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Expression of Toll-Like Receptor 4 Contributes to Corneal Inflammation in Experimental Dry Eye Disease

Hyun Soo Lee,1,2 Takaaki Hattori,1 Eun Young Park,1 William Stevenson,1 Sunil K. Chauhan,1 and Reza Dana1

PURPOSE. To investigate the corneal expression of toll-like receptor (TLR) 4 and determine its contribution to the immunopathogenesis of dry eye disease (DED).

METHODS. Seven to 8-week-old female C57BL/6 mice were housed in a controlled environment chamber and administered scopolamine to induce experimental DED. Mice received intravenous TLR4 inhibitor (Eritoran) to block systemic TLR4-mediated activity. The expression of TLR4 by the corneal epithelium and stroma was evaluated using real-time polymerase chain reaction and flow cytometry. Corneal fluorescein epithelium and stroma was evaluated using real-time polymerase chain reaction. The expression of TLR4 by the corneal stromal cells revealed a significant increase in the expression pattern of TLR4 in DED.

RESULTS. The epithelial cells of normal corneas expressed TLR4 intracellularly; however, DED significantly increased the cell surface expression of TLR4. Similarly, flow cytometric analysis of stromal cells revealed a significant increase in the expression of TLR4 proteins by DED-induced corneas as compared with normal corneas. DED increased the mRNA expression of TLR4 in corneal stromal cells, but not epithelial cells. TLR4 inhibition decreased the severity of CFS and significantly reduced the mRNA expression of IL-1β, IL-6, and TNF. Furthermore, TLR4 inhibition significantly reduced the corneal infiltration of CD11b+ antigen-presenting cells, and lymph node frequency of mature MHC-IIhiCD11b+ cells were assessed.

CONCLUSIONS. These results suggest that DED increases the corneal expression of TLR4 and that TLR4 participates in the inflammatory response to ocular surface desiccating stress. (Invest Ophthalmol Vis Sci. 2012;53:5632–5640) DOI: 10.1167/iovs.12-9547

Dry eye disease (DED), one of the most common ocular complaints, is an immunoinflammatory disorder of the ocular surface; however, the immunopathogenesis of DED has not yet been fully described.1,2 The 2007 Dry Eye WorkShop concluded that tear film instability and hyperosmolarity induce ocular surface inflammation.3 Recent studies have demonstrated that corneal epithelial cells respond to hyperosmolar stress by producing proinflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs).4,5 Furthermore, hyperosmolar stress and proinflammatory cytokines such as interferon (IFN)-γ promote epithelial cell apoptosis.5–8 Toll-like receptors (TLRs) are pattern recognition receptors of the innate immune system that recognize highly conserved microbial structures and products.9,10 To date, 12 murine TLRs have been identified, and TLRs are expressed by a variety of cell types, including epithelial cells, dendritic cells, macrophages, and lymphocytes.10–13 TLR stimulation leads to the activation of nuclear factor-kappaB (NF-kB) that upregulates the production of proinflammatory cytokines and antimicrobial proteins.10,14 The NF-kB signaling pathway is important for the induction of innate and adaptive immune responses.10,14 TLR4 recognizes the Gram-negative bacterial cell wall component lipopolysaccharide (LPS) in association with cofactors such as CD14, LPS-binding protein (LB), and myeloid differentiation factor-2 (MD-2).15,16 It has also been suggested that TLR4 is a receptor for endogenous ligands associated with noninfectious diseases such as myocardial ischemia-reperfusion injury and central nervous system autoimmune disease.17,18 We hypothesized that DED-induced corneal inflammation and injury may lead to the production of endogenous TLR4 ligands that activate the immune system. Therefore, we investigated the corneal expression of TLR4 and sought to determine the expression pattern of TLR4 in DED.

