereafter transferred from CD14 to the TLR4/MD-2 transmembrane coreceptor and triggers the expression of various inflammatory and immunoregulatory genes resulting in the recruitment and activation of immune cells to the site of infection.15–19 The potential relationship between the LPS receptor components of the tear film and those of the corneal epithelium remains to be delineated.

Therefore, the expression and the immune functions of the LPS receptor proteins CD14, LBP, TLR4, and MD-2 were characterized in human tears and corneal epithelia. To investigate the ocular immune response against LPS, human reflex tears were examined for sCD14 and LBP, and the lacrimal gland and the intact corneal epithelium were evaluated as the source. The ability of tears to complement the LPS receptor protein complex components within LPS-challenged corneal epithelial cells was evaluated in vitro for the induction of IL-6 and IL-8. We hypothesize that sCD14 and LBP from human tears can complement the corneal derived repertoire of the LPS receptor complex to induce an innate immune reaction in the epithelium.
METHODS

Subjects
The study was performed in accordance with the tenets of the Declaration of Helsinki and was approved by the Ottawa Hospital Research Ethics Board. The purpose of the research and the procedure for the experiment were explained to all the participants, and their written informed consent was obtained before tear collection. Ninety-one healthy volunteers were recruited randomly at the University of Ottawa Eye Institute. Inclusion criteria were that volunteers were not taking any ocular medication, had no history of eye disease, and had not suffered from dry eye conditions. The study was made up of two groups. The first, consisting of 67 healthy non–contact lens wearers, included 34 women and 33 men; their median age was 48 years (range, 17–86 years). The second group, consisting of 24 healthy contact lens wearers, included 13 women and 11 men; their median age was 27 years (range, 16–54 years).

Tear Fluid Collection
Tear production was stimulated by briefly exposing the eye to vapors of freshly minced onions. Tear samples were then collected with 50-μL flamed-polished disposable microcapillaries (VWR, West Chester, PA), while avoiding ocular surface contact. For the continuous incremental tear flow experiment, onion vapor–stimulated tears from 11 volunteers were collected as 5 successive aliquots of 50 μL each and were transferred into 5 microfuge tubes. All tear samples were frozen at –80°C until analysis.

Human Corneal and Lacrimal Tissues
Ten postmortem human corneas, sepsis free and stored in solution (Optisol; Chiron Vision, Irvine, CA), were obtained from the Eye Bank of Canada. Mean age of the donors was 44.4 years (range, 20–74 years). Epithelia from three human corneas were physically isolated for RNA and protein analysis. Briefly, the epithelium was scraped from the cornea with a corneal knife (Gill; Storz Instruments, Tuttinglen, Germany) under a dissecting microscope, ensuring that the scraping was confined to the corneal region to minimize any conjunctival or stromal contamination. Isolated epithelia were snap frozen in liquid nitrogen and stored at –80°C until analysis.

For RT-PCR analysis, sepsis-free human lacrimal gland biopsy specimens were obtained within 1 hour of surgery from four patients (two men, two women; median age, 37 years; range, 24–53 years) who had medical conditions (inflammation or tumor) requiring surgery. For protein extraction and immunohistologic analysis, healthy human lacrimal glands were obtained from the Department of Cellular and Molecular Medicine Anatomy Program (University of Ottawa) within 48 hours postmortem from four donors (three men, one woman; median age, 71 years; range, 51–89 years). Corneal and lacrimal biopsy specimens were obtained in accordance with the guidelines established by the Ottawa Hospital Research Ethics Board.

Human Corneal Epithelial Cells
The human corneal epithelial cell (HCEC) line, immortalized with a simian virus 40 (SV40) adenovirus recombinant vector, was maintained in keratinocyte serum-free medium (KSFM) (Invitrogen, Carlsbad, CA) supplemented with 0.05 mg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor (Invitrogen). Primary corneal epithelial cells derived from progenitors in the limbal-corneal region of human corneas were maintained in complete KSFM, supplemented with 100 μg/mL penicillin, 100 μg/mL streptomycin, and 10 μg/mL gentamicin (Invitrogen). For differentiation, primary corneal epithelial cells of the HCEC line were grown to 100% confluence for 2 weeks in supplemented hormonal epithelial medium consisting of DMEM (low glucose)/F-12 (Invitrogen), 15% FBS, 10 ng/mL epidermal growth factor, 5 μg/mL insulin, 0.1 μg/mL cholela toxin alpha subunit, 5 mM L-glutamine, 0.5% dimethyl sulfoxide, and gentamicin. All cells were grown at 37°C in a humid environment containing 5% CO2; cell culture media were changed every 2 to 3 days. The stratification of primary corneal epithelial cells and the HCEC line on an air-liquid interface matrix was performed as previously described.

