High-Mobility Group Box 1 in Dry Eye Inflammation

Carolina Lema, Rose Y. Reins, and Rachel L. Redfern

The Ocular Surface Institute, University of Houston, College of Optometry, Houston, Texas, United States

Correspondence: Rachel L. Redfern, The Ocular Surface Institute, University of Houston, College of Optometry, 4901 Calhoun Road, Houston, TX 77204, USA; rredfer2@central.uh.edu.

Submitted: November 10, 2017
Accepted: February 17, 2018

Citation: Lema C, Reins RY, Redfern RL. High-mobility group box 1 in dry eye inflammation. *Invest Ophthalmol Vis Sci*. 2018;59:1741–1750. https://doi.org/10.1167/iovs.17-23363

Purpose. To determine high-mobility group box 1 (HMGB1) expression during experimental dry eye (EDE) and dry eye-like culture conditions and elucidate its role in corneal dry eye-related inflammation.

Methods. EDE was induced in 8- to 12-week-old C57BL/6 mice. Corneal tissue sections and lysates from EDE and untreated mice were evaluated for HMGB1 expression by immunostaining and quantitative real-time PCR (qPCR). For in vitro studies, human corneal epithelial cells (HCEC) were treated with hyperosmolar media, toll-like receptor (TLR) agonists, or proinflammatory cytokines to determine HMGB1 expression. HCEC were also treated with human recombinant HMGB1 (hrHMGB1) alone or in combination with inflammatory stimuli, and TNFα, IL-6, and IL-8 expression evaluated by qPCR and ELISA. Nuclear factor-κB (NF-κB) p65 nuclear translocation was determined by immunostaining.

Results. EDE mice had higher corneal HMGB1 RNA and protein expression compared to untreated animals. In HCEC, hyperosmolar stress and TNFα treatment stimulated HMGB1 production and secretion into culture supernatants. However, in vitro stimulation with hrHMGB1 did not induce secretion of TNFα, IL-6, or IL-8 or NF-κB p65 nuclear translocation. In addition, the inflammatory response elicited by TLR agonists fibroblast-stimulating lipopeptide-1 and lipopolysaccharide was not enhanced by hrHMGB1 treatment.

Conclusions. HMGB1 expression was enhanced by dry eye conditions in vivo as well as in vitro, during hyperosmolar stress and cytokine exposure, suggesting an important role for HMGB1 in dry eye disease. However, no direct inflammatory effect was observed with HMGB1 treatment. Therefore, under these conditions, HMGB1 does not contribute directly to dry eye-induced inflammation and its function at the ocular surface needs to be explored further.

Keywords: dry eye disease, HMGB1, corneal epithelium, inflammation, alarmins

Dry eye disease (DED) is a multifactorial disorder of the tear film and ocular surface.[1] Inadequate tear production or increased evaporation causes tear film instability and ocular surface inflammation that result in symptoms of discomfort, eye dryness, irritation, light sensitivity, itching, and blurry vision, leading to a reduction in quality of life. DED is the most commonly reported reason for seeking medical eye care, placing a significant financial burden on patients' lives and causing reduced productivity at work.[2] Therefore, effective and inexpensive treatment options are urgently needed for DED.[3]

Inflammation is an important response to harmful stimuli, tissue damage, pathogen exposure, or irritants and is characterized by immune cell infiltration and secretion of proinflammatory cytokines. DED-induced inflammation results in the activation of both innate and adaptive immunity.[4–6] The innate immune response is trigged by stress at the ocular surface resulting from altered tear film dynamics and environmental stressors.[7,8] Immature antigen-presenting cells (APCs), including dendritic cells, macrophages, and ocular surface epithelial cells, become activated and produce and release a variety of inflammatory mediators, such as cytokines, chemokines, matrix metalloproteases (MMPs),[9–11] and phospholipases[12] that further intensify the innate inflammatory response.

Damage or danger-associated molecular patterns (DAMPs), also called "alarmins," are endogenous molecules that are released during tissue stress or injury and signal cell damage, promoting the initiation of an inflammatory response.[13] DAMPs have been implicated in the pathogenesis of autoimmune, cardiovascular, metabolic, neurodegenerative, malignant, and infectious diseases[14,15] and are recognized by pattern recognition receptors (PRRs), including toll-like receptors (TLRs). Upon DAMP binding, TLRs activate signaling pathways, such as mitogen-activated protein kinases (MAPKs), leading to nuclear factor-κB (NF-κB) activation and inflammatory mediators production.[10,16] Importantly for the context of DED, TLRs are expressed at the ocular surface and have been implicated in dry eye-related inflammation.[17,18] Therefore, TLR activation by DAMPs may perpetuate ocular surface proinflammatory responses leading to chronic inflammation.

High-mobility group box 1 protein (HMGB1) is an abundant nonhistone nuclear protein that is involved in transcriptional regulation.[19] Upon cell death, HMGB1 is passively released into the extracellular milieu and acts as a DAMP, initiating inflammatory signaling pathways through interactions with PRRs, specifically binding to TLR2, TLR4, and the receptor for advanced glycation end products (RAGE).[19–21] HMGB1 is also actively secreted to trigger inflammation.[22–28] Extracellular HMGB1 binds and activates PRR signaling by itself or via complexes with pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and nucleic acids, or with proinflammatory cytokines and chemokines.[29–31]

In studies of the eye, HMGB1 has been found in the tear fluid during conjunctivitis and blepharitis,[32] in serum of children with vernal keratoconjunctivitis,[33] and in pterygial tissue.[34] Extracellular HMGB1 is also elevated in patients suffering from autoimmune disorders, including primary Sjögren syndrome, who have severe dry eye-related symptoms.[35] In a mouse model of ocular infection, silencing and blocking HMGB1 promoted the resolution of *Pseudomonas aeruginosa* keratitis by decreasing levels of inflammatory mediators and reducing immune cell infiltration.[36,37] These studies suggest that HMGB1 is important during ocular surface inflammation while its mechanism of action remains elusive.

In the current study, a mouse model of experimental dry eye (EDE) was used to examine HMGB1 expression during ocular surface inflammation. We also examined the effect of dry eye-like culture conditions, hyperosmolar stress, and cytokine treatment, on HMGB1 production and secretion by human corneal epithelial cells (HCEC). In addition, we investigated the effect of HMGB1 on inflammatory cytokine secretion to determine the role of HMGB1 in promoting inflammation and dissect its role in DED.

Materials and Methods

Mouse Model of EDE

EDE was induced in 8- to 12-week-old C57BL/6 mice, as previously described.[38] Briefly, mice were housed in an environmentally controlled room that was maintained at ~20% humidity to promote ocular surface desiccation. In addition, mice were exposed to continuous air flow, from fans adjacent to grate-sided cages, and were given subcutaneous scopolamine hydrobromide injections (0.5 mg/0.2 mL; Green Park Compounding Pharmacy, Houston, TX, USA) three times daily for 5 consecutive days. After 5 days of treatment, whole eyes or corneal epithelium were harvested to obtain frozen tissue sections or to extract RNA for quantitative real-time PCR (qPCR) analysis, respectively. Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Houston and adhered to the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Spectralis Spectral-Domain Optical Coherence Tomography (SD-OCT)

Corneal epithelial integrity was evaluated using SD-OCT (Heidelberg Engineering, Heidelberg, Germany), as previously described.[2] Briefly, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (75 mg/7.5 mg/kg body weight) (Vedco, Inc., St. Joseph, MO, USA) and 1.5 μL 1% sodium fluorescein (Sigma-Aldrich Corp., Springfield, MO, USA) was instilled into each eye. This was followed by a 400-μL PBS wash to remove pooled fluorescein and debris. Eyes were immediately imaged using SD-OCT with 488 nm wavelength blue light illumination. For corneal staining image analysis, pixel intensity was measured in a 1.5-mm circular area in the central cornea using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell Culture and Treatment

Telomerase-immortalized human corneal epithelial cells (hTCEpi),[39] SV40-immortalized HCEC,[40] and human monocytes U937[41] were used for these studies, as previously described. hTCEpi were cultured in serum-free keratinocyte basal medium supplemented with Keratinocyte Medium Bullet Kit (KGM-2 Lonza, Walkerville, MD, USA). SV40-HCEC were cultured in supplemental hormonal epithelial medium (SHEM).[42] The human monocyte cell line U937 (ATCC No. CRL-2367) was maintained in RPMI-1640 medium (ThermoFisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum and penicillin/streptomycin/amphotericin. Primary HCEC were cultured from human donor corneal buttons (Saving Sight, Kansas City, MO, USA) and grown in EpiLife medium with 60 μM calcium supplemented with human corneal growth supplement (HCGS; ThermoFisher Scientific), as described previously.[43] All cells were maintained under 5% CO_2 at 37°C and grown to ~80% to 90% confluency before use in experiments.