MATERIALS AND METHODS

Animals

Seven to 8-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used for these experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee, and all animals were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Dry Eye Model

DED was induced by placing mice in a controlled environment chamber (CEC) and administering scopolamine (Sigma-Aldrich, St. Louis, MO) to maximize ocular surface dryness, as previously described.19,20 Mice placed in the CEC were exposed to a relative humidity < 25%, temperature of 20 to 22°C, and airflow of 15 L/min, 24 hours per day. Scopolamine hydrobromide (0.5 mg/0.2 mL) was injected subcutaneously in the dorsal skin of mice three times per day. Age- and sex-matched mice placed in the standard vivarium served as

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normal controls. Mice were euthanized on day 7 or day 9 for cellular and molecular analysis.

**Corneal Fluorescein Staining**

To evaluate the effects of desiccating stress on the ocular surface, corneal fluorescein staining (CFS) was performed at baseline (day 0), day 2, day 4, and day 9. One μL of 1% fluorescein (Sigma-Aldrich) was applied to the inferior-lateral conjunctival sac of the mice, and corneal fluorescein staining was examined with a slit-lamp biomicroscope under cobalt blue light 3 minutes later. Punctate staining was evaluated in a masked fashion using the National Eye Institute grading system, giving a score of 0 to 3 each of the five areas of the cornea.21

**Systemic Administration of TLR4 Inhibitor**

Eritoran tetrasodium (1.1 mg) and vehicle were gifted from Eisai Research Institute (Andover, MA) and reconstituted in endotoxin-free water (Sigma-Aldrich, Monticello, IA). Mice received either 50 μL of Eritoran (5 mg/kg dissolved in vehicle) or vehicle once per day via tail vein injection beginning 1 day prior to dry eye induction.17 Mice were euthanized on day 7 or day 9 for cellular and molecular studies.

**Real-Time Polymerase Chain Reaction**

Total RNA was extracted from corneal tissue or bone marrow–derived dendritic cells (BMDCs) using a commercial reagent (TRizol; Invitrogen, Carlsbad, CA) and an RNA purification kit (RNeasy Micro Kit; Qiagen, Germantown, MD). First-strand cDNA was synthesized with random hexamers using reverse transcriptase (SuperScript III; Invitrogen), and quantitative real-time polymerase chain reaction (PCR) was performed using predesigned primers (Taqman PCR Mastermix and FAM dye-labeled primers; Applied Biosystems, Foster City, CA) for IL-1β (Mm00443228_m1), IL-6 (Mm00446190_m1), TNFα (Mm99999068_m1), CCL2 (Mm00439620_m1), TLR4 (Mm0045273_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1). The GAPDH gene was used as the endogenous reference for each reaction. The results were analyzed by the comparative threshold cycle (Ct) method with commercial analysis software (LightCycler, version 3; Roche Diagnostics Corp., Indianapolis, IN) and the relative expression level of each sample was expressed as fold change from wild-type DED or untreated DED group.

**Western Blot Assay**

Ten corneal epithelial cell sheets were isolated from each group and lysed in extraction buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 20 μg/mL aprotinin, 2 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride) by homogenization. Lysates were centrifuged for 10 minutes at 13,000 g at 4°C. Total protein (100 μg) in each group was separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and subjected to Western blot analysis using TLR4 antibody (eBioscience Inc.). Isotype control was stained with the appropriately matched antibodies (eBioscience Inc.). For intracellular staining of TLR4, the cell fixation/permeabilization kit (eBioscience Inc.) was used as described earlier.22

**Analysis of Cellular Infiltration by Immunohistochemical Staining**

For whole-mount corneal staining of CD11b+ cells, corneas harvested on day 9 were fixed in acetone for 15 minutes and incubated with anti-FcR CD16/CD32 antibody (BD Pharmingen, San Diego, CA) for 45 minutes to block nonspecific staining. Corneas were immunostained with primary or isotype antibody overnight and mounted using a commercial mounting medium with DAPI (4,6-diamidino-2-phenylindole; VectaShield; Vector Laboratories, Burlingame, CA), as previously described.20 The following primary antibodies were used for immunohistochemical staining: fluorescein isothiocyanate (FITC)–conjugated rat anti-mouse CD11b (1:100; monocye/macrophage marker; BD Pharmingen) and FITC-conjugated rat IgG2bk (isotype control; BD Pharmingen). Flat-mount corneas were examined with a confocal microscope (Leica TCS–SP5; Leica Microsystems, Wetzlar, Germany) at ×400 magnification and Z-stack images were taken through the whole thickness of the corneal stroma. FITC-CD11b+ cells in corneal stroma were counted in five to six areas in the periphery (0.5-μm area from the limbus) and two areas in the center of each cornea in a masked fashion using Z-stack images. The mean number of cells was obtained by averaging the cell number in each area examined.

