Desiccating stress worsens alkali burn injury by magnifying caspase-8-induced imbalance of NLRP3 and NLRP6

To the Editor:

Alkali burn (AB), one of the most devastating types of ocular trauma, often develops secondary dry eye, which, in turn, worsens ocular surface disorder.1,2 The corneal epithelium is the first defensive line of the eye involved in pathogen-associated molecular pattern recognition that leads to robust innate immune responses.3,4 However, how damage-associated molecular pattern recognition of eye injuries initiates the innate immune response of the corneal epithelium remains unclear.

In the present study, we used AB and AB-concomitant desiccating stress (DS) models to explore the potential functions and mechanisms of innate immune response in certain diseases (see text in this article’s Online Repository at www.jacionline.org). We observed that tear volume rapidly decreased after AB injury and led to severe secondary dry eye, which further significantly delayed corneal wound healing and worsened the ocular surface disorder.

Nucleotide-binding oligomerization domain–like receptors (NLRs) are cytoplasmic pattern recognition receptors initiating innate immune response during cellular stress.5,6 In the present study, we therefore explored the involvement and potential roles of NLRs in the innate immunity of AB and secondary dry eye after AB injury. NLRP3 is the most versatile and importantly also the most clinically implicated NLR in many inflammatory diseases.7 Our data showed that both the mRNA and protein levels of NLRP3 and adaptor apoptosis-associated speck-like protein containing a CARD (ASC) were significantly higher in the cornea of AB mice than in the cornea of the untreated group (Fig 1, A, B, and E). The activity of caspase-1, the other component of the NLRP3 inflammasome, was also significantly elevated after AB injury (Fig 1, C). Furthermore, DS synergized AB injury to further increase NLRP3 and ASC production. Once NLRP3 was inhibited (for the choice of optimal doses of inhibitors, see the Methods and Results sections and Fig E1 in this article’s Online Repository at www.jacionline.org), ASC expression and caspase-1 activation were subsequently suppressed (Fig 1, B, C, and E). Blocking NLRP3 effectively relieved AB-induced opacification (Fig 1, H) and significantly promoted corneal wound healing (Fig 1, J). Furthermore, NLRP3 inhibition also markedly reduced the dry eye-worsened ocular surface disorder after AB injury and eliminated corneal perforation (Fig 1, G). These results indicated that the activation of NLRP3 inflammasome mediates AB-induced ocular surface injury and DS-worsened AB damage. Thus, the processing of IL-1β and IL-18 was significantly suppressed after NLRP3 inhibition (Fig 1, J), suggesting that NLRP3 drives ocular surface disorder by promoting the processing of active IL-1β and IL-18.

NLRP6 is a newly found NLR, described by recent studies that NLRP6 serves to inhibit the proliferation of intestinal epithelial cells following and its deficiency leads to defective wound healing.8 In contrast to NLRP3, the expression of NLRP6 mRNA and protein was downregulated in both AB and AB combined with DS groups compared with the untreated group (Fig 1, D and E). We further explored the influence of NLRP6 using NLRP6 siRNA to inhibit local NLRP6 production. Our findings demonstrated that NLRP6 inhibition significantly exacerbated the severity of AB- and DS-induced ocular surface damage, including an increased corneal perforation rate, enhanced corneal transparency, and delayed corneal wound healing (Fig 1, G–I), indicating that NLRP6 negatively regulated the inflammation to dampen the damage of these diseases. Our study further disclosed that the production of IL-1β had no significant alternation after blocking NLRP6, but the processing of IL-18 was suppressed (Fig 1, K).

In the present study, our findings first discovered that NLRP6 was significantly upregulated after NLRP3 inhibition (Fig 1, D and E). However, NLRP6 had no effect on NLRP3 production (Fig 1, A and F). These results indicate that AB- and DS-induced NLRP3 upregulation participates in the downregulation of NLRP6 production. Thus, targetedly inhibiting AB- and DS-induced NLRP3 markedly reduced corneal opacification and corneal perforation rate and promoted corneal wound healing via reversing the imbalance of NLRP3 and NLRP6.