For HCEC cell treatments, human recombinant HMGB1 (hrHMGB1) was kindly provided by Alex Kurosky PhD (University of Texas Medical Branch, Galveston, TX, USA). Cells were left untreated (UT) or were stimulated with hrHMGB1 (10 μg/mL) for 4, 8, or 24 hours, with and without addition of TLR agonists (1 μg/mL) (fibroblast-stimulating lipopeptide-1 [FSL-1] and LPS [*Escherichia coli* K2 strain]) (InvivoGen, San Diego, CA, USA) or hrTNFα (10 ng/mL) (R&D Systems, Minneapolis, MN, USA); for 1 or 6 hours, or hrIFNγ (200 U/mL) (R&D Systems) for 18 hours.[44]

For macrophage differentiation, U937 cells were cultured with 200 nM (100 ng/mL) phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Corp.) for 48 hours.[45] Adherent differentiated U937 cells were then treated with 10 μg/mL hrHMGB1 for 4 or 8 hours. LPS (InvivoGen) treatment (1 μg/mL) was used as a positive control.

Hyperosmolar Stress Treatment

To obtain 400, 450, and 500 mOsM media,[46] sterile sodium chloride (1 M) was added to normal cell culture media and osmolarity values were assessed using a 5520 vapor pressure osmometer (Wescor, Inc., South Logan, UT, USA). Cells were incubated with hyperosmolar media for 4, 6, 8, or 24 hours. The 400 to 500 mOsM range was selected based on previous data indicating that the osmolarity in areas of tear breakup can reach up to 560 mOsM.[47]

Cell Viability

Cell viability was analyzed by evaluating the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Corp.) to purple insoluble formazan by mitochondrial succinate dehydrogenase in metabolically active cells.[48] After 24 hours of cell treatment, MTT reagent was added to the culture medium and intracellular formazan crystals were solubilized with dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp.). Absorbance was measured at 570 nm by spectrophotometry. Viability was determined as a percentage of UT cells and 0.02% benzalkonium chloride (BAC; Sigma-Aldrich Corp.) was used as a positive control for cell death.

Enzyme-Linked Immunosorbent Assay (ELISA)

Following treatment, cell supernatants were collected, snap frozen in liquid nitrogen, and stored at −80°C until analyses. ELISAs for IL-6, IL-8, TNFα (BioLegend, San Diego, CA, USA), and HMGB1 (IBL International, Hamburg, Germany) were performed according to the manufacturers' protocols.

HMGB1 and NF-κB p65 Immunostaining

Cells were grown on chamber slides (Nunc LabTek Chamber Slide, ThermoFisher Scientific), treated, and fixed in cold acetone (for HMGB1 staining) or 4% paraformaldehyde (for NF-κB p65 staining). Cells were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Corp.) in PBS (HMGB1) or cold

Investigative Ophthalmology & Visual Science

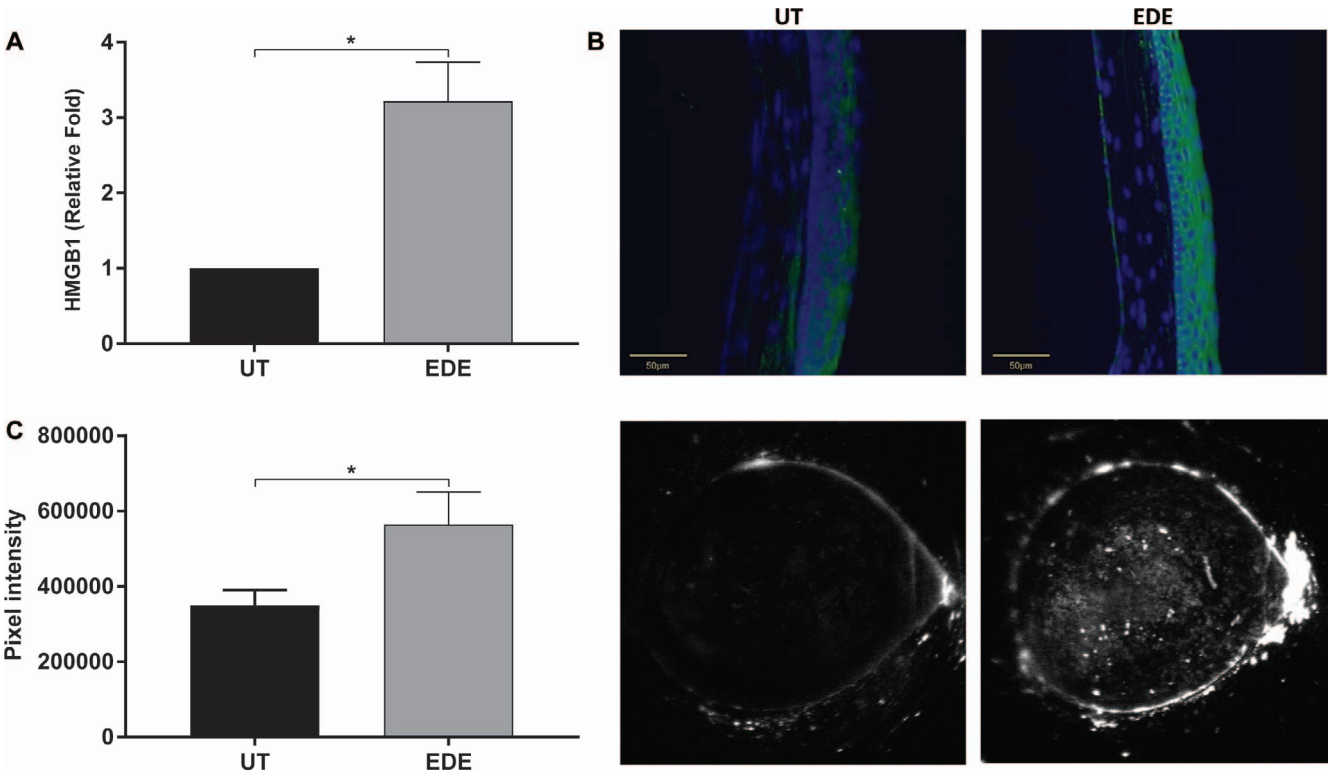

Figure 1. HMGB1 is increased in the corneas of EDE mice. (**A**) Corneal epithelial cells were removed from EDE and UT control mice for evaluation of HMGB1 mRNA expression by qPCR. Graph represents mean ± SEM ($n = 3$), where tissue from three mice was pooled for each sample (nine mice per condition). (**B**) Frozen corneal tissue sections were stained for HMGB1 expression by immunohistochemistry. Images are representative of three mice per condition; *scale bar*: 50 μm. (**C**) Dry eye was assessed by ocular surface fluorescein staining using OCT imaging. Graph represents mean ± SEM pixel intensity quantitated from OCT images (*right panel*) using ImageJ analysis ($n = 6$ for UT and $n = 5$ for EDE). Statistical comparison between UT controls and EDE was performed by unpaired *t*-test. $^*P \leq 0.05$.

methanol (NF-κB p65), followed by blocking with 15% goat or donkey serum (Abcam, Cambridge, MA, USA). Slides were incubated overnight with primary antibodies: rabbit anti-human/mouse HMGB1 (Abcam) or rabbit anti-human NF-κB p65 (Abcam) at 4°C, washed with PBS, and incubated for 1 hour with secondary antibodies (Alexa Fluor 488 goat or donkey anti-rabbit IgG; Invitrogen, Life Technologies, Carlsbad, CA, USA) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Biotium, Hayward, CA, USA). Cover slips were then mounted with Airvol mounting media (courtesy of Alan Burns, PhD, University of Houston, College of Optometry, Houston, TX, USA) and slides imaged with the DeltaVision Imaging System (GE Healthcare, Issaquah, WA, USA).