CD14 and LBP Protein Quantification by ELISA
Collected tear samples were centrifuged for 5 minutes at 10,000g at 4°C to sediment any cellular and insoluble debris. The sCD14 and LBP concentrations in tears, cornea, lacrimal gland, primary cells, and HCEC extracts were measured by ELISA (HyCult Biotechnology, Uden, The Netherlands).

RNA Isolation and RT-PCR Analysis
Messenger RNA was isolated from lacrimal glands, scraped corneal epithelia, confluence-grown primary corneal epithelial cells, and the HCEC line (RNeasy Mini Kit; Qiagen, Chatsworth, CA). To ensure that all genomic DNA was completely removed from the samples, extracts were subjected to DNase digestion with the RNase-free DNase kit (Qiagen). Random primed cDNA synthesis was performed (Superscript First-Strand Synthesis for RT-PCR; Invitrogen) according to the manufacturer’s instructions. Oligonucleotide primers used to amplify CD14, LBP, TLR4, and MD-2 were designed based on the published sequences (GenBank accession numbers X06882, AF105067, U88880, and AB018549, respectively) and were as follows: CD14 sense, ACC-AGCCGAGAACCTTTGAGCTGGA; CD14 antisense, TTAGGAAA-GCCCCGGGCCCTT; LBP sense, GCTGTGGAACCTCTTCCACACAG; LBP antisense, CTGAAATTCAGTATCAGTGTGG; TLR4, sense, AT-GGCCTTCCCTCTCCTGTTGACAC; TLR4 antisense, TCTGTGCAAAACTACTCCAGACAAAT; MD-2 antisense, TTCCATATTTCGAGCTGAGAC; MD-2 sense, GGGCTCCGAGAAATAGCTTCAAC. PCR was performed (REDExtract-N-Amp PCR Readymix; Sigma, St. Louis, MO) and was carried out with 35 cycles of denaturation at 94°C for 45 seconds, annealing at 59°C for 45 seconds, and extension at 72°C for 45 seconds. The resultant products were resolved by electrophoresis in ethidium bromide–stained 1% low-melt agarose gel. To further confirm the veracity of the RT-PCR products, the amplified sequences were purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced by the University of Ottawa Biotechnology Research Institute using the PCR primers.

Immunoblotting
Samples containing CD14 and LBP were resolved by SDS-PAGE under reducing conditions (12% resolving gel) and transferred onto nitrocellulose (Amersham Biosciences, Piscataway, NJ). The membrane was thereafter blocked for 1 hour in 5% dried skim milk in Tris-buffered saline (TBS) with 0.1% Tween-20. CD14 and LBP were probed with the biotinylated polyclonal antibody to human CD14 (0.05 μg/mL in TBS-Tween; R&D Systems, Minneapolis, MN) or with the biotinylated polyclonal antibody to human LBP (0.1 μg/mL in TBS-Tween; R&D Systems). After extensive washing, the membrane was incubated for 1 hour with the antibody horseradish peroxidase (HRP)–conjugated antibody (1:1000; Cell Signaling Technology, Beverly, MA). Antigens were detected using enhanced chemiluminescence (ECL Western Blotting Detection Reagents; Amersham Biosciences).