**Single Cell Isolation from the Cornea and Draining Lymph Nodes**

Excised corneas were incubated with 20 mM EDTA at 37°C to separate the epithelial and stromal layers, and these tissues were subsequently digested in 2 mg/mL collagenase D (Roche Diagnostic Corp.) and 0.5 mg/mL DNase (Roche Diagnostic Corp.) for 2 hours at 37°C. The suspension was filtered through a 70-μm cell strainer (BD Falcon; Becton-Dickinson, Franklin Lakes, NJ). Single-cell suspensions from draining LNs were prepared with a 70-μm cell strainer. Trypan blue exclusion assay confirmed cell viability.

**Flow Cytometric Analysis**

Cells were incubated with Fc blocking antibody in 0.5% BSA at 4°C for 30 minutes. Cells were then immunostained with the following antibodies: FITC-conjugated anti-CD11b, Alexa Fluor 647–conjugated anti-CD11c, allopurinolcin (APC-Cy7)-conjugated anti-IAβ, APC-conjugated anti-CD45, or PE-conjugated anti-TLR4 (UT41; ebioscience Inc.). Isotype control was stained with the appropriately matched antibodies (ebioscience Inc.). For intracellular staining of TLR4, either a cell fixation/permeabilization kit (ebioscience Inc.) or 0.5% Tween-20 was used. Cells were fixed with IC fixation buffer and then incubated with permeabilization buffer and stained with PE-conjugated anti-TLR4 antibody, per the manufacturer’s recommendations. Stained cells were analyzed with a flow cytometer (LSRII; Becton-Dickinson) and a commercial program (Summit v4.3; Dako Colorado, Inc., Fort Collins, CO).

**Immunohistochemical Staining of TLR4-Expressing Corneal Epithelial Cells**

Corneal epithelial cell suspensions were immunostained with Alexa Fluor 488–conjugated anti-TLR4 (UT41; ebioscience Inc.) for 2 hours in 0.5% BSA at room temperature. Isotype control was stained with Alexa Fluor 488–conjugated anti-mouse IgG1 antibody (ebioscience Inc.). After washing these cells, epithelial cells were incubated with plasma membrane stains (CellMask; Invitrogen) for 5 minutes at 37°C in RPMI media (Invitrogen-Gibco), according to the manufacturer’s recommendations. FITC-TLR4 positive cells in corneal epithelial cells were counted in six areas per each group under ×400 magnification in a masked fashion. For intracellular staining of TLR4, the cell fixation/permeabilization kit (ebioscience Inc.) was used as described earlier.

**In Vitro Stimulation of Bone Marrow–Derived Dendritic Cells with Corneal Tissue**

Bone marrow–derived dendritic cells (BMDCs) were generated using a previously described method.22 In brief, femurs and tibiae were collected from freshly euthanized mice, flushed, and the resultant cells were seeded at 1 × 10^6/mL in RPMI 1640 (BioWittacker, Walkersville, MD) supplemented with 10% fetal bovine serum (Gemini Bio-products, West Sacramento, CA), 1% penicillin/strepto-
mycin (Cambrex, East Rutherford, NJ), and 20 ng/mL mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (Biolegend, San Diego, CA) at 37°C with 5% carbon dioxide. The medium was exchanged with 10 mL medium containing 20 ng/mL GM-CSF on day 4. Loosely adherent cells were harvested on day 7 after being thoroughly washed with sterile PBS.