Quantitative Real-Time PCR (qPCR)

Following cell treatments, total RNA was extracted using the RNeasy Mini RNA extraction kit (Qiagen Sciences, Germantown, MD, USA). RNA concentrations were measured with the DeNovix microvolume spectrophotometer (DeNovix, Inc., Wilmington, DE, USA) and cDNA synthesized using the iScript Reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples were tested in triplicate (10 ng/well) and normalized to the housekeeping gene RPL27 (Integrated DNA Technologies, IDT, Coralville, IA, USA). Target genes were amplified using the CFX-96 real-time system with PrimePCR primers (Bio-Rad Laboratories) for human IL-6, IL-8, TLR4, CD14, MD2, and TNFα. Relative quantification of target mRNA was assessed according to the comparative Ct method (ΔΔCt) using CFX Manager software (Bio-Rad Laboratories). Results are presented as relative fold change compared to unstimulated controls.

Statistical Analyses

Data derived from in vitro and in vivo experiments was analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). *T*-test and analysis of variance (ANOVA) was used to test for statistical significance with Bonferroni's test for multiple comparisons. Results were expressed as mean ± standard error of the mean (SEM) of two or three independent experiments. For all results, a P value ≤ 0.05 was used for statistical significance.

Results

HMGB1 is Upregulated in Response to EDE Conditions

To determine if HMGB1 expression is modulated during dry eye, C57BL/6 mice were subjected to EDE for 5 days and whole corneas harvested to assess mRNA expression of HMGB1. HMGB1 levels increased in EDE compared to untreated (non-EDE) controls (Fig. 1A). Immunohistochemical analysis on frozen tissue sections was also performed to assess HMGB1 protein expression. HMGB1 was localized mainly to the superficial surface of the corneal epithelium, with a lower amount of HMGB1 staining in the stroma (Fig. 1B). EDE conditions resulted in an increase in HMGB1 expression in the corneal epithelium. To verify that our EDE model resulted in dry eye, as described previously, ocular surface damage was determined by fluorescein staining using SD-OCT imaging. As expected, EDE mice had greater corneal staining compared with UT controls (Fig. 1C).

Investigative Ophthalmology & Visual Science

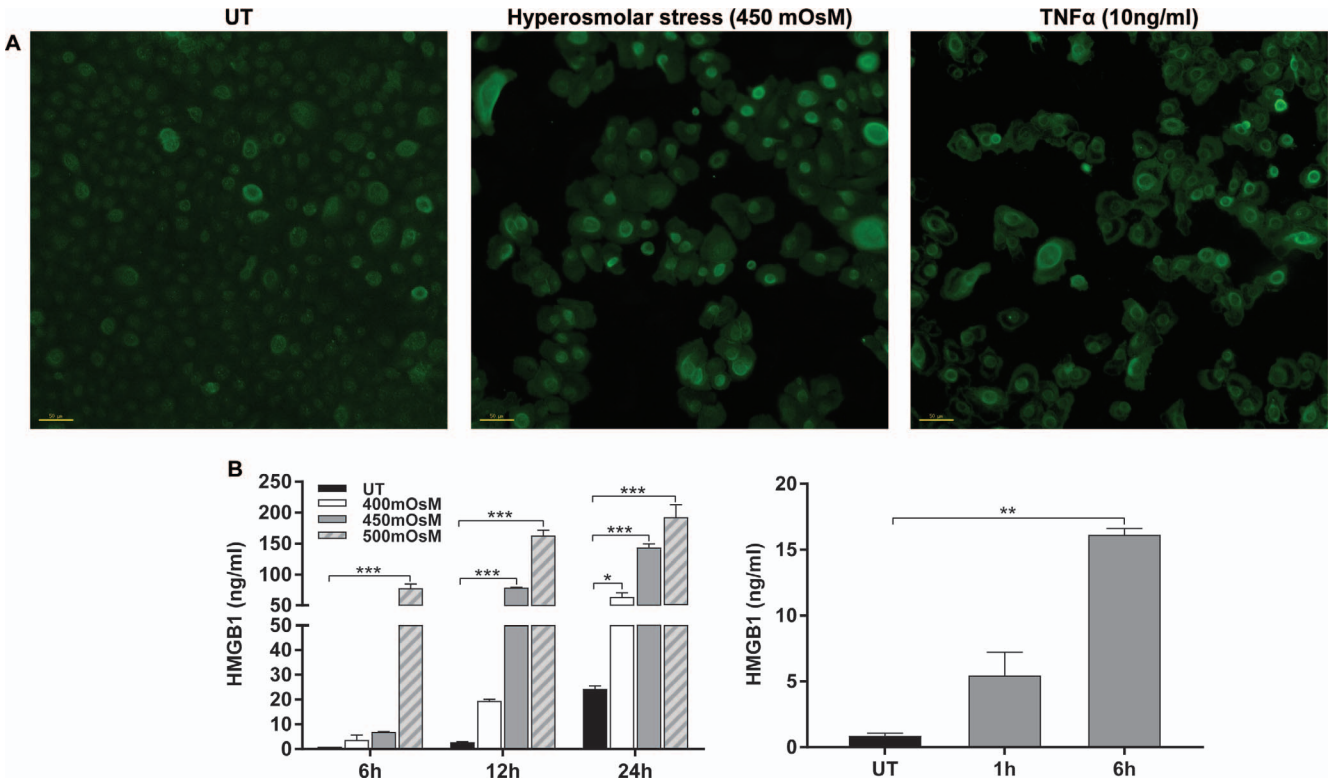

Figure 2. Hyperosmolar stress and TNFα increase HMGB1 cellular expression and secretion in HCEC. (**A**) hTCEpi were cultured with 450 mOsM media or in the presence of TNFα (10 ng/mL). After 6 hours, both hyperosmolar stress and TNFα induced increase of nuclear and cytoplasmic HMGB1 expression when compared to the UT control. Images are representative of $n = 2$ independent experiments; *scale bar*: 50 μm. (**B**) SV40 HCEC were cultured for 6, 12, or 24 hours in hyperosmolar media (400, 450, or 500 mOsM) (*left graph*). hTCEpi were stimulated with TNFα (10 ng/mL) for 1 or 6 hours (*right graph*). HMGB1 was measured in cell culture supernatants by ELISA. Graphs represent mean ± SEM of $n = 2$ independent experiments. ANOVA was used to test for statistical significance with Bonferroni's test for multiple comparisons. $^{***}P \leq 0.0001$; $^{**}P \leq 0.001$; $^{*}P \leq 0.05$.

Hyperosmolar Stress and TNFα Stimulation Increase HMGB1 Expression in HCEC

As HMGB1 was increased in EDE, we next wanted to further examine its role during ocular surface inflammation using cultured corneal epithelial cells. hTCEpi were treated with hyperosmolar media (450 mOsM) or TNFα (10 ng/mL) for 6 hours to mimic the inflammatory environment that occurs with DED. Both treatments resulted in an increase in intracellular HMGB1 expression when compared to the UT control (Fig. 2A). In addition, we used SV40 HCEC, an established cell line extensively used in dry eye studies to assess HMGB1 secretion under hyperosmolar conditions. SV40 HCEC secreted HMGB1 into culture supernatants in response to hyperosmolar stress in a dose and time-dependent manner (Fig. 2B). After 6 hours, HMGB1 secretion was observed only at 500 mOsM. At 12 hours, HMGB1 was secreted with 450 and 500 mOsM. At the longest time point tested, HCEC secreted HMGB1 at 400, 450, and 500 mOsM after 24 hours of hyperosmolar stress (Fig. 2B). Similarly, HMGB1 secreted levels increased by almost 6- and 16-fold following TNFα treatment after 1 and 6 hours, respectively (Fig. 2B). Importantly, neither hyperosmolar media nor TNFα treatment affected the viability of the corneal epithelial cells (Supplementary Fig. S1). These results demonstrate that during inflammatory stimulation and stress, HMGB1 expression is augmented and furthermore, HMGB1 is secreted into the extracellular environment.

