Inflammatory Response to Lipopolysaccharide on the Ocular Surface in a Murine Dry Eye Model

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PURPOSE. Toll-like receptor 4 (TLR4) alerts cells to the presence of bacteria by initiating an inflammatory response. We hypothesize that disruption of the ocular surface barrier in dry eye enhances TLR4 signaling. This study determined whether dry eye enhances expression of inflammatory mediators in response to topically applied TLR4 ligand.

METHODS. A single dose of lipopolysaccharide (LPS) or vehicle (endotoxin-free water) was applied to the cornea of nonstressed (NS) mice or mice subjected to 5 days of desiccating stress (DS). After 4 hours, corneal epithelium and conjunctiva were extracted to analyze expression of inflammatory mediators via PCR. Protein expression was confirmed by immunobead assay and immunostaining.

RESULTS. Topically applied LPS increased expression of inflammatory mediators IL-1β, CXCL10, IL-12a, and IFN-γ in the conjunctiva, and IL-1β and CXCL10 in the cornea of NS mice compared to that in untreated controls. LPS in DS mice produced 3-fold increased expression of IL-1β in cornea and 2-fold increased expression in IL-12a in conjunctiva compared to that in LPS-treated control mice.

CONCLUSIONS. LPS increased expression of inflammatory cytokines on the ocular surface. This expression was further increased in dry eye, which suggests that epithelial barrier disruption enhances exposure of LPS to TLR4+ cells and that the inflammatory response to endotoxin-producing commensal or pathogenic bacteria may be more severe in dry eye disease.

Keywords: conjunctiva, cornea, cytokines, dry eye, inflammation, lipopolysaccharide, TLR4, toll-like receptors

Toll-like receptors (TLRs) are known as the "first responders" of the immune system, recognizing pathogen-associated molecules and then alerting immune cells to produce an inflammatory response. TLRs have been linked to a number of autoimmune diseases[1,2] and are known to be highly expressed on tissues exposed to the environment, such as the gut and lung.[3] TLRs are also expressed by epithelial and immune cells in the eye and are involved in response to infection and induction of inflammation on the ocular surface.[4] Dry eye (DE) is a common, potentially debilitating condition, with a prevalence reported to be as high as 33%.[5] Evidence suggests that chronic dry eye is a localized autoimmune disease[6] characterized by increased levels of inflammatory and T-cell–associated cytokines on the ocular surface.[7,8] Of particular interest to the pathology of dry eye is TLR4, a toll-like receptor subtype activated by lipopolysaccharide (LPS), a component of gram-negative bacteria. LPS activation of TLR4 leads to production of inflammatory cytokines, such as IL-1β and TNF-α.[9,10] These cytokines are elevated in DE,[7,11] but it is unknown whether TLR4 ligands stimulate their production. In addition, increased conjunctival expression of TLR4 has been reported in our mouse model of dry eye,[12] yet, how this increased expression relates to inflammation that develops in DE is unclear. Despite correlations between TLR4 and DE, the role of TLRs in dry eye disease has not been fully explored.

TLR4 is expressed mainly by wing and basal, not superficial, corneal epithelial cells.[13] Corneal epithelial apoptosis and barrier disruption have been demonstrated in murine models of dry eye,[8,14,15] which could lead to exposure of TLR4 in the deeper layers of the corneal epithelium. Theoretically, this could give commensal and pathogenic bacteria and their products greater access to TLR4, contributing to the vicious cycle of inflammation observed in DE. The purpose of this study was to compare inflammation triggered by topically administered LPS in nonstressed eyes with dry eyes that were subjected to experimental desiccating stress.

MATERIALS AND METHODS

Mice

Female mice (C57BL/6J strain) 6 to 8 weeks old were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Female TLR4KO mice used in the study came from a colony established in our vivarium that was started with (C.C3-Tlr4^Lps-d/J strain) mice purchased from Jackson Laboratory. Animal studies were approved by the Institutional Animal Care and Use Committee at the Baylor College of Medicine. All studies adhered to the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Visual Research.

Of the total number of animals used in each group, 5 to 6 were used for PCR, 4 for immunobead assay, 3 for immunofluorescence (interleukin-12a [IL-12a]), and 3 for immunohisto-

chemistry (IL-1β). The groups in the immunostaining experiments were untreated plus LPS (UT+LPS), UT+water, desiccating stress day 5 (DS5)+LPS, and DS5+water. Additionally, 6 UT and 2 DS5 controls without topical treatment were used in the immunohistochemistry experiment. Three UT and 2 DS5 controls without topical treatment were used for immunofluorescent staining. In the cell culture studies, 5 TLR4KO mice and 7 C57BL/6J mice were used in total.

Murine Model of Dry Eye

Mice were exposed to desiccating stress (DS) to create a murine model of dry eye, similar to methods previously described.[8,14,16] DS was induced by housing the mice in an environmentally controlled room with relative humidity ≤30%. Mice were placed in customized cages with two sides constructed of wire to allow exposure to air drafts created by fans. Tear secretion was pharmacologically inhibited by subcutaneous injection of scopolamine hydrobromide (0.5 mg/0.2 mL; Sigma-Aldrich Corp., St. Louis, MO, USA) 4 times daily (8:30 AM, 11:00 AM, 1:00 PM, and 4:30 PM). Mice were exposed to DS5. Nonstressed (NS) control mice were housed in a vivarium with 50% to 75% relative humidity, no exposure to air drafts, and no scopolamine injections.