BMDCs ($5 \times 10^5$ cells) were seeded on the bottom surface of a 24-well plate with either 100 ng/mL vehicle or Eritoran. Corneal epithelium and stroma harvested from naïve and dry eye–induced mice were homogenized in 500 mL of sterile fresh culture medium. Supernatants were centrifuged to remove undissolved particles and added to the top of a tissue-culture insert with 1.0 mm pores (Transwell; BD Falcon, Franklin Lakes, NJ). After BMDC pretreatment with Eritoran or vehicle for 1 hour, BMDCs were incubated with homogenized corneal tissue for 18 hours after which they were collected in TRIzol for real-time PCR analysis.

**RESULTS**

**DED Increases TLR4 Expression on Corneal Epithelial and Stromal Cells**

The first experiment aimed to elucidate whether TLR4 expression on the cell surface of corneal epithelial and stromal cells (including CD45 negative and positive cells) is modified in DED. Corneal epithelial and stromal cells from dry eye–induced or naïve corneas were stained with anti-CD45 and anti-TLR4 antibody. Dry eye induction significantly increased the frequency of TLR4 expression on CD45 negative epithelial cells ($P = 0.0039$, Fig. 1A) and increased the mean fluorescence intensity (MFI) of TLR4 expression on CD45 negative stromal cells (8.05 ± 2.17 in naïve vs. 15.5 ± 4.44 in DED, $P = 0.048$, Fig. 1B) and CD45 positive stromal cells (23.07 ± 7.09 in naïve vs. 43.23 ± 12.24 in DED, $P = 0.034$, Fig. 1C).

**DED Causes Corneal Epithelial Cells to Translocate Cytoplasmic TLR4 to the Cell Surface**

Previous studies have reported that human corneal epithelial cells express TLR4 protein intracellularly, but not on the cell surface. In our study, extracellular and intracellular analysis

**Statistical Analyses**

Data are expressed as the mean ± SEM of at least three trials. The significance of the difference between groups was analyzed with the two-tailed Student’s t-test using commercial analytical software (Prism, version 5.0; GraphPad, San Diego, CA). $P < 0.05$ was considered statistically significant.
of TLR4 with flow cytometry revealed that most TLR4 was primarily located intracellularly in epithelial cells of the normal cornea (data not shown), consistent with a previous report involving human corneal epithelial cells.\textsuperscript{12} Interestingly, DED increased the expression of TLR4 mRNA in the stroma ($P = 0.035$) after 7 days of dry eye induction, but not in the epithelium. Furthermore, DED led to a significant increase in the cell surface expression of TLR4 by corneal epithelial cells as compared with those in the normal cornea as measured by flow cytometry and immunohistochemical examination ($P = 0.0003$, Fig. 2A); however, there was no increase in either the mRNA or protein levels of TLR4 in dry eye corneal epithelium (Figs. 2B, 2C), suggesting that TLR4 is located intracellularly in normal corneal epithelial cells and DED induces the translocation of cytoplasmic TLR4 to the epithelial cell surface.

**Figure 2.** Translocation of cytoplasmic TLR4 in DED corneal epithelial cells. (A) Immunohistochemical staining of cell surface and cytoplasmic TLR4 on corneal epithelial cells from naive and DED-induced corneas with commercial-conjugated (Alexa Fluor 488) anti-TLR4 (green), commercial plasma membrane (CellMask, dark red), and DAPI nuclear staining (blue). Representative confocal images depicting increased epithelial cell surface expression of TLR4 in DED corneas as compared with naive corneas. FITC-TLR4 positive cells in corneal epithelial cells were counted at two areas per slide for each group (two mice per group) under $3400$ magnification. Data are presented as the mean $\pm$ SEM of three independent experiments for a total of six mice per group. (B) Real-time PCR analysis revealed that TLR4 mRNA expression was increased in the stroma, but not epithelium, of DED corneas on day 7. Data are presented as the mean $\pm$ SEM of three experiments with three mice per group. (C) Western blot analysis demonstrating that DED did not increase the expression of TLR4 protein relative to RasGAP, as an internal control, in the epithelium of DED corneas as compared with naive corneas ($^{*}P < 0.05$; $^{**}P < 0.01$).
Systemic TLR4 Inhibition Attenuates Dry Eye–Induced Corneal Inflammation