HMGB1 Does Not Increase IL-6, IL-8, or TNFα Expression in hTCEpi

Upon release, HMGB1 has been shown to propagate inflammatory signaling. Therefore, we wanted to determine if HMGB1 treatment could upregulate the production of proinflammatory cytokines in HCEC. Based on the observed levels of HMGB1 in cell culture supernatants, hTCEpi were initially stimulated with HMGB1 (0.001–1 μg/mL) followed by quantitation of inflammatory cytokines at the protein and mRNA levels; however, no stimulatory effect was observed even in the presence of TLR ligands (data not shown). Although most studies conducted in epithelial cells report ≤1 μg/mL HMGB1 as able to elicit inflammation, the use of as high as 10 μg/mL in human[49] and mouse APCs[50,51] is not uncommon.

Therefore, given the negative results observed at lower doses, hTCEpi were stimulated with HMGB1 at 10 μg/mL; and IL-6, IL-8, and TNFα levels were determined in both RNA (cell lysates) and cell culture supernatants. No change in expression was observed for any of the analyzed inflammatory cytokines at either the RNA (Fig. 3A) or protein (Fig. 3B) levels. Conversely, with the same HMGB1 treatment conditions, macrophage-differentiated U937 (Mϕ-U937) cells, confirmed by CD14 expression (Supplementary Fig. S2), responded by increasing the expression of IL-6 after both 4 and 8 hours (Fig. 3C). At the protein level, IL-6 concentrations increased by 23.5-fold and IL-8 by 36.1-fold after 4 hours (Fig. 3D). After 8 hours of HMGB1 treatment, all three cytokines were increased: IL-6 (51.3-fold); IL-8 (35.4-fold); and TNFα (8.5-fold) (Fig. 3D). These results

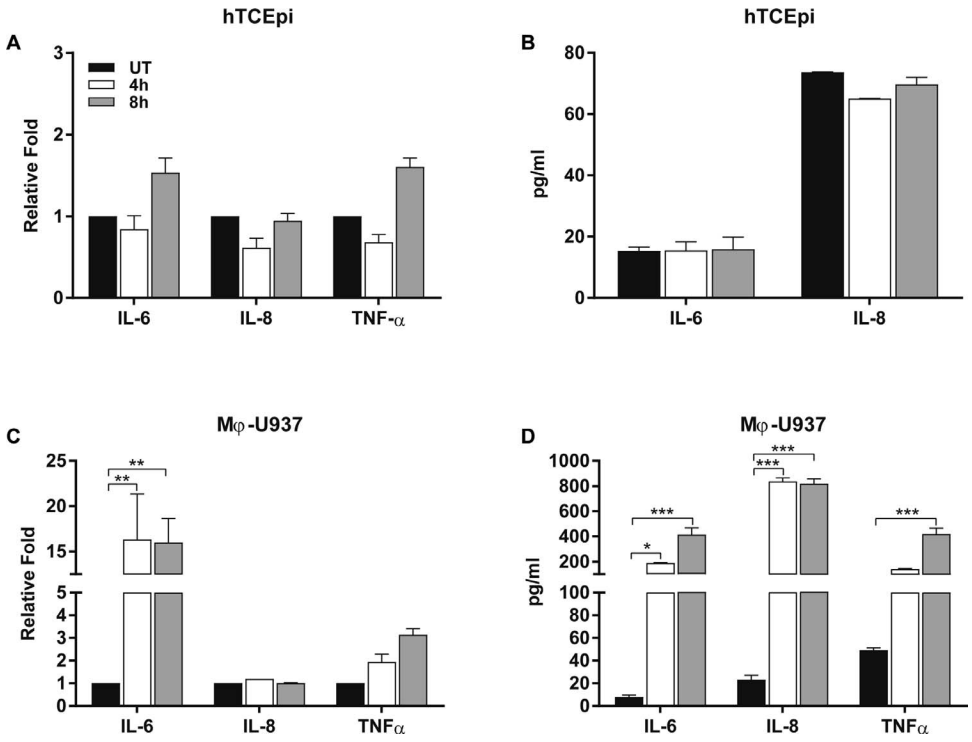

FIGURE 3. HMGB1 does not induce secretion of inflammatory cytokines in HCEC. hTCEpi (**A**, **B**) and macrophage-differentiated U937 (Mϕ-U937) cells (**C**, **D**) were stimulated with HMGB1 (10 μg/mL) for 4 or 8 hours. mRNA expression (*left graphs*) and secreted IL-6, IL-8, and TNFα (*right graphs*) were measured by qPCR and ELISA, respectively. Graphs represent mean ± SEM of $n = 2$ (Mϕ-U937) and $n = 4$ (HTCEpi) independent experiments. ANOVA was used to test for statistical significance with Bonferroni's test for multiple comparisons. $^{***}P \leq 0.0001$; $^{**}P \leq 0.001$; $^{*}P \leq 0.01$.

validate the proinflammatory activity of the hrHMGB1 used in the present study, despite the inability of HCEC to respond to HMGB1 treatment.

HMGB1 Does Not Synergize With TLR Agonists to Increase Inflammatory Cytokines in HCEC

In addition to acting as an alarmin on its own, HMGB1 also acts in coordination with various TLR ligands, PAMPs, to initiate inflammation and induce cytokine production. As HMGB1 treatment was not able to induce cytokine expression in HCEC, we examined HMGB1's ability to enhance the HCEC response to TLR2 and TLR4 ligands. hTCEpi and primary HCEC were cultured with rhHMGB1, FSL-1 (TLR2 agonist), or both for 24 hours and levels of IL-6 and IL-8 were measured in cell culture supernatants. Simultaneously, in order to confirm that results were not inherent to the cell line used, parallel treatments were conducted in primary cultures of HCEC prepared from corneas derived from human donors. In hTCEpi, FSL-1 alone increased the secretion of IL-6 and IL-8 (Fig. 4A). In primary HCEC, only IL-8 was increased by FSL-1 treatment (Fig. 4B). When HMGB1 was added with FSL-1, no change was observed in IL-6 or IL-8 levels above FSL-1 treatment alone, in either hTCEpi or primary HCEC.

To examine the ability of HMGB1 to synergize with the TLR4 agonist, LPS, hTCEpi were stimulated with HMGB1 (10 μg/mL), LPS (1 μg/mL), or both for 8 hours. RNA was extracted from cellular lysates and the relative mRNA expression of IL-6, IL-8, and TNFα was determined by qPCR. No significant change in expression was observed for any of the inflammatory

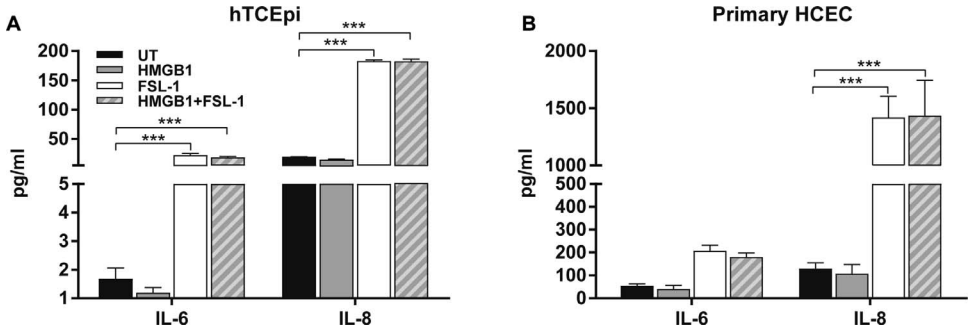

FIGURE 4. HMGB1 does not synergize with FSL-1 to increase inflammatory cytokines in HCEC. hTCEpi (**A**) and primary HCEC (**B**) were cultured in the presence of HMGB1 (10 μg/mL) or FSL-1 (1 μg/mL) or both for 24 hours and levels of IL-6 and IL-8 were measured in culture supernatants. Graphs represent mean ± SEM of $n = 3$ independent experiments. ANOVA was used to test for statistical significance with Bonferroni's test for multiple comparisons. $^{***}P \leq 0.0001$.