LPS Treatment

To determine whether TLR4 could be activated on the ocular surface of untreated mice, NS mice were treated with ultrapure LPS from *Salmonella enterica* serovar Minnesota mutant R595 (Invivogen, San Diego, CA, USA). Mice were treated topically (5 μL/eye) with LPS dissolved in endotoxin-free water (Sigma-Aldrich Corp.) at a dose of 1 μg/μL or 10 μg/μL. The higher dose of LPS (10 μg/μL) was used only in gene expression experiments, not in experiments for protein analysis. Five microliters of endotoxin-free water per eye was used as a vehicle control. Mice were held in place for 1 minute to allow eye drops to distribute. Untreated NS mice were used as controls. After 4 hours, mice were euthanized for PCR or immunostaining experiments. An additional experiment for PCR analysis was conducted using this method and endotoxin-free saline (NaCl 0.9%; Enzo Life Sciences, Farmingdale, NY, USA) as the vehicle. For protein analysis using an immunobead assay, conjunctiva was extracted after 24 hours.

To determine whether DE led to increased expression of inflammatory cytokines after TLR4 activation, DS5 mice were treated with 5 μL/eye LPS (1 μg/μL) or water on day 5 of desiccating stress. Untreated NS and DS5 mice were used as control groups. After 4 hours, mice were euthanized to extract corneal epithelium and conjunctiva for RNA analysis. An identical protocol was used for immunostaining experiments of mice exposed to desiccating stress.

RNA Isolation

Corneal epithelium and conjunctiva were extracted to measure gene expression of inflammatory mediators via PCR. Corneal epithelium was scraped with a scalpel and conjunctiva was surgically excised. Tissue samples from cornea and conjunctiva were pooled separately from both eyes to give one cornea and one conjunctiva sample per mouse. RNA was extracted using the RNeasy Plus Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Concentration of isolated RNA was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). After RNA isolation, cDNA was synthesized using Ready-To-Go You-Prime First-Strand beads (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) as previously reported.[17]

PCR

After cDNA synthesis, PCR was run on a StepOnePlus real-time PCR system (Applied Biosystems, Grand Island, NY, USA). Gene expression was analyzed using the comparative threshold cycle ($\Delta\Delta C_T$) method. C_T values for each gene were normalized to the C_T values of the housekeeping gene for each sample. The housekeeping gene used for these experiments was hypoxanthine guanine phosphoribosyl transferase (HPRT). Fold differences in expression were calculated after comparing values for each gene to the those in the untreated group. Each experiment in this study was completed with its own specific group of untreated mice from the same batch of mice used in that particular experiment. Primers (Life Technologies, Grand Island, NY, USA) used in this study included interferon-γ (IFN-γ; ABI assay ID Mm00801778_m1), IL-1β (ABI assay ID Mm00434228_m1), IL-6 (ABI assay ID Mm00446190_m1), IL-12a (ABI assay ID Mm00434165_m1), CXCL10 (ABI assay ID Mm00445235_m1), tumor necrosis factor-α (TNF-α; ABI assay ID Mm00443260_g1), and HPRT (ABI assay ID Mm00446968_m1).

Protein Isolation and Analysis

Conjunctiva was surgically excised for protein analysis. Two conjunctiva samples were obtained for each treatment group. Each sample contained pooled conjunctiva from 4 eyes/2 mice. Tissue was extracted and placed in 100 μL of radioimmunoprecipitation assay buffer (Sigma-Aldrich Corp.) treated with a cOmplete, EDTA-free protease inhibitor cocktail tablet (Roche, Basel, Switzerland). Excised tissue was chopped with surgical scissors, sonicated, and kept on ice for 30 minutes. Afterward, samples were centrifuged at 14,000 rpm for 20 minutes at 4°C. Supernatant was stored at −80°C. Samples were normalized for total protein concentration by using Pierce BCA protein assay kit (Life Technologies). Then, concentrations of CXCL10 were analyzed using an immunobead assay (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The assay was run using a Luminex 100 system (Luminex, Austin, TX, USA).

Immunofluorescent Staining

Immunofluorescent staining was used to visualize IL-12a protein in the conjunctiva after administration of LPS. Tissue sections (one slide with 2 samples per animal) were fixed in acetone at −20°C for 10 minutes. Then, nonspecific sites on the samples were blocked for 60 minutes with 20% goat serum (Sigma-Aldrich Corp.) in phosphate-buffered saline (PBS). The primary antibody (IL-12a ab203031; Abcam, Cambridge, MA, USA), diluted 1:100 in 5% goat serum in PBS, was then added for 1 hour. After being washed with PBS, sections were incubated with Alexa Fluor 488 AffiniPure goat anti-rabbit immunoglobulin G (IgG; H+L; product 111545144; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted in PBS for 1 hour in the dark. Samples were washed and then counterstained with propidium iodide. Slides without primary antibody were used as a negative control. Pictures were taken at 40× magnification using an Eclipse E400 microscope equipped with a digital camera (DS-Qi1Mc; Nikon Instruments Inc, Melville, NY, USA).

Immunohistochemistry

Immunohistochemistry was used to analyze protein expression of IL-1β. Briefly, tissue sections were fixed in acetone at −20°C for 10 minutes. After washing in PBS, samples were incubated with 0.3% hydrogen peroxide in PBS to quench endogenous

peroxidases and washed again in PBS. Sections were then treated with an avidin/biotin blocking kit (catalog SP-2001; Vector Laboratories, Burlingame, CA, USA). After being rinsed in PBS, the samples were blocked for 1 hour with 20% goat serum (Sigma-Aldrich Corp.) in PBS. The primary antibody (rabbit anti-mouse IL-1β; D4T2D; Cell Signaling Technology, Danvers, MA, USA) was then diluted 1:50 in 5% goat serum in PBS and applied to the samples for 1 hour. After being washed, the secondary antibody, biotinylated goat anti-rabbit IgG (product 550338; BD Pharmingen, Franklin Lakes, NJ, USA) was applied to the samples for 30 minutes. Sections were washed and treated with a Vectastain Elite ABC kit (catalog no. PK-6100; Vector Laboratories) for 30 minutes. Then, the sections were washed again and incubated with solution from a Vector NovaRED peroxidase substrate kit (catalog no. SK-4800; Vector Laboratories) for 15 minutes. The reaction was terminated, and samples were washed in distilled water. Then the sections were counterstained for 2 minutes with Mayer hematoxylin (using Lillie modification) (ScyTek Laboratories, Logan, UT, USA). After this, the sections were dehydrated in ethanol and incubated in xylene. Finally, Permount (Fisher Scientific, Pittsburgh, PA, USA) was applied, and a coverslip was attached. Slides without primary antibody were used as a negative control. Slides were examined and photographed at ×40 magnification, using an Eclipse E400 microscope equipped with a digital camera (model DS-Fi1; Nikon). A second set of images were recorded at increased magnification, using an A1P MP upright microscope with a motorized stage equipped with a color camera (model DS-U3; Nikon) and a 100×/1.45 oil (plan apochromat OFN25 DIC; Nikon) objective.