To explore the function of TLR4 in the immunopathogenesis of murine DED, we evaluated DED severity in mice treated with systemic TLR4 inhibitor. TLR4 inhibitor was injected intravenously every day beginning 1 day prior to dry eye induction. TLR4 inhibitor–treated mice displayed significantly lower CFS at day 4 (P = 0.029 vs. untreated; P = 0.012 vs. vehicle) and day 9 (P = 0.0039 vs. untreated; P = 0.0025 vs. vehicle, Fig. 3A), and reduced expression of TNF (P = 0.0020 vs. vehicle) in DED corneal epithelium and IL-1β (P = 0.0027 vs. vehicle), IL-6 (P = 0.0291 vs. vehicle), TNF (P = 0.001 vs. untreated; P = 0.0501 vs. vehicle), and CCL2 (P = 0.0150 vs. untreated; P = 0.1019 vs. vehicle) in DED corneal stroma as compared with untreated and/or vehicle-treated mice on day 9 (n = 4 corneas per group). Data are presented as the mean ± SEM of three or four experiments (*P < 0.05; **P < 0.01; ***P < 0.001).

DED Induces Corneal Expression of Endogenous TLR4 Ligands

To provide evidence that an endogenous TLR4 ligand is involved in the inflammatory response to DED, we evaluated the activation of BMDCs following coculture with homogenized corneal tissues exposed to either vehicle or TLR4 inhibitor. Proinflammatory cytokine expression by DCs is an important indicator of DC activity23,24 and our data for mRNA expressions of these cytokines on BMDC after coculture are provided in Supplemental Figure S1 (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9547/-/DCSupplemental). BMDCs expressed significantly higher mRNA levels of IL-1β (P = 0.0003 for the corneal stroma) and TNF (P = 0.001 for the corneal epithelium; P = 0.024 for the corneal stroma) in response to homogenized dry eye corneal tissue, as compared with naïve corneal tissue (Fig. 5).

DISCUSSION

Dry eye disease is a multifactorial, immune-mediated disorder of the ocular surface. Numerous studies have demonstrated...
**Figure 4.** Systemic TLR4 inhibition suppressed inflammatory cell activity in DED. (A) Representative confocal images showing CD11b+ cell (grayscale) infiltration of the central cornea. Intravenous TLR4 inhibition significantly decreased the number of CD11b+ cells in the periphery and center of dry eye corneas as compared with untreated and vehicle-treated corneas at day 9. Data are presented as the mean ± SEM of three or four repeated experiments, involving three to four corneas per group (*P < 0.05; **P < 0.01). (B) Representative flow data demonstrating that systemic TLR4 inhibition decreased the number of mature MHC-II^hi^CD11b+ and MHC-II^hi^CD11c+ APCs in the draining lymph nodes of dry eye mice, compared with untreated and vehicle-treated mice. Representative flow data from two trials with pooled cells from four mice per group.
that DED increases the ocular surface expression of pro-
flammatory cytokines such as IL-1β, IL-6, and TNF. These cytokines activate resident APCs and promote the corneal infiltration of additional CD11b+ cells. Activated APCs subsequently stimulate an adaptive immune response, as evidenced by the proliferation of CD4 T cells in the draining lymphatics. Furthermore, APCs prime pathogenic ocular surface-specific CD4+ T cells (e.g., T helper 17 cells), supporting the theory that DED has a autoimmune component. Autoreactive CD4+ T cells are recruited to the ocular surface by chemokines such as CCL2, CCL5, and CXCL9-11, leading to immune-mediated ocular surface damage.

TLR4 ligation results in the production of proinflammatory cytokines such as IL-1β, IL-6, and TNF that promote the activation of leukocytes and lymphocytes. The ligation of TLR4 on immature dendritic cells (DCs) leads to the downregulation of CCR6 and upregulation of CCR7, thereby enhancing the migration of DCs to draining lymph nodes. TLR4 signaling is unique in that it involves the MyD88-dependent and -independent pathways that increase the intensity and diversity of responses to LPS.