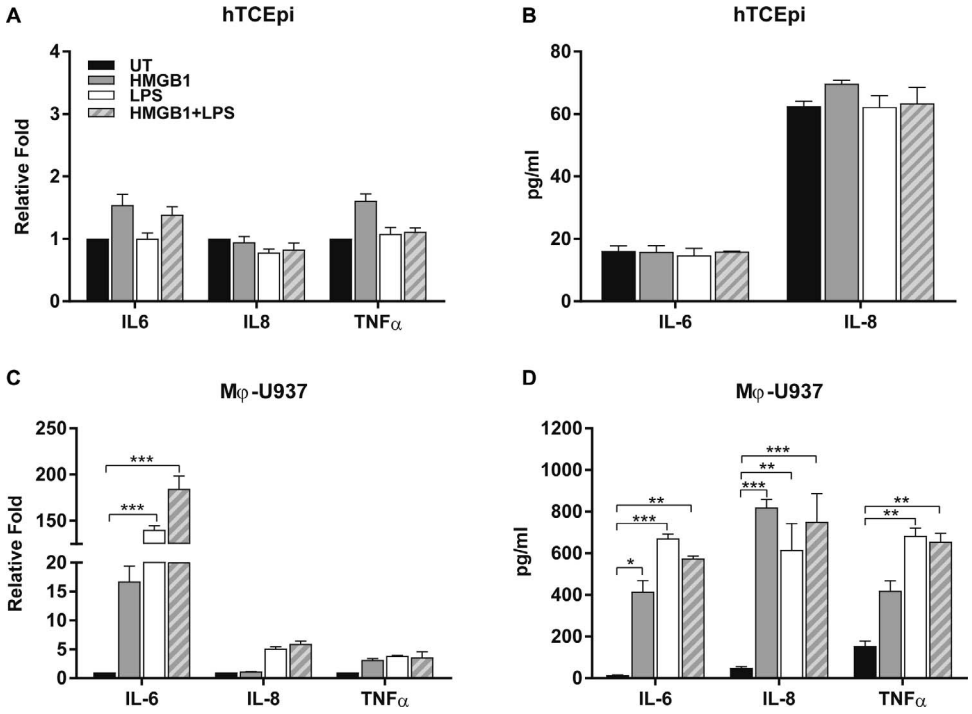

Figure 5. HMGB1 does not synergize with LPS to increase inflammatory cytokines in HCEC. hTCEpi (**A**, **B**) and macrophage-differentiated U937 (Mϕ-U937) cells (**C**, **D**) were stimulated with HMGB1 (10 μg/mL) or LPS (1 μg/mL) or both for 8 hours. mRNA expression (*left graphs*) and secreted cytokines (*right graphs*) were measured by qPCR and ELISA, respectively. Graphs represent mean ± SEM of $n = 2$ (Mϕ-U937) and $n = 4$ (hTCEpi) independent experiments. ANOVA was used to test for statistical significance with Bonferroni's test for multiple comparisons. $^{***}P \leq 0.0001$; $^{**}P \leq 0.001$; $^{*}P \leq 0.01$.

cytokines analyzed after 8 hours (Fig. 5A) and 24 hours in primary HCEC and SV40 HCEC (data not shown). Similar results were obtained with secreted levels of IL-6, IL-8, and TNFα in cell culture supernatants (Fig. 5B). Parallel experiments were conducted on Mϕ-U937 cells. At the mRNA level, LPS and HMGB1 increased IL-6 expression (Fig. 5C); this increase was even greater in the presence of both. In cell supernatants, Mϕ-U937 cells responded to both HMGB1 and LPS by secreting IL-6, IL-8, and TNFα (Fig. 5D). However, no synergistic effect was observed.

IFNγ Did Not Improve Cell Responsiveness to LPS or HMGB1

In order to determine whether the unresponsiveness of HCEC to LPS and HMGB1 was due to deficient TLR signaling ability, expression of the TLR4 accessory molecule, myeloid differentiation protein-2 (MD2) was evaluated and induced by IFNγ. Primary HCEC were stimulated with IFNγ prior to treatment with HMGB1, LPS, or both for 8 hours. Cell supernatants were then collected for assessment of IL-6, IL-8, and TNFα expression. Although IFNγ treatment increased MD2 expression (Supplementary Fig. S3), augmenting the signaling capability of TLR4, it did not improve the ability of HCEC to respond to LPS or HMGB1 with IL-6, IL-8, or TNFα induction (Fig. 6).

HMGB1 Does Not Induce NF-κB Translocation in hTCEpi

The NF-κB pathway is a prototypical inflammatory signaling pathway that is activated in response to stress and immune

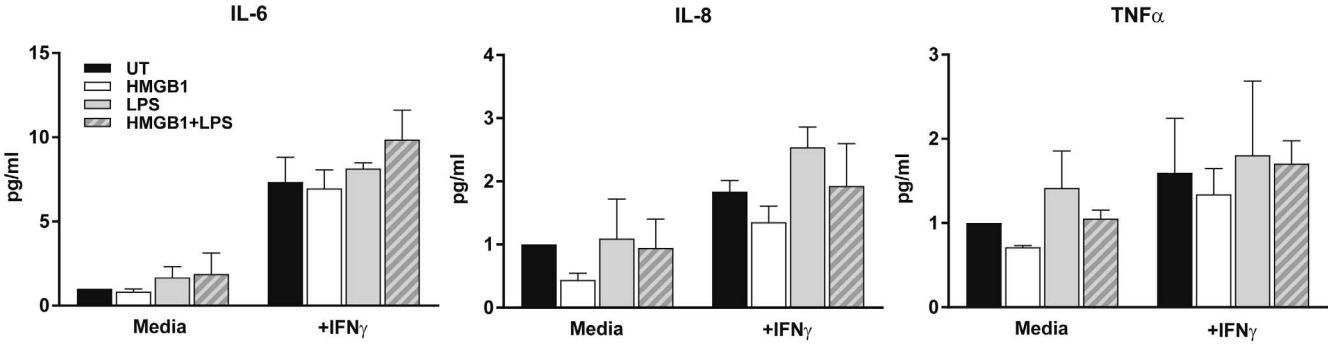

Figure 6. IFNγ does not improve HCEC responsiveness to LPS or HMGB1. Primary HCEC were stimulated with IFNγ (200 U/mL) prior to treatment with HMGB1, LPS, or both for 8 hours. Cell lysates were collected for RNA extraction and qPCR determination of relative IL-6, IL-8, and TNFα mRNA expression. Graphs represent mean ± SEM of $n = 2$ independent experiments.

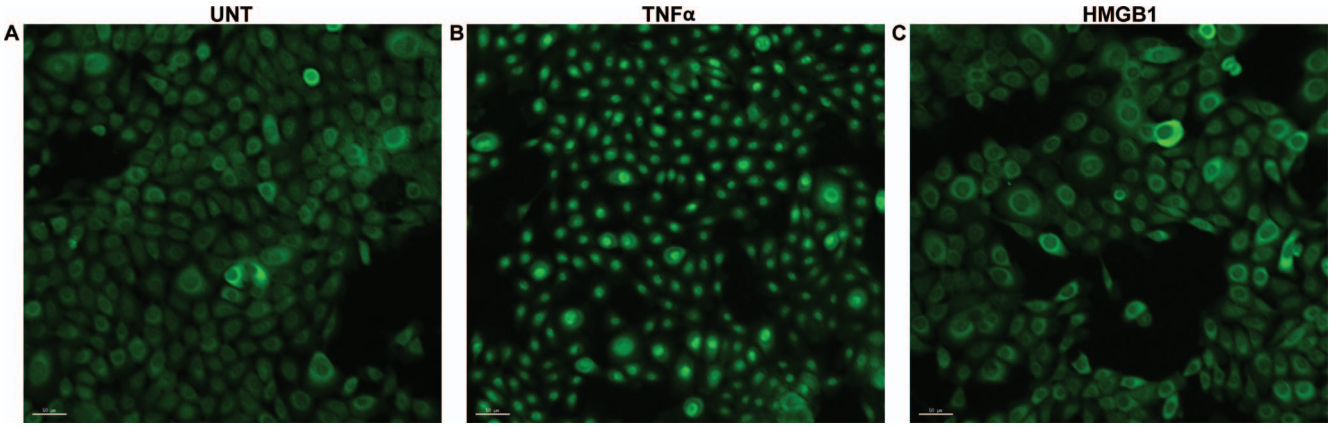

FIGURE 7. HMGB1 does not induce NF-κB translocation in HCEC. hTCEpi were cultured in the presence of HMGB1 (50 ng/mL) for 2 hours and immunostained for NF-κB p65 expression. TNFα (10 ng/mL) was used as a positive control for NF-κB p65 cytoplasm-nucleus translocation. No translocation of NF-κB p65 was observed with HMGB1 treatment. Images are representative of $n = 2$ independent experiments; *scale bar*: 50 μm.

system activation. Upon signaling initiation, the NF-κB p65 subunit is released from a complex of regulators and translocates to the nucleus, where it binds DNA regulatory elements and induces transcription of proinflammatory genes. To determine if HMGB1 was able to induce NF-κB translocation, hTCEpi were cultured with HMGB1 (50 ng/mL) for 2 hours. Cells were then immunostained for NF-κB p65 to examine cytoplasm-nuclear translocation. TNFα (10 ng/mL) treatment was used as positive control. HMGB1 did not activate the NF-κB signaling pathway as no translocation of NF-κB p65 was observed (Figs. 7A–C).