Immunofluorescence Staining
Human corneal epithelial cells were grown on a sterile microscope coverslip placed in a 6-well tissue culture plate. After reaching 70% to 80% confluence, cells were fixed with 3.7% paraformaldehyde for 20 minutes at room temperature (RT) and were permeabilized with 0.2% Triton X-100 for 20 minutes at RT. Human corneas and lacrimal glands were fixed overnight at 4°C in 4% paraformaldehyde. After overnight equilibration in 30% sucrose, they were incubated at RT for 2 hours in a 1:1 solution of 30% sucrose/optimus cutting temperature (OCT) embedding medium. Samples were flash frozen in sucrose/OCT embedding medium, cryosectioned to 10 μm on a Shandon cryostat
For immunostaining, corneal and lacrimal cryosections were rehydrated in PBS for 10 minutes. All slides were blocked in 5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) for 1 hour at 37°C. For histologic localization of CD14 and LBP, the slides were incubated overnight at 4°C with 27.5 μg/mL mouse monoclonal anti-CD14 (MY4; Beckman Coulter, Miami, FL) in PBS containing 5% normal donkey serum and 0.3% Triton-X. Sections were thereafter overlaid with 0.7 μg/mL anti–LBP rabbit polyclonal antibody (HyCult Biotechnology) for 2 hours at 37°C. After extensive washing, the slides were incubated for 2 hours at 37°C with 30 μg/mL fluorescein isothiocyanate (FITC)–conjugated anti–mouse IgG and 30 μg/mL rhodamine red-X (RRX)–conjugated anti-rabbit IgG (Jackson ImmunoResearch). Corneal sections were further incubated with 2-minute fluorescence counterstaining (DAPI; Inno- genex, San Ramon, CA) followed by mounting (Vectashield mounting medium; Vector Laboratories, Burlingame, CA) of the microscope slide. Sections were observed with a fluorescence microscope (Axioskop 2; Carl Zeiss, Thornwood, NY).

Immunohistologic localization of TLR4 and MD-2 was performed by blocking the rehydrated cryosections with 5% normal donkey serum (Jackson ImmunoResearch). TLR4 and MD-2 proteins were localized with 2 μg/mL biotinylated goat polyclonal anti-TLR4 (R&D Systems) and 8 μg/mL rabbit polyclonal anti–MD-2 (FL-160; Santa Cruz Biotechnology, Santa Cruz, CA) incubated for 2 hours at 37°C. After 3 washes in PBS, the sections were further incubated for 2 hours at 37°C with 32 μg/mL FITC-conjugated mouse monoclonal anti-biotin and 30 μg/mL RRX-conjugated anti–rabbit IgG (Jackson ImmunoResearch). Nuclei were visualized with DAPI staining, and the slides were mounted as described.

**LPS Biologic Assays on Cultured Human Corneal Epithelial Cells**

To quantify cytokine production, cultured primary corneal epithelial cells and the HCEC line were plated in 24-well flat-bottom tissue culture plates. After reaching approximately 70% to 80% confluence, the cells were left untreated or were preincubated with various doses of filter-sterilized pooled human tears, 500 ng/mL recombinant human sCD14 (R&D Systems), 150 ng/mL recombinant human LBP (R&D Systems), 10 μg/mL anti–human CD14 monoclonal antibody MY4 (mouse IgG2b; Beckman Coulter, Miami, FL), 10 μg/mL anti–human LBP monoclonal antibody 6G3 (mouse IgG1; HyCult Biotechnology), and 10 μg/mL of their isotype-matched controls MOPC-141 and MOPC-21, respectively (Sigma-Aldrich) for 2 hours at 37°C. To eliminate any potentially unwanted LPS contaminants affecting cytokine production, an assay of the gelatin of Limulus amebocyte lysate (Associates of Cape Cod, East Falmouth, MA), and mounted on Super-frost slides (Fisher Scientific, Nepean, ON, Canada). After they were air dried for 2 hours, slides were stored at -80°C until use.

**TABLE 1. Levels of sCD14 and LBP in Human Tears According to Sex and Contact Lens Wear**

<table>
<thead>
<tr>
<th>Study Groups*</th>
<th>sCD14† (ng/mL)</th>
<th>LBP† (ng/mL)</th>
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<tbody>
<tr>
<td>Women (n = 34)</td>
<td>562 ± 337</td>
<td>140 ± 132</td>
</tr>
<tr>
<td>Men (n = 33)</td>
<td>597 ± 194</td>
<td>125 ± 90</td>
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<tr>
<td>Non-contact lens wearers (n = 67)</td>
<td>579 ± 274</td>
<td>133 ± 113</td>
</tr>
<tr>
<td>Contact lens wearers (n = 24)</td>
<td>509 ± 300</td>
<td>143 ± 90</td>
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</tbody>
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*The first study group was composed of 67 healthy non-contact lens wearers (34 women, 33 men; median age, 48 years; range, 17–86 years). The second group was composed of 24 healthy contact lens wearers (13 women, 11 men; median age, 27 years; range, 16–54 years). Tear production was stimulated by onion vapors, and reflex tears were collected with flamed-polished disposable microcapillaries.†Quantification of sCD14 and LBP in tears was performed by ELISA using commercially available kits.
MA) was performed to measure endotoxin levels in all reagents before they were added to the cells. Any reagent that tested positive for LPS was treated with END-X (Associates of Cape Cod) to remove residual endotoxin. With sCD14, LBP, tears, and antibodies still present in the medium, LPS derived from Pseudomonas aeruginosa serotype 10 (Sigma) was added at a concentration of 100 ng/mL to activate the cells. The medium was harvested after 24 hours, centrifuged to remove cellular debris, and stored at –70°C until analysis. Culture supernatants were analyzed by ELISA for IL-8 (Sanguin Reagents, Amsterdam, The Netherlands) and IL-6 (eBioscience, San Diego, CA).