Cell Culture

Cell culture studies were performed to identify which cell types on the ocular surface responded to LPS stimulation. LPS was added directly to either epithelial or dendritic cell (DC) cultures in vitro.

For epithelial cell cultures, cornea or conjunctiva explants were extracted from female C57BL/6J or TLR4KO mice. Conjunctiva explants were cultured using methods similar to those described previously.[18] Conjunctiva explants were excised from the forniceal conjunctiva of 6- to 8-week-old female C57BL/6 mice or TLR4KO mice and left for 15 minutes at 37°C in keratinocyte serum-free medium (KSFM) (Thermo Fisher Scientific) supplemented with 3% fetal bovine serum, 1.25 μg/mL amphotericin B (product 15290-018; Thermo Fisher Scientific), 0.5 μL/mL gentamicin (product 15750-060; Thermo Fisher Scientific), and 5 μg/mL dispase II (product 04942078001; Roche). For cornea cultures, explants were left for 30 minutes in supplemented hormone epithelium medium (SHEM) containing Dulbecco modified Eagle medium/F12 medium (product D8437; Sigma-Aldrich Corp.) supplemented with 5 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin A, 0.5% dimethyl sulfoxide, 50 μg/mL gentamicin, 1.25 μg/mL amphotericin B, 5% fetal bovine serum, and 5 μg/mL dispase II (Roche). Corneal and conjunctival explants were then plated at one explant per well in 24-well plates. Conjunctiva cultures received 500 μL/well of KSFM with 80 ng/mL mouse EGF (BD Biosciences, San Jose, CA, USA) and cornea cultures received 500 μL/well SHEM. Culture plates were then placed in a 37°C incubator with 5% CO_2. After 3 days, cornea cultures received 500 μL of fresh SHEM/well for a total of 1 mL medium/well; medium was changed every 3 days. For conjunctiva cultures, wells received another 500 μL of KSFM on day 7 for a total of 1 mL of medium/well. On day 11, wells were left untreated or treated with 1 μL of 1 μg/μL LPS dissolved in endotoxin-free water.

After 4 hours, well contents were collected for PCR analysis. One well was collected for each cornea sample. Two to three wells were collected for each conjunctiva sample.

Direct stimulation of DCs by LPS was also tested in vitro. DCs were cultured using previously published methods.[19,20] Briefly, DCs were isolated from C57BL/6J mouse bone marrow and cultured for 10 days using RPMI 1640 medium (Thermo Fisher Scientific). On day 10, cells were left untreated or treated with 1 μL of 1 μg/μL LPS dissolved in endotoxin-free water. After 4 hours, DC cultures were collected for PCR analysis.

Statistical Analysis

All statistical analyses were performed using Prism version 6.01 software (GraphPad, Inc, La Jolla, CA, USA). Within each experiment for PCR and immunobead assay analysis, 1-way ANOVA was used to determine overall statistical significance, and the Holm-Sidak multiple comparison test was performed to find differences between groups. Two-way ANOVAs were used to analyze statistical significance between experiments, with Sidak multiple comparisons test used to analyze the significance between groups of different experiments. For the PCR experiments where saline was used as the vehicle and for PCR analysis of the cell culture studies, an unpaired Student *t* test with Welch's correction was used to determine statistical significance among groups. For C57BL/6J mouse cell culture experiments, the number of samples per group were 4 samples per DC group, 4 samples per untreated conjunctiva cell group, and 5 samples per LPS-treated conjunctiva cell group. For the cornea, 2 untreated and 3 LPS-treated samples were used to test for IL-1β. Six untreated and 4 LPS-treated cornea culture samples were used to test for TNF-α. C_T values were set at 40 for untreated DC samples that did not contain enough IL-1β to quantify. For the TLR4KO mouse cell culture experiments, 5 samples were used in each cornea and conjunctiva group.