Discussion

DED is characterized by increased tear osmolarity, ocular surface damage, and chronic inflammation. Following cell injury during inflammation, the alarmin HMGB1 is released and activates an innate immune response. The present study demonstrates that HMGB1 levels are increased in the corneal epithelium in EDE and in dry eye-like cell culture conditions. However, we did not observe a direct inflammatory effect of HMGB1 on HCEC, suggesting that HMGB1 has a different function on HCEC during inflammation.

In a recent study, we found that HMGB1 levels were increased in the tears of dry eye subjects as well as a positive correlation between HMGB1 concentration in the tears and Ocular Surface Disease Index (OSDI) score, suggesting HMGB1 levels may be associated with ocular discomfort.[52] In the current study, EDE animals had ocular surface damage and increased corneal HMGB1 expression, shown by immunostaining and qPCR analyses, in comparison to animals housed in a normal, non-EDE environment. Tear film osmolarity was not determined in the EDE mice; however, previous studies have shown that C57BL/6 mice with EDE have elevated tear film osmolarity (300 mOsM) compared to UT mice (177 mOsM).[53]

A study using a mouse model of Sjögren syndrome demonstrated that blocking HMGB1, by subconjunctival administration of anti-HMGB1 antibody, resulted in decreased corneal epithelial erosions, increased tear secretion, and higher goblet cell density.[54] These results suggest a proinflammatory role for HMGB1 during ocular surface damage and dry eye-related inflammation.

In DED, tear film instability and environmental stresses, such as low humidity, produce changes in tear osmolarity that lead to chronic corneal epithelium stress, inflammation, and ocular irritation. When HCEC were cultured in hyperosmolar media (450 mOsM), we observed increased levels of HMGB1 protein in both nucleus and cytoplasm, as well as HMGB1 protein release into the cell culture supernatant. Likewise, when HCEC were stimulated with TNFα, HMGB1 expression was increased, both at the RNA and protein levels. TNFα is one of the main inflammatory cytokines produced by the corneal epithelium in response to hyperosmolarity.[46] In addition, the clinical severity of DED has been correlated with TNFα levels,[55] and TNFα has been found to be elevated in tears from DED patients[56,57] and in tear-washings of dry eye mice.[58] These results demonstrate that HMGB1 production is enhanced by a dry eye-like environment.

Based on these results of increased HMGB1 in human DED subjects and EDE, we hypothesized that secreted HMGB1 augmented inflammatory signaling during desiccating stress and dry eye culture conditions. To evaluate this, we treated HCEC with hrHMGB1 and measured IL-6, IL8, and TNFα levels in cell culture supernatants. hrHMGB1-treated cells did not show any change in inflammatory cytokine expression compared to the UT controls, at any HMGB1 concentration tested. On the contrary, Mϕ-U937 cells responded to hrHMGB1 stimulation by upregulating these cytokines, demonstrating that the hrHMGB1 used was biologically active.

It has been shown that HMGB1 can interact with soluble PAMPs and bind to TLRs to initiate signaling.[59,60] In addition to RAGE,[19] HMGB1 binds to TLR2,[61] TLR4,[51,61,62] and TLR9.[63] We have previously demonstrated that HCEC secrete inflammatory cytokines and chemokines when stimulated by TLR agonists[17,64] and have shown TLR upregulation in the cornea, conjunctiva, and lacrimal gland in EDE mice.[18] We have also reported an increase in TLR4 expression in DED subjects.[17] Therefore, as HCEC did not produce cytokines in response to HMGB1 treatment alone, we examined a possible synergism between HMGB1 and LPS (TLR4 agonist) or FSL-1 (TLR2 agonist). When HCEC were stimulated with LPS or FSL-1, there was increased production of IL-6 and IL-8. However, the addition of hrHMGB1 did not further augment cytokine expression.

Although HCEC express TLR4,[65] they have been reported to be low responders to LPS due to insufficient expression of MD2,[66] an accessory molecule required for HMGB1-TLR4 signaling.[67] Therefore, we used IFNγ to stimulate MD2 expression in primary HCEC, confirming higher expression by qPCR. However, IFNγ priming did not induce IL-6, IL-8, or TNFα secretion by HCEC treated with LPS or hrHMGB1. We also examined cytoplasm-nuclear translocation of NF-kB p65 in HCEC by immunohistochemistry, as HMGB1 has been shown

to activate NF-kB signaling in other cell types.[23] After 2 hours, we did not observe NF-kB p65 translocation in HCEC, which was in agreement with the lack of stimulated inflammatory cytokine production.

Our data suggest that although its expression is increased, HMGB1 does not mediate DED-related inflammation in the corneal epithelium by directly inducing inflammatory cytokine production. However, secreted HMGB1 might have a direct inflammatory effect on other corneal cell types, such as dendritic cells or macrophages. The human cornea hosts mature and immature resident bone marrow-derived APCs, demonstrating that this important tissue actively participates in the immune response to foreign antigens and invading pathogens.[68] Therefore, HMGB1 might stimulate PRRs on these APCs, influencing inflammatory signaling and shaping the initiation of adaptive immune responses. The ability of HMGB1 to influence immune cells in the corneal epithelium has previously been reported during *P. aeruginosa* infection, where the use of siHMGB1 or a neutralizing antibody to HMGB1 resulted in decreased mononuclear cell infiltration and improved clinical score in infected C57BL/6 mice.[37] Similarly, the HMGB1 inhibitor glycyrrhizin reduced IL-1β expression and cellular infiltrates in the corneal stroma of PA-infected mice.[36] Future in vitro and in vivo studies investigating blocking of secreted HMGB1 via neutralizing antibodies, HMGB1 inhibitors, or reactive oxygen scavengers might also provide new insights into its role in dry eye inflammation.

In addition to immune cells, epithelial-derived HMGB1 might target other cells of the cornea. Alarmins, released from necrotic corneal epithelial cells, have been shown to induce CCL11 (eotaxin-1) and vascular cell adhesion molecule-1 secretion by keratocytes,[69] which are known to play a key role in the recruitment of inflammatory immune cells into the cornea during innate and adaptive immune responses.[70–72]

The absence of IL-6, IL-8, and TNFα production by HCEC when stimulated with HMGB1 in our study agrees with a recent report by Fukuda et al. (2017) that investigated the ability of necrotic HCEC-derived alarmins to upregulate inflammatory cytokines in separate HCEC cultures.[73] In this study, the supernatant derived from necrotic cells induced upregulation of IL-6 and IL-8; however, when added individually, HMGB1 failed to produce this inflammatory response. This result also suggests that HMGB1's proinflammatory role within the corneal epithelium is not direct, and it could be dependent on the presence of other alarmins, inflammatory molecules, or may activate immune cells directly.

Extracellular HMGB1 might stimulate immune cells at the ocular surface, having important implications during dry eye. HMGB1 has been shown to activate dendritic cells through both autocrine and paracrine mechanisms, inducing their maturation and migration to lymph nodes, where their ability to stimulate naïve T cells is also influenced by HMGB1.[49,74–76] Mature dendritic cells secrete HMGB1 resulting in upregulation of the chemokine receptor CCR7, which is necessary for migration upon stimulation.[76] Importantly, dendritic cell CCR7 plays an important role in ocular surface inflammation and mediates APC trafficking and the induction of a Th17 response in a mouse model of EDE.[77,78] Blockade of CCR7 ameliorated EDE and reduced EDE-severity and the Th17 response.[76] Therefore, it will be critical to evaluate HMGB1's role in the context of dendritic cell activation during dry eye, which may provide a mechanism into HMGB1's inflammatory action at the ocular surface.