Statistical Analysis

Experiments in this study were performed in triplicate to confirm the reproducibility of the results. Values are represented as mean ± SD. The statistical significance of differences between two or more means was evaluated using ANOVA; P < 0.01 (indicated by asterisks) was considered statistically significant.

RESULTS

Quantification of sCD14 and LBP in Human Tears

Both sCD14 and LBP proteins in onion vapor–stimulated tears from healthy donors were found to be present in all tear samples (n = 91), with a mean concentration of 561.1 ± 281.6 ng/mL for sCD14 (intra-assay variability, 2.3% CV and 135.8 ± 106.6 ng/mL for LBP (intra-assay variability, 4.8% CV). sCD14 and LBP levels varied from 35.4 to 1249.5 ng/mL and from 23.5 to 307.4 ng/mL, respectively. Linear regression and ANOVA revealed that age, sex, contact lens wear, and continuous tear stimulation had no influence on sCD14 and LBP values in reflex tears (Fig. 1; Table 1). Western blot analysis of representative tear samples showed that sCD14 exhibited an apparent molecular weight of 50 kDa (Fig. 2A, lane 4), characteristic of the secretory form of the protein.23 Immunoblotting also revealed the presence of the 60 kDa LBP protein in human tears, which corresponded to the same apparent molecular mass as serum LBP (Fig. 2A, lanes 4 and 2, respectively).

CD14 and LBP Expression in Human Lacrimal Glands

Immunoblotting analysis was performed on human lacrimal gland extracts (n = 4) to determine the source of these tear immune proteins. Two polypeptides with molecular masses of 50 kDa and 55 kDa were detected with the anti–CD14 antibody, whereas a 60-kDa protein was identified as LBP with the anti-LBP antibody (Fig. 2A, lane 3). CD14 and LBP expression were further detected in human lacrimal glands by the amplification of both transcripts by RT-PCR (Fig. 2B) using human gene-specific primers. Partial sequencing of the RT-PCR products matched the CD14 and LBP published sequences (data not shown). Immunofluorescence detection of both proteins in human lacrimal glands (n = 4) showed CD14 and LBP to be localized in the cytoplasm of acinar cells.

FIGURE 2. The LPS receptor complex, CD14, LBP, TLR4, and MD-2 are expressed by the lacrimal gland and the corneal epithelium. (A) Lacrimal gland, tears, and corneal epithelium were analyzed by immunoblotting with anti-human CD14 and anti-human LBP polyclonal antibodies. (B) Detection of CD14, LBP, TLR4, and MD-2 mRNAs by RT-PCR from human lacrimal gland, isolated corneal epithelium, cultured corneal epithelial primary cells, and the immortalized human corneal epithelial HCEC-cells. Reactions were performed with and without the reverse transcriptase (RT) step to confirm the cDNA origin of the amplification.

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constituting the alveoli of the serous gland (Figs. 3A–C). Control sections omitting primary antibodies (Figs. 3I–L) and cross-reactivity analysis of the secondary antibodies (Figs. 3M–P) confirmed the specificity of labeling.

Lacrimal Acinar Cells Express TLR4 and MD-2

Besides synthesizing and secreting sCD14 and LBP in tears, lacrimal acinar cells were found to express TLR4 and MD-2, as shown by RT-PCR (Fig. 2B). For TLR4, however, three products were amplified using primers flanking the region between exons 1 and 4 of the gene. Sequencing of the amplified fragments confirmed the nature of the three splicing variants of human TLR4 (data not shown). The TLR4 transcript containing exons 1, 3, and 4, encoding the proper functional TLR4,24 was predominant in human lacrimal gland (Fig. 2B). The two less abundant alternative splicing forms were unlikely to encode functional TLR4 because of premature translational termination of the protein.24 In lacrimal acinar cells, TLR4 and MD-2 proteins were found to be colocalized in situ by immunofluorescence (Figs. 3E–G).