RESULTS

TLR4 Activation in Nonstressed Mice

In the first set of experiments, LPS eye drops were administered to nonstressed mice to determine whether TLR4 activation on the ocular surface could lead to the release of proinflammatory mediators. Gene expression was measured via RT-qPCR 4 hours after application of LPS. In general, LPS administration led to a dose-dependent increase in inflammatory mediator expression on the ocular surface. Concentrations of 0.1 μg/μL and 0.01 μg/μL were also tested (results not shown). LPS concentrations of 10 μg/μL and 1 μg/μL led to enhanced expression of certain genes (Figs. 1, 2). In most cases, only small differences in expression were observed between the 1 μg/μL and 10 μg/μL concentrations, indicating that maximal TLR4 activation was reached at the 1 μg/μL concentration; however, higher concentrations were not tested. In the cornea, application of 1 μg/μL LPS significantly increased expression of IL-1β (2.17 ± 0.31-fold) and CXCL10 (36.93 ± 7.75-fold) (Fig. 1). In the conjunctiva, a wider variety of inflammatory mediators were induced. In addition, the conjunctiva displayed more dramatic increases in gene expression after LPS administration. For instance, 1 μg/μL LPS enhanced IL-1β (21.27 ± 9.61-fold) and CXCL10 (185 ± 41.28-fold). IFN-γ (13.45 ± 4.49-fold), and IL-12a (3.99 ± 0.27-fold) were also significantly increased in the conjunctiva after application of 1 μg/μL LPS. TNF-α (7.02 ± 3.37-fold) and IL-6 (20.12 ± 10.48-fold) were also increased after 1 μg/μL LPS, but the results did not reach statistical significance. Increases in IL-
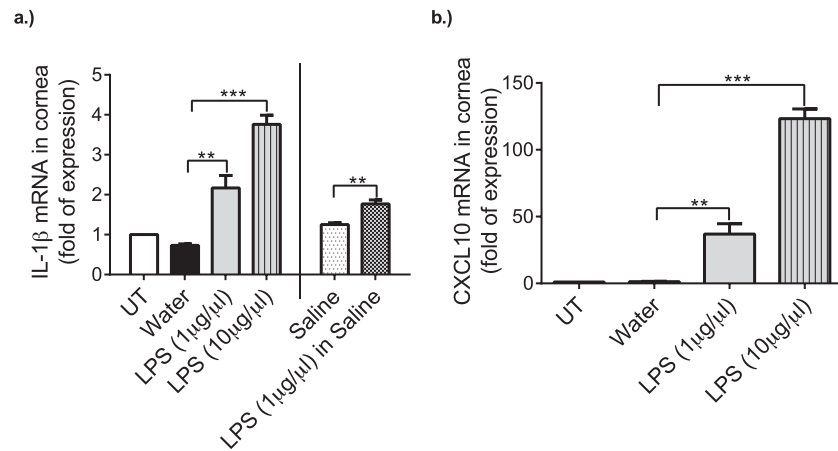
FIGURE 1. LPS increases mRNA expression of IL-1β and CXCL10 in the cornea. NS mice were treated with LPS or endotoxin-free water and euthanized after 4 hours to analyze cytokine/chemokine expression. IL-1β and CXC10 expression in the cornea significantly increased after LPS administration. Average of two experiments is shown ($n = 3$ per group in each experiment). *Error bars* are SDs. (**a**) Endotoxin-free saline was used as an alternate vehicle for LPS. In this segment of the graph, *bars* depict one experiment ($n = 5$ per group) and *error bars* are shown as SEM. LPS administration led to a significant increase in IL-1β when dissolved in either water or saline. ***$P \leq 0.001$; **$P \leq 0.01$.
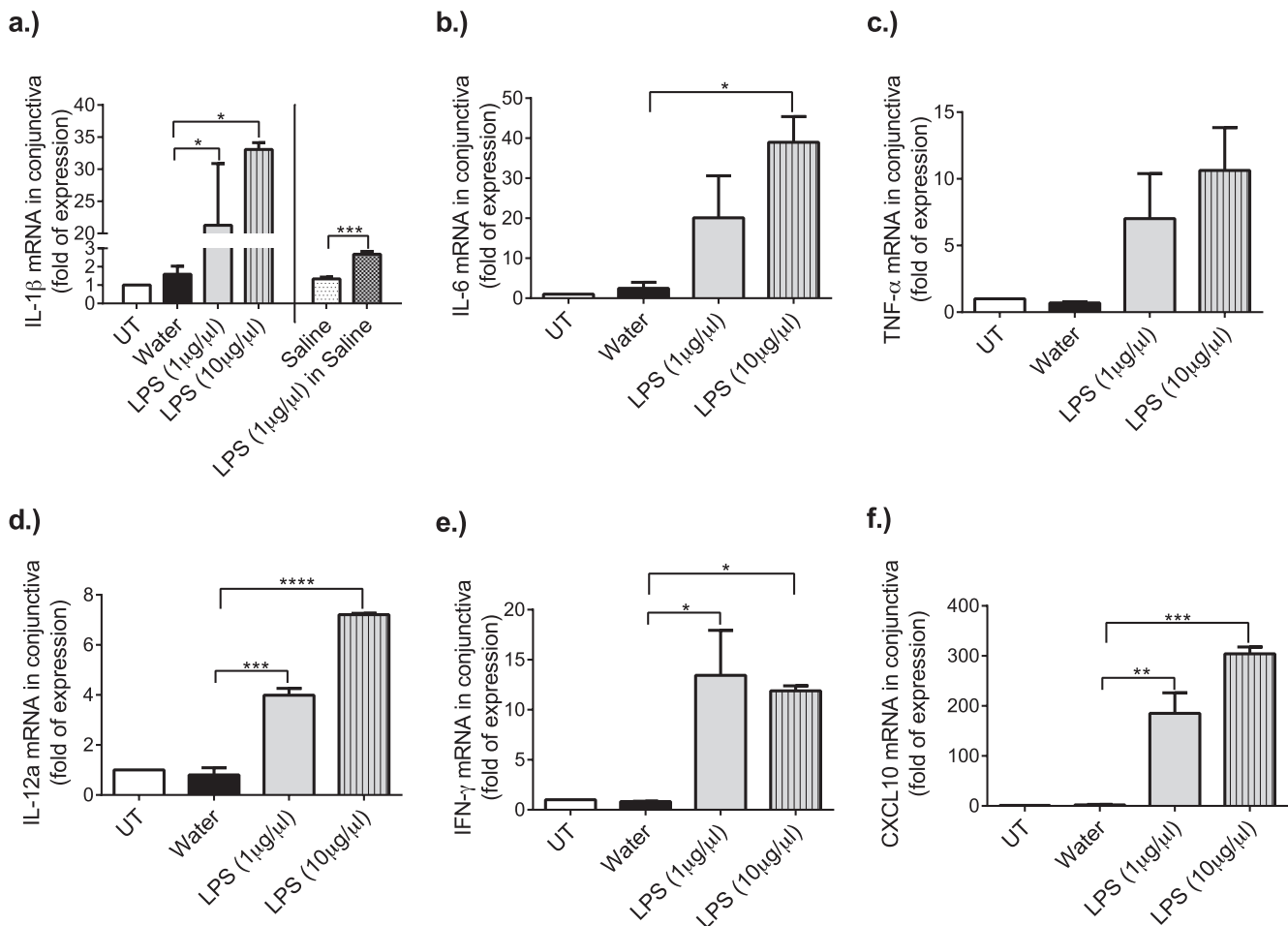