There is some controversy regarding the expression of TLR4 by corneal epithelial cells. Song et al. have reported that human corneal epithelial cells (HCEs) express TLR4 and secrete proinflammatory mediators in response to stimulation with LPS. However, Ueta et al. have reported that HCEs express TLR2 and TLR4 only at the intracellular level, and the intracellular transfer of LPS in vitro does not elicit an inflammatory response. This unresponsiveness may have been caused by deficient expression of LPS cofactors such as MD-2, LBP, or CD14, and MD-2-deficient mice do not experience LPS-mediated endotoxic shock, a featured they share with TLR4-deficient mice.

In the present study, we found that TLR4 proteins are primarily expressed intracellularly in normal murine corneal epithelial cells, consistent with previous reports involving HCEs. DED induction increased the cell surface expression of TLR4 on corneal epithelial cells and stromal cells. Interestingly, these increases occurred without corresponding increases in the expression of TLR4 mRNA or protein. These findings suggest that the cell surface expression of TLR4 increases through the translocation of cytoplasmic TLR4. Previous studies have shown that proinflammatory cytokines such as TNF, IFN-γ, and IL-17 can increase the expression of TLR4, MD-2, and LPS-dependent cytokines by synovial tissue and intestinal epithelial cells; however, Ueta et al. have reported that the stimulation of HCEs with IL-1β or TNF did not increase the expression of cell surface TLR2 or TLR4. Furthermore, oxidative stress causes the translocation of TLR4 in alveolar macrophages, thereby permitting the augmentation of LPS responsiveness. Based on the results of the present experiment, we conclude that TLR4 expression can be upregulated by extrinsic factors such as DED-associated inflammation. Further investigation will be necessary to identify which factors specifically induce the corneal epithelial and stromal cell surface expression of TLR4 in response to DED.

Systemic TLR4 blockade decreased the severity of DED as compared with both untreated and vehicle-treated mice. Previous studies have demonstrated that TLR4 deficiency diminishes immunoinflammatory responses to nonocular injury, as well as the severity of autoimmune diseases. TLR4-deficient mice express significantly lower levels of proinflammatory mediators including TNF, IL-6, CCL2, CCL5, and CXCL10 in a model of retinal ischemia. Furthermore, TLR4-deficient mice demonstrate decreased infiltration of inflammatory cells, particularly Th17 cells, and reduced production of IL-17 in a rheumatoid arthritis model. TLR4 inhibition also diminishes the production of chemokines such as IL-8/CXCL8 and CCL2, and reduces the infiltration of inflammatory cells into the cornea in response to LPS stimulation.

TLR4 is an important mediator of immune responsiveness not only to LPS, but also a variety of endogenous ligands including heat shock proteins, high-mobility group box 1, and extracellular matrix degradation products. Recent studies have demonstrated that the degradation of extracellular matrix components including fibronectin, hyaluronic acid, and heparan sulfate generates endogenous bioactivators of TLR4. MMP-9 activity is significantly increased in DED, and MMP-9 is involved in the degradation of corneal epithelial tight junctions and basement membrane proteins. Furthermore, endogenous TLR4 ligand (e.g., S100 A8 and A9) levels are increased in the tears of patients with DED, and S100A8/A9 levels have been correlated with clinical disease severity.

Therefore, we propose that DED-associated ocular surface inflammation leads to the generation of endogenous TLR4.
bioactivators, although the identity of these ligands remains to be determined.

Herein, we present evidence that systemic TLR4 blockade ameliorates the clinical signs of DED in association with reduced corneal expression of proinflammatory cytokines, reduced ocular surface APC infiltration, and decreased maturation of APCs that migrate to the draining lymph nodes. Taken together, these findings suggest that TLR4 expression is upregulated in DED and TLR4 contributes to the inflammatory response to desiccating stress at the ocular surface.

References