LPS-Receptor Complex Expression in the Human Corneal Epithelium

To examine whether LPS receptor proteins could complement tear contribution in the cornea, the expression of these proteins was investigated in this stratified tissue. Immunohistologic analysis of human corneas (n = 7) revealed that LBP is strongly expressed in the squamous superficial epithelium and in some columnar basal cells (Fig. 4B). Negligible LBP staining was observed among wing cells composing the intermediate layer of the corneal epithelium. Corneal CD14 expression was sparse and mainly limited to the basal epithelial cells with an absence of staining in the superficial and wing cells (Fig. 4A). In the basal epithelium, LBP and CD14 were not colocalized, suggesting they were expressed by different epithelial cells (Fig. 4C). CD14 and LBP could also be detected by Western blotting in isolated corneal epithelia (Fig. 2A, lane 5), further confirming the immunofluorescence results. In contrast, TLR4 and MD-2 expression were polarized with ubiquitous staining along the basal and wing epithelial cells, with little or no expression in the superficial differentiated epithelium (Figs. 4E–F). Image overlay indicated that both proteins were expressed by the same cells (Fig. 4G). To elucidate the origin of these proteins, the respective CD14, LBP, TLR4, and MD-2 mRNAs were detected by RT-PCR in the isolated human corneal epithelia (n = 3) (Fig. 2B). Three splicing forms of human TLR4 were amplified from corneal epithelial cDNA, with the functional transcript composed of exons 1, 3, and 4 the most abundant.

Partial Expression of the LPS-Receptor Complex by Human Corneal Epithelial Cells and HCEC Line

To test the hypothesis that HCECs may express LPS receptor components to complement tear sCD14 and LBP, their expression was analyzed in vitro. CD14 mRNA was amplified by RT-PCR from freshly isolated primary corneal epithelial cells and from the HCEC line (Fig. 2B). LBP mRNA was not detected in primary or HCEC cells (Fig. 2B). These results were supported by immunofluorescence findings by which CD14, but not LBP, showed some reactivity in HCECs (Figs. 5A–C) and primary corneal epithelial cells (data not shown). ELISA on total cell lysate and culture supernatant indicated that CD14 was expressed by corneal epithelial cells but was
not secreted (data not shown). LBP was not detected by ELISA in corneal epithelial cell lysate or culture supernatant, which suggested that it was not expressed by primary or HCEC cells in vitro. Cultures of primary corneal epithelial cells and HCECs differentiated in supplemented hormonal epithelial medium or stratified on an air–liquid interface matrix failed to induce LBP expression (data not shown). On the other hand, TLR4 and MD-2 were expressed in vitro, as confirmed by RT-PCR (Fig. 2B) and immunofluorescence (Figs. 5E–G).

Corneal Response to LPS in Presence of Tear CD14 and LBP

To assess the biologic relevance of tear sCD14 and LBP in the initiation of an endotoxin immune response, the secretion of proinflammatory molecules was analyzed in primary human corneal epithelial cells and the HCEC line challenged with *Pseudomonas*-derived LPS in the presence of sCD14, LBP, or human tears. In vitro cultures of primary corneal epithelial cells and the HCEC line expressed the LPS receptor components (Figs. 2B, 5A, 5B, 5E, 5F) similarly to basal and wing epithelial cells in vivo (Figs. 4A, 4B, 4E, 4F), suggesting that they may represent an adequate in vitro model system to study LPS responsiveness in amplifying cells. LPS alone had little effect on IL-6 and IL-8 secretion by primary corneal epithelial cells (Figs. 6A, 6B) and the HCEC line (Figs. 6C, 6D), as previously observed, whereas the addition of recombinant sCD14 or LBP increased IL-6 and IL-8 production (Fig. 6). However, no major difference was detected in IL-6 or IL-8 production whether sCD14 and LBP were added alone or were combined. This indicated that the two soluble proteins increased, individually or in concert, the efficiency of the LPS-induced response. Recombinant sCD14 and LBP-mediated activation was significantly abrogated by the neutralizing anti-CD14 MY4 and anti-LBP 6G3 antibodies, thus suggesting that both proteins contributed to this activity (Fig. 6). Pooled human tears were also tested for enhancing LPS-mediated cytokine production. Tear sCD14 and LBP mediated the activation of primary and HCEC cells by endotoxin in a dose-dependent manner to produce IL-6 (Figs. 7A, 7C) and IL-8 (Figs. 7B, 7D). The tear-induced production of IL-6 and IL-8 in primary corneal epithelial cells (Figs. 7A, 7B) and the HCEC line (Figs. 7C, 7D) was significantly inhibited by anti-CD14 MY4 and anti-LBP 6G3 antibodies, but not by their isotype-matched controls, therefore supporting the biologic activity of sCD14 and LBP in tears for mediating innate immunity.