FIGURE 2. LPS increases mRNA expression of inflammatory mediators in the conjunctiva. NS mice were treated with LPS or vehicle and euthanized after 4 hours for gene expression analysis. LPS administration increased expression of multiple inflammatory mediators in the conjunctiva. Average of two experiments is shown ($n \approx 3$ per group in each experiment). *Error bars* are SDs. (**a**) Endotoxin-free saline was used as an alternate vehicle for LPS. In this segment of the graph, *bars* depict one experiment ($n = 5$ per group), and *error bars* are SEM. ****$P \leq 0.0001$; ***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$.
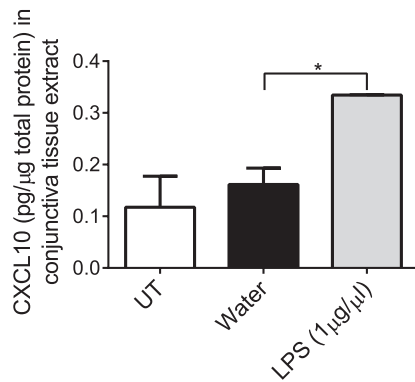
Figure 3. LPS increases protein expression of CXCL10 in the conjunctiva. NS mice were treated with LPS. Conjunctiva was extracted after 24 hours and analyzed for CXCL10 by using an immunobead assay. LPS administration led to a significant increase in CXCL10 protein expression. *Error bars* are SDs. *$*P \leq 0.05$.

6 expression (38.97 ± 6.44-fold) in the conjunctiva after 10 µg/µL LPS did reach statistical significance (Fig. 2).

Because water may affect the ocular surface as a hypotonic solution, endotoxin-free saline was evaluated as an alternative vehicle to LPS. The choice of vehicle may affect gene expression; however, the increase in IL-1β remained significant when saline was used as the diluent. Figures 1a and 2a show that 1 µg/µL LPS dissolved in saline led to a significant increase in IL-1β in both the cornea and the conjunctiva.

TLR4 activation by LPS was verified by repeating identical experiments in TLR4KO mice (results not shown). Nonstressed TLR4KO mice were treated with 1 µg/µL LPS and euthanized 4 hours later to measure expression of inflammatory mediators by PCR. Increases in gene expression by LPS were abolished or greatly diminished in TLR4KO mice. A slight and nonsignificant increase in IL-1β was observed in the conjunctiva of LPS-treated TLR4KO mice, which may suggest noncanonical inflammasome activation by LPS.[21,22] Because no significant increases in gene expression were observed, these findings indicate the stimulatory effects of LPS are mediated by TLR4-dependent pathways on the ocular surface.

In order to verify that increased gene expression of inflammatory mediators also resulted in an increase in protein expression, concentrations of CXCL10 were measured by an immunobead assay in conjunctival tissue extracts obtained 24 hours after application of LPS. CXCL10 was selected because it led to the largest increases in gene expression. A significant increase ($P \leq 0.05$) in CXCL10 protein was found in the conjunctiva of NS mice treated with 1 µg/µL LPS compared to NS mice treated with vehicle (endotoxin-free water) (Fig. 3).

Protein expression was also verified by immunostaining (Fig. 4). Nonstressed mice were given LPS (1 µg/µL) or vehicle and euthanized after 4 hours. Immunohistochemical staining demonstrated that IL-1β increased in the cornea of NS mice treated with LPS compared to NS mice treated with water (Fig. 4a). In Figure 4b, higher magnification ($\times 100$) was used to display increased IL-1β specifically in the corneal epithelium of NS+LPS mice. Immunofluorescent staining was used to detect protein expression of IL-12a in the conjunctiva. Figure 4c shows an increase in IL-12a-positive cells in NS mice treated with LPS.

LPS was also tested in vitro to determine which cell types may be responsible for the expression of inflammatory mediators observed in the in vivo studies. Figure 5 shows the effect of LPS when it is added to explant cell cultures from non-stressed mice. Addition of LPS to cell culture medium led to an increase in IL-1β and TNF-α in cornea (Fig. 5a), conjunctiva

(Fig. 5b), and DC (Fig. 5c) cultures. No increase in expression was observed when lipopolysaccharide was added to cornea and conjunctiva cultures from TLR4KO mice (results not shown).

TLR4 Activation in Experimental Dry Eye

After TLR4 activation was demonstrated on the ocular surface in nonstressed mice, the next set of experiments was designed to determine whether TLR4 activation is enhanced during dry eye disease. Mice were exposed to DS for 5 days, then an LPS drop was administered to the eye. Corneal epithelium and conjunctiva were extracted after 4 hours for analysis of gene expression by RT-qPCR. Figure 6 demonstrates that TLR4 activation by LPS was greater during dry eye disease. Compared to control NS eyes, LPS increased expression of IL-1β (≈ 3-fold) in the cornea and IL-12a (≈ 2-fold) in the conjunctiva of dry eyes.

In a separate experiment using the same methods, protein expression was analyzed in DS mice by immunostaining. Figures 4a and 4b further support the hypothesis that LPS administration leads to enhanced expression of inflammatory mediators during dry eye disease. Enhanced protein expression of IL-1β was observed in DS5 mice treated with LPS compared to NS mice treated with LPS (Figs. 4a, 4b). Figure 4a shows enhanced expression of IL-1β in the corneal stroma of DS5+LPS mice, and Figure 4b demonstrates the same increase in the corneal epithelium. In addition, an increase in IL-12a–positive cells was noted in DS5 mice treated with LPS compared to DS controls (Fig. 4c).