In addition to dendritic cells, macrophages can actively secrete HMGB1.[79] Macrophages respond to extracellular HMGB1 through production of inflammatory and angiogenic factors, such as TNFα, IL-8, and VEGF,[80] which we also demonstrated in the current study. An increase in proinflammatory mediators leads to further production and release of HMGB1 and recruitment of more immune cells to the site of stress or injury.[81] Macrophages are resident cells in the ocular surface conjunctiva, and there is evidence that their inflammatory activation markers are increased during EDE.[82] Therefore, it will also be important to evaluate HMGB1's effect on these immune cells in the context of ocular surface inflammation.

In summary, our results showed that HMGB1 was released by corneal epithelial cells in DED, both in vitro and in vivo, demonstrating an important biological role for this alarmin at the ocular surface. However, HMGB1 alone, or in combination with TLR agonists, did not elicit direct influence on inflammatory pathways and cytokine production in HCEC. Therefore, the role of HMGB1 and its role during dry eye-related inflammation in stimulating immune cells remain to be investigated further.

Acknowledgments

The authors thank Alex Kurosky, PhD (University of Texas Medical Branch, Galveston, TX, USA) for providing the rhHMGB1 and Alison McDermott, PhD (University of Houston, College of Optometry, Houston, TX, USA) for providing the hTCEpi, SV40 HCEC, and U937 cells.

Supported by National Institutes of Health/National Eye Institute Grant R01 EY023638 (RR) and National Institutes of Health P30 EY07551 (UHCO core grant).

Disclosure: **C. Lema**, None; **R.Y. Reins**, None; **R.L. Redfern**, None

References

1. Craig JP, Nichols KK, Akpek EK, et al. TFOS DEWS II definition and classification report. *Ocul Surf*. 2017;15:276–283.
2. Moss SE, Klein R, Klein BE. Prevalence of and risk factors for dry eye syndrome. *Arch Ophthalmol*. 2000;118:1264–1268.
3. Jones L, Downie LE, Korb D, et al. TFOS DEWS II management and therapy report. *Ocul Surf*. 2017;15:575–628.
4. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Arch Ophthalmol*. 2012;130:90–100.
5. Stern ME, Schaumburg CS, Pflugfelder SC. Dry eye as a mucosal autoimmune disease. *Int Rev Immunol*. 2013;32:19–41.
6. Calonge M, Enriquez-de-Salamanca A, Diebold Y, et al. Dry eye disease as an inflammatory disorder. *Ocul Immunol Inflamm*. 2010;18:244–253.
7. Bron AJ, de Paiva CS, Chauhan SK, et al. TFOS DEWS II pathophysiology report. *Ocul Surf*. 2017;15:438–510.
8. Willcox MDP, Argueso P, Georgiev GA, et al. TFOS DEWS II tear film report. *Ocul Surf*. 2017;15:366–403.
9. Baudouin C. The pathology of dry eye. *Surv Ophthalmol*. 2001;45(suppl 2):S211–S220.
10. Li DQ, Chen Z, Song XJ, Luo L, Pflugfelder SC. Stimulation of matrix metalloproteinases by hyperosmolarity via a JNK pathway in human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2004;45:4302–4311.
11. De Paiva CS, Corrales RM, Villarreal AL, et al. Corticosteroid and doxycycline suppress MMP-9 and inflammatory cytokine expression, MAPK activation in the corneal epithelium in experimental dry eye. *Exp Eye Res*. 2006;83:526–535.
12. Aho VV, Nevalainen TJ, Saari KM. Group IIA phospholipase A2 content of tears in patients with keratoconjunctivitis sicca. *Graefes Arch Clin Exp Ophthalmol*. 2002;240:521–523.

13. Erlandsson Harris H, Andersson U. Mini-review: the nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol*. 2004;34:1503–1512.
14. Land WG. The role of damage-associated molecular patterns (DAMPs) in human diseases: part II: DAMPs as diagnostics, prognostics and therapeutics in clinical medicine. *Sultan Qaboos Univ Med J*. 2015;15:e157–e170.
15. Land WG. The role of damage-associated molecular patterns in human diseases: part I: promoting inflammation and immunity. *Sultan Qaboos Univ Med J*. 2015;15:e9–e21.
16. Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. *Invest Ophthalmol Vis Sci*. 2004;45:4293–4301.
17. Redfern RL, Barabino S, Baxter J, Lema C, McDermott AM. Dry eye modulates the expression of toll-like receptors on the ocular surface. *Exp Eye Res*. 2015;134:80–89.
18. Redfern RL, Patel N, Hanlon S, et al. Toll-like receptor expression and activation in mice with experimental dry eye. *Invest Ophthalmol Vis Sci*. 2013;54:1554–1563.
19. Kang R, Chen R, Zhang Q, et al. HMGB1 in health and disease. *Mol Aspects Med*. 2014;40:1–116.
20. Keyel PA. How is inflammation initiated? Individual influences of IL-1, IL-18 and HMGB1. *Cytokine*. 2014;69:136–145.
21. Yang H, Antoine DJ, Andersson U, Tracey KJ. The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. *J Leukoc Biol*. 2013;93:865–873.
22. Shimizu S, Kouzaki H, Kato T, Tojima I, Shimizu T. HMGB1-TLR4 signaling contributes to the secretion of interleukin 6 and interleukin 8 by nasal epithelial cells. *Am J Rhinol Allergy*. 2016;30:167–172.
23. Kim DE, Min KJ, Kim JS, Kwon TK. High-mobility group box-1 protein induces mucin 8 expression through the activation of the JNK and PI3K/Akt signal pathways in human airway epithelial cells. *Biochem Biophys Res Commun*. 2012;421:436–441.
24. Wolfson RK, Chiang ET, Garcia JG. HMGB1 induces human lung endothelial cell cytoskeletal rearrangement and barrier disruption. *Microvasc Res*. 2011;81:189–197.
25. Wu X, Mi Y, Yang H, Hu A, Zhang Q, Shang C. The activation of HMGB1 as a progression factor on inflammation response in normal human bronchial epithelial cells through RAGE/JNK/NF-kappaB pathway. *Mol Cell Biochem*. 2013;380:249–257.
26. Fiuza C, Bustin M, Talwar S, et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood*. 2003;101:2652–2660.
27. Sappington PL, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP. HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. *Gastroenterology*. 2002;123:790–802.
28. Treutiger CJ, Mullins GE, Johansson AS, et al. High mobility group 1 B-box mediates activation of human endothelium. *J Intern Med*. 2003;254:375–385.
29. Harris HE, Andersson U, Pisetsky DS. HMGB1: a multifunctional alarmin driving autoimmune and inflammatory disease. *Nat Rev Rheumatol*. 2012;8:195–202.
30. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5:331–342.
31. Yanai H, Taniguchi T. Nucleic acid sensing and beyond: virtues and vices of high-mobility group box 1. *J Intern Med*. 2014;276:444–453.
32. Cavone L, Muzzi M, Mencucci R, et al. 18beta-glycyrrhetic acid inhibits immune activation triggered by HMGB1, a pro-inflammatory protein found in the tear fluid during conjunctivitis and blepharitis. *Ocul Immunol Inflamm*. 2011;19:180–185.
33. Zicari AM, Zicari A, Nebbioso M, et al. High-mobility group box-1 (HMGB-1) and serum soluble receptor for advanced glycation end products (sRAGE) in children affected by vernal keratoconjunctivitis. *Pediatr Allergy Immunol*. 2014;25:57–63.
34. Han SJ, Min HJ, Yoon SC, et al. HMGB1 in the pathogenesis of ultraviolet-induced ocular surface inflammation. *Cell Death Dis*. 2015;6:e1863.
35. Dupire G, Nicaise C, Gangji V, Soyfoo MS. Increased serum levels of high-mobility group box 1 (HMGB1) in primary Sjogren's syndrome. *Scand J Rheumatol*. 2012;41:120–123.
36. Ekanayaka SA, McClellan SA, Barrett RP, Kharotia S, Hazlett LD. Glycyrrhizin reduces HMGB1 and bacterial load in Pseudomonas aeruginosa keratitis. *Invest Ophthalmol Vis Sci*. 2016;57:5799–5809.
37. McClellan S, Jiang X, Barrett R, Hazlett LD. High-mobility group box 1: a novel target for treatment of Pseudomonas aeruginosa keratitis. *J Immunol*. 2015;194:1776–1787.
38. Redfern RL, Patel N, Hanlon S, et al. Toll-like receptor expression and activation in mice with experimental dry eye. *Invest Ophthalmol Vis Sci*. 2013;54:1554–1563.
39. Robertson DM, Li L, Fisher S, et al. Characterization of growth and differentiation in a telomerase-immortalized human corneal epithelial cell line. *Invest Ophthalmol Vis Sci*. 2005;46:470–478.
40. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36:614–621.
41. Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer*. 1976;17:565–577.
42. Redfern RL, Reins RY, McDermott AM. Toll-like receptor activation modulates antimicrobial peptide expression by ocular surface cells. *Exp Eye Res*. 2011;92:209–220.
43. McDermott AM, Redfern RL, Zhang B, Pei Y, Huang L, Proske RJ. Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2003;44:1859–1865.
44. Talreja J, Dileepan K, Puri S, et al. Human conjunctival epithelial cells lack lipopolysaccharide responsiveness due to deficient expression of MD2 but respond after interferon-gamma priming or soluble MD2 supplementation. *Inflammation*. 2005;29:170–181.
45. Sintiprungrat K, Singhto N, Sinchaikul S, Chen ST, Thongboonkerd V. Alterations in cellular proteome and secretome upon differentiation from monocyte to macrophage by treatment with phorbol myristate acetate: insights into biological processes. *J Proteomics*. 2010;73:602–618.
46. Li DQ, Luo L, Chen Z, Kim HS, Song XJ, Pflugfelder SC. JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res*. 2006;82:588–596.
47. Pflugfelder SC. Tear dysfunction and the cornea: LXVIII Edward Jackson Memorial Lecture. *Am J Ophthalmol*. 2011;152:900–909.
48. Cappiello F, Casciaro B, Kolar SS, Baidouri H, McDermott AM, Mangoni ML. Methods for in vitro analysis of antimicrobial activity and toxicity of anti-keratitis peptides: bacterial viability in tears, MTT, and TNF-alpha release assays. *Methods Mol Biol*. 2017;1548:395–409.
49. Messmer D, Yang H, Telusma G, et al. High mobility group box protein 1: an endogenous signal for dendritic cell maturation and Th1 polarization. *J Immunol*. 2004;173:307–313.