Yamada et al8 observed that antagonism of IL-1 after AB injury markedly reduces corneal inflammation and enhances corneal transparency. Caspase-8 is a classical cysteine-aspartic acid protease in apoptotic signals whose new paradigm of nonapoptotic role in the innate immunity has been disclosed by us.9 Our previous study demonstrated that caspase-8 activation is involved in the innate immune response of retina by promoting the processing of IL-1β.9 Therefore, we hypothesized that the increased IL-1β production in AB may also be due to the participation of caspase-8 activation. Our data showed that AB injury significantly activated caspase-8 compared with untreated corneas. Furthermore, we disclosed that DS synergized AB, inducing a higher caspase-8 activity (Fig 2, A; see Fig E2 in this article’s Online Repository at www.jacionline.org). Inhibiting caspase-8 significantly attenuated AB-induced corneal pacification as well as DS-worsened AB injury, promoted the corneal wound healing, and resulted in no corneal perforation, indicating that caspase-8 signaling was actively involved in AB- and DS-induced ocular surface disorder (Fig 2, B–D). Blocking caspase-8 activation significantly reduced the processing of IL-1β and IL-18 (Fig 2, E–G), suggesting that caspase-8 signaling drove the processing of IL-1β and IL-18 to accelerate ocular surface disorder induced by AB and worsened by DS and targetedly blocking caspase-8 relieved the damage.

We then determined the correlation of caspase-8 and NLRs. Notably, inhibiting caspase-8 signaling suppressed the production of NLRP3, ASC, and caspase-1 in both AB and AB combined DS groups (Fig 2, H, I, K, and L). However, NLRP6 was upregulated after caspase-8 inhibition compared with noninhibition (Fig 2, J and K). Thus, NLRP3 and NLRP6 had no effect on the activation of caspase-8 (Fig 2, A). Together, these results revealed a previously unidentified correlation of NLRP3/NLRP6 and caspase-8 in AB and AB with DS. Caspase-8 signaling is upstream of NLRP3 and NLRP6. Caspase-8 activation induced by AB and DS leads to the upregulation of NLRP3 and downregulation of NLRP6, which further regulates the processing of IL-1β and IL-18 involved in AB and DS-worsened AB injury.
FIG 1. AB-induced imbalance of NLRP3/NLRP6 magnified by DS and NLRP3 protected against the ocular surface disorder induced by AB and DS injury. A, B, D-F, NLRP3, ASC, and NLRP6 gene and protein expression treated with AB combined with or without DS and with or without NLRP3 inhibitor or NLRP6 siRNA. Graph of the relative densities of NLRP3, NLRP6, and ASC normalized to β-actin for WB results. C, NLRP3 promoted caspase-1 activity. G, Rate of corneal perforation. H and I, Representative images of corneas and corneal wounds closure. J and K, NLRP3 and NLRP6 regulated the processing of IL-1β and IL-18. P3 inh, NLRP3 inhibitor; glybenclamide; P6 si, NLRP6 siRNA; UT, untreated; WB, Western blot. Representative images are shown. Data are presented as mean ± SD. Experiments were repeated 3 times with similar results. *P < .05. **P < .001.
FIG 2. Caspase-8 activation regulated the imbalance of NLRP3 and NLRP6 in AB-induced and DS-worsened ocular surface injury. **A**, Caspase-8 activity treated with AB combined with or without DS and with or without caspase-8 inhibitor, NLRP3 inhibitor, or NLRP6 siRNA. **B and C**, Representative pictures of cornea and corneal wounds closure. **D**, Rate of corneal perforation. **E-G**, Caspase-8 promoted the processing of IL-1β and IL-18. Graph of the densities of pro–IL-1β, IL-1β, pro–IL-18, and IL-18 normalized to β-actin. **H-K**, Caspase-8 regulated NLRP3, NLRP6, and ASC expression. Graph of the densities of NLRP3, NLRP6, and ASC normalized to β-actin. **L**, Caspase-8 drove caspase-1 activity. **C8 inh**, Caspase-8 inhibitor Z-IETD-fmk; **UT**, untreated. Representative images are shown. Data are presented as mean ± SD. The experiments were repeated 3 times with similar results. *P < .05. **P < .001.
In summary, our investigations reveal new discoveries on the innate immune response of AB and DS-worsened AB damage. Exposure to DS magnifies the imbalance of NLRP3 and NLRP6, causing increased corneal perforation rate, decreased opacity, and delayed wound healing after AB injury. The key to the severity of ocular damage is that greater NLRP3 activity suppresses the negative innate immune regulator NLRP6. Furthermore, we have shed light that caspase-8 activation regulates the imbalance of NLRP3 and NLRP6. Inhibition of caspase-8 and NLRP3 alleviates AB and DS-worsened AB damage because of the decreased production of IL-1β and IL-18 and recovers the balance of NLRP3 and NLRP6. Uncovering this relationship not only helps us understand the mechanisms but also paves the way to disclose new treatments for AB and exacerbation of DS after AB injury.