Discussion

Dry eye is a costly disease and is highly prevalent in the elderly.[5] With an aging population and limited treatment options available for dry eye, it is important to define all the factors that can contribute to inflammation on the ocular surface. Toll-like receptors are known to promote inflammation, but their role in dry eye has not been fully explored.

Previously published studies suggest a link between TLR4 and dry eye. TLR4 expression was found to increase in the conjunctiva in our mouse model of dry eye.[12] Moreover, inhibition of TLR4 decreases expression of some inflammatory mediators during experimental DE.[23] However, additional research is needed to understand inflammatory mediators associated with TLR4 activation on the ocular surface. Theoretically, commensal or pathogenic LPS-producing bacteria, as well as endogenous TLR4 ligands, could make a significant contribution to the inflammation observed in dry eye. Importantly, gram-negative bacterial sequences have been found in the normal ocular microbiome.[24] Endogenous TLR4 ligands are thought to include extracellular matrix breakdown components,[25] fibrinogen cleavage products,[26] and heat shock proteins.[27] Heat shock protein 70 has been linked to DE,[28] but the presence of other endogenous TLR4 ligands has not yet been fully investigated. Potentially, disruption of ocular surface homeostasis in dry eye could lead to generation of endogenous TLR4 ligands. Despite this possibility, the ability of TLR4 to produce a proinflammatory response in the eye during normal and diseased states has not been fully explored.

This study indicates that TLR4 is present on the eye and that TLR4 activation can lead to increased production of proin-flammatory mediators. In fact, LPS application to the ocular surface can lead to the release of several inflammatory mediators linked to dry eye. The greatest variety of inflammatory mediators and most dramatic rises in gene expression were noted in the conjunctiva (Fig. 2). This was expected, as
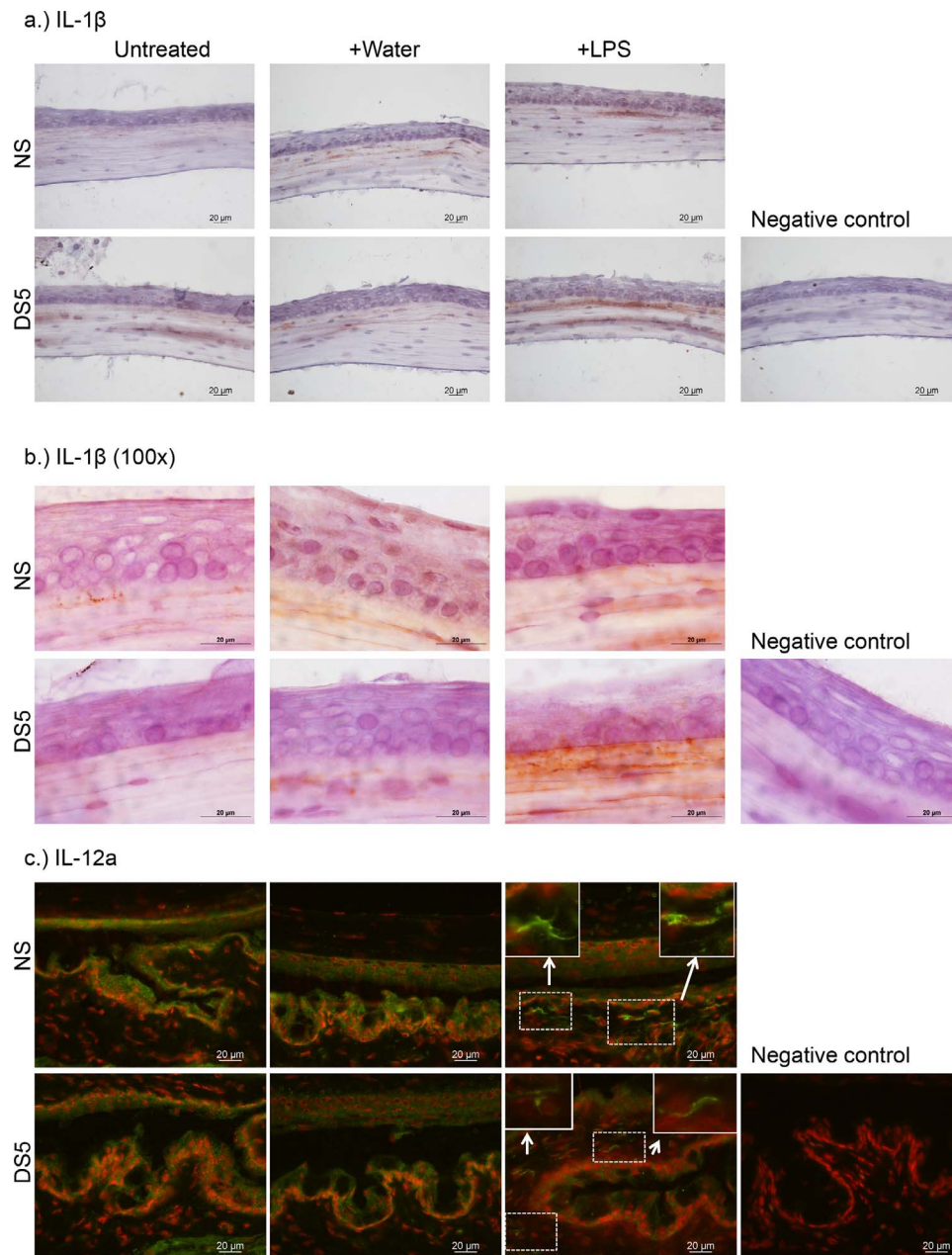
FIGURE 4. Increased protein expression of IL-1β and IL-12a after LPS administration. (**a**) Immunohistochemical staining shows increased IL-1β expression (*red*) in the central cornea (*stroma*) after administration of LPS (1 μg/μL) to nonstressed mice. Expression of IL-1β observed in DS5+LPS mice is enhanced compared to that in NS+LPS mice. Hematoxylin was used as a nuclear stain (*dark blue*). (**b**) Higher magnification (×100) was used to examine enhanced expression of IL-1β in the corneal epithelium of NS+LPS and DS5+LPS mice. (**c**) Immunofluorescent staining demonstrates that application of LPS (1 μg/μL) increases expression of IL-12a in the conjunctiva of nonstressed and DS5 mice. The *white boxes* indicate IL-12a-positive cells (*green*), which are shown enlarged in the *upper corners*. Propidium iodide was used as a counterstain (*red*). Slides without primary antibody are shown as negative controls. *Scale bars*: 20 μm.