**DISCUSSION**

In this study, sCD14 and LBP—two LPS receptor proteins—were identified in tears and found to mediate an LPS-induced innate immune response within amplifying corneal epithelial cells. Although sCD14 has been identified in most human secretory fluids, the presence of LBP in tears is remarkable because it was undetectable or was present in trace amounts in other secretions. The dual presence of both proteins in tears was consistent with those of other tear innate immune proteins, such as lactoferrin and lysozyme. In
nonstimulated conditions, basal tears have been demonstrated to contain slightly higher levels of other immune proteins, such as lactoferrin, secretory IgA, and lysozyme, compared with reflex tears. Therefore, we suspect that sCD14 and LBP levels in basal tears may be slightly higher or equivalent to those measured in reflex tears, potentially making sCD14 and LBP constantly present on the surface of the eye, not only when reflex tears are induced.

The mRNA and protein detection of CD14 and LBP in the lacrimal gland suggests that acinar epithelial cells are a source of both proteins in human tears. Although LBP in reflex tears could also originate from additional sources, such as superficial epithelial cells, the fact that reflex tears flow voluminously and quickly suggests that such a contribution would be minimal. In addition, the sole presence of the 50-kDa sCD14 isoform in human tears makes potential contributions from serum exudate unlikely because of the presence of two serum sCD14 isoforms, 50 kDa and 55 kDa. The 50-kDa sCD14 isoform in tears is likely to originate from the cleavage of a 55-kDa precursor that was present, in addition to the 50-kDa soluble isoform, in the lacrimal protein extract.

sCD14 and LBP within tears may serve a combination of roles. They may protect the lacrimal gland against Gram-negative infections. The abundance and variety of antimicrobial products synthesized and secreted by the lacrimal gland could help explain the infrequency of infections in this serous gland. Besides expressing sCD14 and LBP, lacrimal acinar cells express TLR4 and MD-2, enabling them to respond to Gram-negative insults threatening to compromise its function. In a similar manner, tear sCD14 and LBP may protect the downstream lacrimal sac and nasal epithelium against infection by tear drainage through the nasolacrimal duct. On the ocular surface, tear LBP and sCD14 may prevent the adherence of pathogens to the intact corneal epithelium. In keratitis, *P. aeruginosa* adheres to corneal epithelial cells through adhesins such as LPS, which is known to bind to specific corneal epithelial molecules such as asialo GM1 glycolipids, galectin-3, and CFTR. With their known opsonizing and neutralizing activities, tear LBP and sCD14 could bind and neutralize LPS on the surfaces of *P. aeruginosa*, thereby inhibiting its adhesion to the cornea. Furthermore, tear sCD14 and LBP could prevent shed LPS monomers from interacting with the intact corneal epithelium by transferring them to lactoferrin or lipoproteins, two proteins also found in human tears. The lactoferrin- and lipoprotein-bound LPS would no longer be available to interact with corneal epithelial cells and could be removed from the ocular surface by tears, eyelid blinking, and hydrodynamic flushing. In addition, LBP alone may mediate LPS detoxification by forming large LBP-LPS complexes that would subsequently be washed away with tears. Finally, as another mechanism, tear-derived LPS and sCD14 may function in combination with the epithelium to deliver LPS to the exposed inner epithelial layers of the breached corneal epithium to mediate a stronger immune response, as described here.