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This work was supported by the National Natural Science Foundation of Key Projects of China (grant no. 81530028), National Natural Science Foundations of China (grant nos. 81570830, 81170829, 81371019, 81670816, and 81400375), Tianjin Research Program of Application Foundation and Advanced Technology (grant nos. 12JCJBC15400 and 15JCYBJC34600), and Supporting Project for Key Specialty from Bureau of Health, Tianjin.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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http://dx.doi.org/10.1016/j.jaci.2017.04.018
METHODS

Unilateral AB

After systemic anesthesia with an intraperitoneal injection combined with 0.5% proparacaine topical anesthesia (SomnoSuite; Kent Scientific, Torrington, Conn), a unilateral AB was created on the right eye of 6- to 8-week-old C57BL/6 mice. Whatman filter paper (2.5 mm diameter) was soaked in 1 N NaOH and placed on the central cornea for 10 seconds, followed by extensive rinsing with balanced salt solution (Alcon, Fort Worth, Tex), as previously described.12 Precautions were taken to avoid damage to the peripheral cornea, conjunctiva, and lids.

A separate group of mice that received unilateral AB was also subjected to DS for 5 days after anesthetic recovery. The AB combined DS group was injected with 0.5 mg/ml scopoline (Sigma-Aldrich, St Louis, Mo) and exposed to a drafty low humidity (<30% relative humidity) environment for 5 days as previously described.13 The contralateral eyes served as untreated controls.

Animal care and use strictly conformed to the standards in the ARVO Statement for the use of animals in Ophthalmic and Vision Research, and the study was approved by the Committee of Animal Care of the Zhongshan Ophthalmic Center (2014-003).

Treatment regimen

The experimental eyes were injected with 10 µL of caspase-8 inhibitor Z-IETD-fmk (20, 40, and 60 µM, Calbiochem, Gibbstown, NJ), the NLRP3 inhibitor glibenclamide (30, 60, and 120 µM, Sigma-Aldrich), BBS vehicle (balanced salt solution, Alcon), NLRP6 siRNA (10, 50, and 100 nM, Invitrogen, Shanghai, China), or control siRNA using lipofectamine 2000 transfection (Invitrogen) into subconjuctival tissue 1 mm from the limbus at 6 and 12 o’clock positions before surgery once daily. In addition, the experimental eyes were topically treated with 2 µL Z-IETD-fmk, glibenclamide, NLRP6 siRNA, vehicle, or control siRNA 4 times per day. Effective inhibition was reached at doses of 40 µM of caspase-8 inhibitor Z-IETD-fmk, 120 µM of NLRP3 inhibitor glibenclamide, and 50 nM of NLRP6 siRNA, and these concentrations were therefore used for subsequent experiments (Fig E1).

Corneal perforation

All eyes in each treatment group were examined daily under a microscope (SMZ 1500; Nikon, Melville, NY) for the presence of corneal perforation. Corneal edema and opacity were observed using a microscope equipped with a color digital camera (DS-Fi1; Nikon) using the method described by Yoeurek et al.14

Quantitative real-time PCR

Total RNA, reverse transcription, and quantitative real-time PCR were performed as previously described.15 TaqMan gene expression assays used for this study included mGAPDH (Mm99999915_g1), mNLRP3 (Mm00409004_m1), mNLRP6 (Mm00460229_m1), mASC (Mm00445747_m1), mL-1β (Mm00434228_m1), and mL-18 (Mm00434225_m1).