the greater permeability of the conjunctival epithelium[29] may make TLR4-positive cells more accessible to LPS than in the cornea. IL-6 is one proinflammatory cytokine that was expressed in the conjunctiva after LPS treatment (Fig. 2). IL-6 promotes the Th17 response, which is relevant in dry eye pathogenesis.[6] In fact, Th17 cells contribute to corneal epithelial barrier disruption during DE.[8] Additionally, TNF-α appeared to increase in a dose-dependent manner, though it did not reach statistical significance. TNF-α is also important in Th17 differentiation[30] and has been shown to stimulate MMP-9 production in vitro,[31] which can lead to further corneal

epithelial disease. Both IL-6 and TNF-α have been found to be increased in mouse models of dry eye[32] and in humans[33] with the disease.

In addition to generating Th17 pathway cytokines, LPS administration also led to mediators associated with the Th1 response. Importantly, IFN-γ was upregulated in the conjunctiva after LPS treatment (Fig. 2). IFN-γ is the defining cytokine of the Th1 response, which contributes to ocular surface disease and tear dysfunction.[6] Upregulation of IFN-γ by LPS is important as IFN-γ expression is increased in mice and humans with DE.[8] Furthermore, IFN-γ has been linked to goblet cell

a.) Cornea Culture
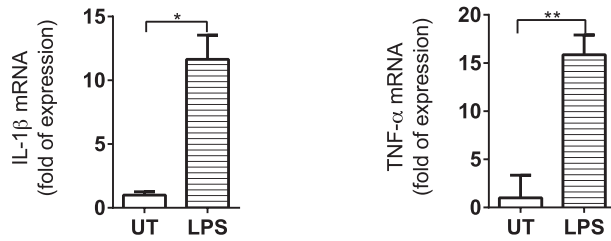
b.) Conjunctiva Culture
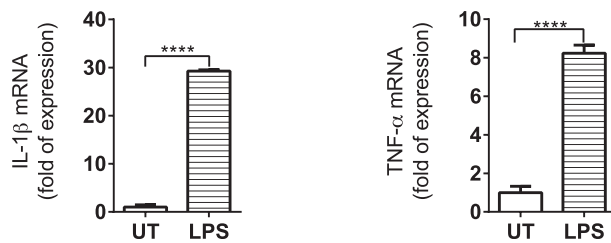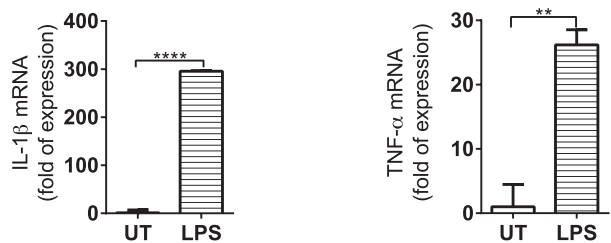

c.) Dendritic Cell Culture

FIGURE 5. LPS increases mRNA expression of inflammatory mediators in murine cell culture. Increased expression levels of IL-1β and TNF-α are demonstrated after addition of 1 μg of LPS to cornea (**a**), conjunctiva (**b**), and dendritic (**c**) cell cultures. Cells were collected 4 hours after the addition of LPS. *Error bars* are SEM. ****$P \leq 0.0001$; **$P \leq 0.01$; *$P \leq 0.05$.

loss and is capable of inducing apoptosis in the conjunctival epithelium.[34] Increased production of IFN-γ after treatment with LPS is also significant, in part, because of the ability of IFN-γ to amplify the immune response. IFN-γ increases production of Th1 chemokines (CXCL9, CXCL10, and CXCL11), which, in turn, attract greater numbers of IFN-γ–producing cells.[35] Also, IFN-γ increases expression of CD80 and CD86 on dendritic cells, which is required for T cell activation.[35] Finally, IFN-γ leads to increased IL-12 receptor expression.[35] Interestingly, IL-12a, the main cytokine responsible for Th1 differentiation, was also significantly upregulated in the conjunctiva after LPS stimulation (Fig. 2). Upregulation of IL-12a, in conjunction with IFN-γ, suggests LPS stimulation can promote the Th1 response that is critical to dry eye pathology.

It is particularly interesting that application of LPS to non-stressed mice leads to production of inflammatory mediators in the cornea (Fig. 1), as well as the conjunctiva. There are some conflicting findings in the literature as to whether[36,37] or not[38,39] human corneal epithelial cells can respond to LPS in vitro. Figure 5 demonstrates cultured mouse cornea epithelial cells do respond to LPS in vitro. In vivo, wounded mouse corneas have been demonstrated to respond to LPS stimulation.[40,41] This study suggests that normal, unwounded mouse corneas can also respond to TLR4 activation in vivo.