Investigative Ophthalmology & Visual Science

50. Kim S, Kim SY, Pribis JP, et al. Signaling of high mobility group box 1 (HMGB1) through toll-like receptor 4 in macrophages requires CD14. *Mol Med*. 2013;19:88–98.
51. Yang H, Lundback P, Ottosson L, et al. Redox modification of cysteine residues regulates the cytokine activity of high mobility group box-1 (HMGB1). *Mol Med*. 2012;18:250–259.
52. Alven A. *The Role of Damage Associated Molecular Patterns in Dry Eye Inflammation* [master's thesis]. Houston, TX: College of Optometry, University of Houston; 2016.
53. Stewart P, Chen Z, Farley W, Olmos L, Pflugfelder SC. Effect of experimental dry eye on tear sodium concentration in the mouse. *Eye Contact Lens*. 2005;31:175–178.
54. Kim KH, Kim DH, Jeong HJ, et al. Effects of subconjunctival administration of anti-high mobility group box 1 on dry eye in a mouse model of Sjgren's syndrome. *PLoS One*. 2017;12: e0183678.
55. Mrugacz M, Ostrowska L, Bryl A, Szulc A, Zelazowska-Rutkowska B, Mrugacz G. Pro-inflammatory cytokines associated with clinical severity of dry eye disease of patients with depression. *Adv Med Sci*. 2017;62:338–344.
56. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea*. 2009;28:1023–1027.
57. Bohm D, Keller K, Boehm N, et al. Antibody microarray analysis of the serum proteome in primary breast cancer patients. *Cancer Biol Ther*. 2011;12:772–779.
58. Chen Y, Zhang X, Yang L, et al. Decreased PPAR-gamma expression in the conjunctiva and increased expression of TNF-alpha and IL-1beta in the conjunctiva and tear fluid of dry eye mice. *Mol Med Rep*. 2014;9:2015–2023.
59. Qin Y, Chen Y, Wang W, et al. HMGB1-LPS complex promotes transformation of osteoarthritis synovial fibroblasts to a rheumatoid arthritis synovial fibroblast-like phenotype. *Cell Death Dis*. 2014;5:e1077.
60. Ibrahim ZA, Armour CL, Phipps S, Sukkar MB. RAGE and TLRs: relatives, friends or neighbours? *Mol Immunol*. 2013; 56:739–744.
61. Park JS, Svetkauskaite D, He Q, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem*. 2004;279:7370–7377.
62. Yang H, Hreggvidsdottir HS, Palmblad K, et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci U S A*. 2010;107:11942–11947.
63. Ivanov S, Dragoi AM, Wang X, et al. A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood*. 2007;110:1970–1981.
64. Reins RY, Courson J, Lema C, Redfern RL. MyD88 contribution to ocular surface homeostasis. *PLoS One*. 2017;12:e0182153.
65. Redfern RL, McDermott AM. Toll-like receptors in ocular surface disease. *Exp Eye Res*. 2010;90:679–687.
66. Zhang J, Kumar A, Wheater M, Yu FS. Lack of MD-2 expression in human corneal epithelial cells is an underlying mechanism of lipopolysaccharide (LPS) unresponsiveness. *Immunol Cell Biol*. 2009;87:141–148.
67. Yang H, Wang H, Ju Z, et al. MD-2 is required for disulfide HMGB1-dependent TLR4 signaling. *J Exp Med*. 2015;212:5–14.
68. Hamrah P, Dana MR. Corneal antigen-presenting cells. *Chem Immunol Allergy*. 2007;92:58–70.
69. Fukuda K, Ishida W, Tanaka H, et al. Alarmins from corneal epithelial cells upregulate CCL11 and VCAM-1 in corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2013;54:5817–5823.
70. Wilson SE, Mohan RR, Mohan RR, Ambrosio R Jr, Hong J, Lee J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res*. 2001;20:625–637.
71. Hong JW, Liu JJ, Lee JS, et al. Proinflammatory chemokine induction in keratocytes and inflammatory cell infiltration into the cornea. *Invest Ophthalmol Vis Sci*. 2001;42:2795–2803.
72. Lee SK, Choi BK, Kang WJ, et al. MCP-1 derived from stromal keratocyte induces corneal infiltration of CD4+ T cells in herpetic stromal keratitis. *Mol Cells*. 2008;26:67–73.
73. Fukuda K, Ishida W, Miura Y, et al. Cytokine expression and barrier disruption in human corneal epithelial cells induced by alarmin released from necrotic cells. *Jpn J Ophthalmol* 2017;61:415–422.
74. Rovere-Querini P, Capobianco A, Scaffidi P, et al. HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Rep*. 2004;5:825–830.
75. Dumitriu IE, Baruah P, Valentinis B, et al. Release of high mobility group box 1 by dendritic cells controls T cell activation via the receptor for advanced glycation end products. *J Immunol*. 2005;174:7506–7515.
76. Dumitriu IE, Bianchi ME, Bacci M, Manfredi AA, Rovere-Querini P. The secretion of HMGB1 is required for the migration of maturing dendritic cells. *J Leukoc Biol*. 2007;81: 84–91.
77. Kodati S, Chauhan SK, Chen Y, et al. CCR7 is critical for the induction and maintenance of Th17 immunity in dry eye disease. *Invest Ophthalmol Vis Sci*. 2014;55:5871–5877.
78. Saban DR. The chemokine receptor CCR7 expressed by dendritic cells: a key player in corneal and ocular surface inflammation. *Ocul Surf*. 2014;12:87–99.
79. Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999;285:248–251.
80. van Beijnum JR, Dings RP, van der Linden E, et al. Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood*. 2006;108: 2339–2348.
81. Bianchi ME, Crippa MP, Manfredi AA, Mezzapelle R, Rovere Querini P, Venereau E. High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair. *Immunol Rev*. 2017;280:74–82.
82. You IC, Coursey TG, Bian F, Barbosa FL, de Paiva CS, Pflugfelder SC. Macrophage phenotype in the ocular surface of experimental murine dry eye disease. *Arch Immunol Ther Exp (Warsz)*. 2015;63:299–304.

Investigative Ophthalmology & Visual Science