Immunofluorescence staining

Indirect immunofluorescence staining was performed as previously described.16 Primary antibody against NLRP3 (NB1P-77080), caspase-1 (NB100-56565), caspase-8 (NB100-56116, Novus Biologicals, Littleton, Colo), NLRP6 (PA5-21022), IL-1β (P420B, Thermo Scientific, Rochester, NY), IL-18 (P420A, Thermo Scientific, Rochford, Ill), ASC (N-15, SC-22514-R), and IL-18 (6C-6179, Santa Cruz Biotechnology, Dallas, Tex) were used. Propidium iodide or 4’, 6-diamidino-2-phenylindole was used for nuclear counterstaining.

Western blot

Western blot analysis was performed as previously described.16 The primary antibodies consisted of NLRP3, ASC, NLRP6, pro–caspase-1, caspase-1 (1:200), caspase-8 (1:200), IL-1β (1:200), IL-18 (1:200), and β-actin (1:1000).

Caspase-1 and caspase-8 activity assay

The corneal and conjunctival tissue from AB and AB combined DS mice were lysed in RIPA (Radio Immunoprecipitation Assay) buffer for the Caspase-1 and Caspase-8 Fluorometric Assay Kits (BioVision, Milpitas, Calif) to test caspase activity according to the manufacturer’s protocol as previously described.17

Statistical analysis

Student t test was used to compare differences between 2 groups. One-way ANOVA test was used for comparisons among 3 or more groups, followed by Dunnett post hoc test. P < .05 was considered statistically significant. These tests were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc, La Jolla, Calif).

RESULTS

To determine the effective doses of caspase-8 inhibitor, NLRP3 inhibitor, and NLRP6 siRNA, a preliminary experiment was performed. As shown in Fig E1, A, caspase-8 activity was largely inhibited at the doses of 40 and 60 µM Z-IETD-fmk compared with the BBS vehicle in the AB group and a dose of 40 µM was therefore used for subsequent experiments. The protein level of NLRP3 was primarily inhibited by NLRP3 inhibitor until the dose reached 120 µM (Fig E1, B). Different doses of NLRP6 siRNA (10, 50, and 100 nM) were used and the doses of 50 and 100 nM nearly completely knocked down the levels of NLRP3 protein expression in AB compared with the group transfected with control siRNA (Fig E1, C).

To further confirm caspase-8 inhibition on the protein level, we performed additional Western blot experiments. As shown in Fig E2, caspase-8 inhibitor, Z-IETD-fmk (40 µM), successfully suppressed the expression of caspase-8 protein compared with BBS.

REFERENCES

The effects of caspase-8 inhibitor Z-IETD-fmk, NLRP3 inhibitor glybenclamide, and NLRP6 siRNA at different doses. A, Caspase-8 activity was suppressed by caspase-8 inhibitor Z-IETD-fmk at different doses (20, 40, and 60 μM). Caspase-8 activation was largely inhibited at doses of 40 and 60 μM and a dose of 40 μM was therefore used for subsequent experiments. B, Different doses of NLRP3 inhibitor, glybenclamide (30, 60, and 120 μM), were used to determine the effective dose. A dose of 120 μM glybenclamide primarily inhibits the expression of NLRP3 as evaluated by WB. C, Different doses of NLRP6 siRNA (10, 50, and 100 nM) were used and the doses of 50 and 100 nM largely knocked down NLRP6, compared with control siRNA. A dose of 50 nM was chosen for subsequent experiments. C8 inh, Caspase-8 inhibitor Z-IETD-fmk; UT, untreated; WB, Western blot. The experiment was repeated 3 times with similar results. Representative images are shown. Data are presented as mean ± SD of the fold increase compared with controls. *P < .05. **P < .001.
FIG E2. Inhibition of caspase-8 by caspase-8 inhibitor Z-IETD-fmk. **A**, Representative WB images showed that caspase-8 protein levels were significantly elevated in the AB group compared with the untreated group, were magnified by DS combined AB, and were largely suppressed after caspase-8 inhibition. **B**, Graph of the densities of caspase-8 normalized to β-actin. C8 inh, Caspase-8 inhibitor Z-IETD-fmk; UT, untreated. The experiment was repeated 3 times with similar results. Representative images are shown. Data are presented as mean ± SD of the fold increase compared with controls. *P < .05. **P < .001.