Dry eye disease (DED) is a multifactorial disorder of the tear film and ocular surface. 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. Therefore, effective and inexpensive treatment options are urgently needed for DED.

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. The innate immune response is triggered by stress at the ocular surface resulting from altered tear film dynamics and environmental stressors. 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 metalloproteinases (MMPs), and phospholipases 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. DAMPs have been implicated in the pathogenesis of autoimmune, cardiovascular, metabolic, neurodegenerative, malignant, and infectious diseases 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 including IL-6, IL-8, and TNFα production.

High-mobility group box 1 (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
In studies of the eye, HMGB1 has been found in the tear fluid during conjunctivitis and blepharitis, and in serum of children with vernal keratoconjunctivitis, and in pterygial tissue. Extracellular HMGB1 is also elevated in patients suffering from autoimmune disorders, including primary Sjögren syndrome, who have severe dry eye-related symptoms. 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. 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. 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 chloride (1 M) was added to normal cell culture media and 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.

**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.) for 4, 8, 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.

**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, TNF-α (BioLegend, San Diego, CA), 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
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).

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 ≤ 0.05.
Hyperosmolar Stress and TNFα Stimulation Increase HMGB1 Expression in HCEC

As HMGB1 was increased in DED, 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 (0.001–10 μg/mL) followed by quantification 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 10 μg/mL HMGB1 as able to elicit inflammation, the use of as high as 10 μg/mL in human49 and mouse APCs50,51 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 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 < 0.0001; **P < 0.001; *P < 0.05.
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 cytokines.

**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 ≤ 0.0001; **P ≤ 0.001; *P ≤ 0.01.

**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 ≤ 0.0001.
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 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 ≤ 0.0001; **P ≤ 0.001; *P ≤ 0.01.

**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.
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 a 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. 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). 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. 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. In addition, the clinical severity of DED has been correlated with TNFα levels, and TNFα has been found to be elevated in tears from DED patients and in tear-washings of dry eye mice. 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, IL-8, 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, Mq-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. In addition to RAGE, HMGB1 binds to TLR2, TLR4, and TLR9. We have previously demonstrated that HCEC secrete inflammatory cytokines and chemokines when stimulated by TLR agonists and have shown TLR upregulation in the cornea, conjunctiva, and lacrimal gland in EDE mice. We have also reported an increase in TLR4 expression in DED subjects. 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, they have been reported to be low responders to LPS due to insufficient expression of MD2, an accessory molecule required for HMGB1-TLR4 signaling. 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-κB p65 in HCEC by immunohistochemistry, as HMGB1 has been shown...
to activate NF-κB signaling in other cell types. After 2 hours, we did not observe NF-κB 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. 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. Similarly, the HMGB1 inhibitor glycyrrhizin reduced IL-1β expression and cellular infiltrates in the corneal stroma of P. aeruginosa-infected mice. 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, which are known to play a key role in the recruitment of inflammatory immune cells into the cornea during innate and adaptive immune responses. 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. 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. Mature dendritic cells secrete HMGB1 resulting in upregulation of the chemokine receptor CCR7, which is necessary for migration upon stimulation. 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. Blockade of CCR7 ameliorates EDE-severity and the Th17 response. 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. Macrophages respond to extracellular HMGB1 through production of inflammatory and angiogenic factors, such as TNFα, IL-8, and VEGF, 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. Macrophages are resident cells in the ocular surface conjunctiva, and there is evidence that their inflammatory activation markers are increased during DED. 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.

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