In the cornea (and conjunctiva), LPS administration significantly increased expression of CXCL10 and IL-1β in NS mice (Fig. 1). Both the CXCL10 protein and mRNA are known to increase in a mouse model of dry eye.[17] Increased CXCL10 expression has also been found in human dry eye.[42] Similarly, IL-1β, a proinflammatory cytokine, is increased in mice with experimental dry eye[43] and in humans with the disease.[11] Blockade of IL-1 pathways during dry eye has been shown to be efficacious in murine models of DE.[44,45] In the end, LPS was found to enhance expression of cytokines and chemokines on a nonstressed ocular surface, which demonstrates that TLR4 activation could contribute to homeostatic immune surveillance.

The cell culture studies demonstrate that mouse cornea and conjunctiva epithelial cells, as well as dendritic cells, can respond to LPS on the ocular surface. The production of inflammatory mediators observed in the in vivo studies may be from a combination of direct and indirect stimulation. Figure 5 suggests that direct activation of epithelial and dendritic cells by LPS is possible. Indirect stimulation of epithelial cells by LPS-stimulated DCs (or other immune cells) may also contribute to production of proinflammatory
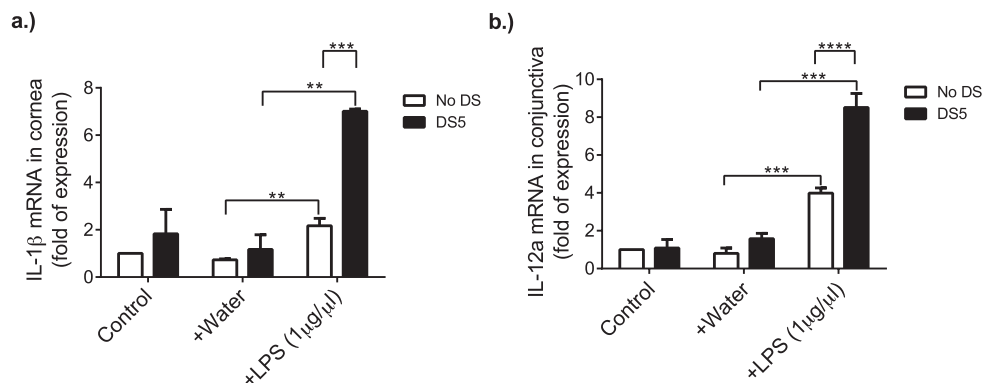

FIGURE 6. Expression of inflammatory mediators is enhanced during dry eye disease. LPS administration after 5 days of desiccating stress led to increased expression of IL-1β (cornea) and IL-12a (conjunctiva). Expression levels are higher in mice exposed to desiccating stress than in those in nonstressed mice. DS and NS studies were separate experiments. Each series represents an average ± SD of two experiments ($n \approx 3$ per group in each experiment). Separate sets of NS untreated mice were used to calculate fold increase for each series (NS control represents two separate sets of mice for each series). ****$P \leq 0.0001$; ***$P \leq 0.001$; **$P \leq 0.01$.

mediators. Figure 5 demonstrates that IL-1β and TNF-α, both of which may stimulate additional mediator release from epithelial cells, were upregulated in LPS-stimulated DC culture. Indeed, IL-1β has been shown to increase production of IL-1β and IL-6 in limbal epithelial stem cells,[46] and TNF-α has been shown to increase CXCL10 production in human corneal epithelial cells.[47]

Although LPS stimulation can lead to inflammation in a normal eye, this study suggests that TLR4 activation is enhanced during dry eye disease. Figures 4 and 6 display increased expression of IL-1β in the cornea after LPS administration when mice are exposed to DS. This finding supports our hypothesis that disruption of the corneal epithelial barrier during DE leads to greater exposure of TLR4 located in the wing and basal layers of the corneal epithelium. TLR4 is then more accessible in the cornea, leading to greater activation by LPS. Disruption of the corneal epithelial barrier has been established in our mouse model.[48] However, other mechanisms are still a possibility, including increased cell surface expression of TLR4 in the corneal epithelium during DE.[23] Nevertheless, enhanced expression of IL-1β in the cornea during DE after LPS stimulation may impact clinical severity. For instance, IL-1β has been shown to upregulate MMP-9,[31] which can lead to disruption of the corneal epithelium.[49] In turn, this could lead to even greater exposure of TLR4 and contribute to a damaging inflammatory cycle.

Surprisingly, Figure 6 also shows that LPS administration during DE increases expression of IL-12a in the conjunctiva. IL-12a is a subunit of IL-12 and IL-35.[50] More research needs to be conducted to assess the biological activity of IL-35 on the ocular surface. However, IL-12 is the main promoter of the Th1 response. Upregulation of IL-12 by LPS may indicate that TLR4 activation could be important in the early stages of dry eye disease. Microbial stress may lead to enhanced release of IL-12 at the initiation of DE, amplifying the intensity of the disease. TLR4 stimulation could potentiate the induction of the DE immune response by changing the cytokine milieu and activating Th1-polarizing dendritic cells. Additional research is needed to determine the mechanism behind the increase in IL-12a. In theory, it could be because of upregulation of TLR4 in the conjunctiva during DS.[12] However, enhanced expression of other cytokines was not observed. IL-12a is mainly released from dendritic cells and macrophages. Considering this, increased sensitivity of those cell types to LPS during DS is a possibility that needs further investigation.

In conclusion, this study demonstrates that not only is TLR4 capable of producing a proinflammatory response on the ocular surface but that response is enhanced during DE disease. TLR4 activation during DE appears to be important, especially in the initial stages of DE. In the cornea, disruption of the epithelium may expose additional TLR4 molecules, increasing susceptibility to LPS stimulation. In the conjunctiva, enhanced expression of IL-12a after TLR4 activation may promote Th1 differentiation, leading to more severe disease. Further research into amplification of inflammation by TLR4 activation in DE is needed. The role of commensal bacteria and endogenous TLR4 ligands in activation of TLR4 during DE also needs further exploration. Despite this, the proinflammatory reactions produced by TLR4 on the ocular surface illustrate that TLR4 is a potential pharmaceutical target for dry eye inflammation.

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