The corneal epithelium expresses the LPS receptor complex in a manner that could minimize LPS responsiveness on its intact structure. Indeed, all the LPS receptor constituents required for an endotoxin response are expressed by basal and wing epithelial cells located in the inner corneal epithelial layers, with no CD14, TLR4, or MD-2 expression evident in the squamous apical layers. Such polarized expression has been noted for TLR5 and was proposed to prevent host detrimental inflammatory responses of the corneal epithelium against non-pathogenic ocular flora. The minute cytokine response mediated exclusively by LPS also correlated with previous find-
This observation could be attributed to the limited presence of CD14 or TLR4 on the surfaces of corneal epithelial cells or to their intracellular localization, minimizing their biologic response to LPS. In addition, superficial corneal epithelial cells expressing LBP may prevent *P. aeruginosa* adherence by releasing LBP in the outer corneal environment and saturating the LPS adhesins of the bacteria. LBP expressed by the apical epithelial layer of the cornea could also play an important role in LPS recognition in the injured corneal surface. As with IL-1α, LBP stored in the cytoplasm of superficial epithelial cells may be passively released by the rupture of the cellular membrane caused by pathogens or trauma. LBP released by the damaged superficial cells could increase the LBP concentration in the wound microenvironment and improve LPS delivery to the exposed wing and basal epithelial cells to trigger an innate immune response. Alternatively, the receptor complex components, apparently strategically arranged within the corneal epithelium, may function in conjunction with those identified in tears for a more refined and regulated immune response.

Because of its anatomic localization, the corneal epithelium is constantly exposed to microorganisms and their virulence factors. A highly sensitive inflammatory response against pathogens, though beneficial in other organs, may affect corneal transparency. The infiltrating polymorphonuclear leukocytes (PMNs) and plasma proteins in the cornea can diffract and obstruct the visual axis, thus causing the focused image to be cast away from the retina. Persistent inflammation may also lead to permanent corneal destruction by scarring, perforation, and blindness. Thus, a system that minimizes hyperinflammation in response to minor infections yet is sufficiently sensitive to potentially dangerous infections would be advantageous for the corneal epithelium. The localization of LBP components within the layers of the corneal epithelium and the complementation of tears may mediate this response. Although the ocular surface is relatively resistant to microbial invasions, corneal surface injury disrupts the epithelial integrity, allowing the infiltration of pathogens into the epithelium. Proliferating primary corneal epithelial cells demonstrate some properties of amplifying basal epithelial cells in

**Figure 6.** Enhanced LPS response of primary corneal epithelial cells and HCEC line in the presence of sCD14 and LBP. Primary corneal epithelial cells (A, B) and HCEC line (C, D) were pretreated with sCD14 and LBP (500 ng/mL and 150 ng/mL, respectively, the average concentrations found in human tears), anti-CD14 monoclonal antibody MV4 (10 μg/mL), anti-LBP monoclonal antibody G6 (10 μg/mL), and their isotype-matched controls (IgG2b and IgG1, respectively, at 10 μg/mL each) before the addition of LPS from *P. aeruginosa* (100 ng/mL). The data shown correspond to three independent experiments. Statistically significant differences were determined using ANOVA (*P < 0.05; **P < 0.005).
Within primary epithelial cells and HCECs, tears CD14 and LBP were required for an efficient delivery of endotoxin to the LPS receptor molecules expressed by corneal epithelial cells and for the release of IL-6 and IL-8 cytokines. Extracellular LBP and sCD14 can complement and facilitate the transfer of LPS to CD14 and TLR4/MD-2. Previous studies performed in vivo on abraded mouse cornea showed that LPS induced cytokine expression, mediated neutrophil recruitment, and induced inflammation at the wound site. These studies support our data suggesting that LPS immune activation may occur when there is a breach in the squamous epithelial layer, allowing tear sCD14 and LBP to deliver LPS to TLR4/MD-2 located in the inner epithelial layers, thereby allowing them to generate an adequate immune response in conjunction with the innate properties of the stromal keratocytes.

In summary, this study suggests that the combined roles of tear sCD14 and LBP, along with the polarized expression of the LPS receptor complex in the corneal epithelium, could explain ways in which corneal epithelial cells induce immune responses against infiltrating Gram-negative pathogens. Further understanding of the molecular interactions among tear sCD14 and LBP, Gram-negative pathogens, and corneal epithelial cells may lead to the development of efficient treatments to enhance ocular innate immune defenses and to prevent the sight-threatening consequences of severe Gram-negative infections.

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