MyD88 Deficiency Protects Against Dry Eye–Induced Damage

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PURPOSE. Dry eye disease (DED) is a multifactorial disease associated with ocular surface inflammation. Toll-like receptors (TLRs) are integral in the initiation of inflammatory signaling. Therefore, we evaluated the effect of TLR-deficiency on dry eye–related ocular surface damage and inflammation using a mouse model of experimental dry eye (EDE).

METHODS. C57BL/6 wild-type (WT), MyD88−/−, and IL-1R−/− mice were exposed to EDE conditions for 5 days. Tear production was measured by phenol red thread test and ocular surface damage assessed with fluorescein staining. Corneal homogenates were obtained for matrix metalloproteinase (MMP) and cytokine expression analysis by Luminex assay and quantitative PCR. In addition, whole eyes and eyelids were dissected and goblet cells and Meibomian glands were imaged, respectively.

RESULTS. Following 5 days of EDE, WT mice had extensive ocular surface staining, while MyD88−/− mice had no increased staining above non-EDE conditions. Similarly, MyD88−/− mice did not have increased corneal MMP-2, 3, or 8 concentrations, as seen with WT mice. MyD88-deficiency also resulted in decreased corneal cytokine levels. In addition, MyD88−/− mice had significantly lower conjunctival goblet cell counts compared with both WT (EDE) and IL-1R−/− (non-EDE) mice. However, there was no difference in Meibomian gland morphology between WT, IL-1R−/−, and MyD88−/− mice.

CONCLUSIONS. These studies demonstrate the importance of TLR signaling in dry eye development. Mice lacking TLR signaling, MyD88−/−, were protected from EDE-induced ocular surface damage and inflammatory mediator expression, warranting further investigation into TLR inhibition as a potential therapeutic for DED.

Keywords: Toll-like receptors, MyD88, dry eye disease, ocular surface inflammation

Dry eye disease (DED) is a common multifactorial inflammatory condition that results in chronic ocular discomfort, visual disturbances, and significant reduction in the quality of life. With millions of individuals diagnosed each year in the United States alone, the health care costs are staggering, confirming that DED is a major public health concern. While the pathogenesis of the disease is still not fully understood, it is well established that ocular surface inflammation is a major contributor to both the development and the propagation of dry eye signs and symptoms. Therefore, studies that aim to further dissect inflammatory pathways involved in DED are important and necessary, with the goal of developing strategies to dampen ocular surface inflammation.

One contributing aspect to dry eye–induced inflammation is elevated tear osmolality in dry eye patients. Hyperosmolar stress (HOS) and tear film defects or deficiency lead to increased levels of proinflammatory molecules in the cornea and conjunctiva. Ocular surface antigen-presenting cells (APC) become activated, which stimulate naïve T lymphocytes in the draining lymph nodes. Effector Th1 and Th17 cells then home to the ocular surface where they secrete cytokines and matrix metalloproteinases (MMPs), resulting in damage and the promotion of chronic inflammation.

Toll-like receptors (TLR) are important sensors of the innate immune system that initiate inflammatory signaling pathways upon activation. As pattern recognition receptors, TLRs recognize not only microbial ligands, important for the defense against infection, but also endogenous moieties on proteins that are released during cellular damage and inflammation, termed “damage-associated molecular patterns” (DAMPs). Upon engagement, TLRs initiate inflammatory signaling pathways, leading to nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF) transcription factor activation and cytokine production. Importantly, TLRs are present at the ocular surface, where they increase proinflammatory mediator production and have been implicated in the pathogenesis of DED and activation of APCs.

Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein that is essential in coupling TLR-ligand binding to initiation of intracellular signaling. While there are also MyD88-independent TLR signaling pathways, MyD88 is the primary pathway culminating in NF-κB activation and cytokine production. MyD88 is also used by the IL-1 receptor (IL-1R) for signal transduction. In order to determine the influence of TLR signaling on dry eye–induced ocular surface damage and inflammation, in the current study, we used a mouse model of experimental dry eye (EDE) to evaluate and compare ocular surface staining, proinflammatory mediator production, goblet cell counts, and Meibomian gland structure in MyD88-deficient mice.
MyD88-Dependent TLR Signaling and Dry Eye

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was measured in a 1.5-mm circular area in the central cornea illumination. For corneal staining image analysis, pixel intensity was imaged using SD-OCT with 488-nm wavelength blue light to remove pooled fluorescein and debris. Eyes were immediately instilled with fluorescein (Sigma-Aldrich, Springfield, MO, USA) was instilled following injection of ketamine/xylazine (75 mg/7.5 mg/kg body weight; Telazol, Abbott Laboratories, North Chicago, IL, USA) administered three times a day (Zone-Quick; Oasis, Glendora, CA, USA). Mice were held firmly by the scruff of the neck to prevent blinking and the end of a cotton swab was placed on the cornea to collect tear wicking. Mice were euthanized by the method of the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Mouse Model of Experimental Dry Eye

To study DED in vivo, C57BL/6 (WT), IL-1R1/−, and MyD88−/− mice were subjected to EDE conditions as previously described.19 Mice were housed in an environmentally controlled room, designated for dry eye conditions, with humidity maintained at approximately 20% and temperature approximately 70°F. Cages were modified with an open grate on each side to allow for continuous airflow from fans adjacent to each cage. In addition to low humidity and airflow, reduced tear production was induced by subcutaneous scopolamine hydrobromide injections (0.5 mg/0.2 mL; Green Park Compounding Pharmacy, Houston, TX, USA) administered three times a day for 5 consecutive days at 8:00 AM, 12:00 PM, and 4:00 PM. Control mice (non-EDE) were kept in a normal humidity environment (~65%) without blowing fans or modified cages.

Phenol Red Thread Test

Following five days of EDE conditions, tear volume was measured using phenol red impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA). Mice were held firmly by the scruff of the neck to prevent blinking and the end of a phenol red thread was placed on the palpebral conjunctiva for a count of 15 seconds. The amount of tear wicking was determined by measuring the length of red-colored thread. Measurements were taken from six animals, both eyes, per experimental group.

Spectrals Spectral Domain Optical Coherence Tomography (SD-OCT)

Corneal epithelial integrity was evaluated using SD-OCT (Heidelberg Engineering, Heidelberg, Germany), as previously described.13 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 of 1% sodium fluorescein (Sigma-Aldrich, 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 (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Luminex Multiplex Assay

For mouse tissue inflammatory mediator quantification, corneal epithelial and conjunctival samples were removed following experimental conditions (6 mice per sample, n = 3 experiments) and pooled tissue was homogenized in 0.2% Triton X-100/PBS containing a protease inhibitor cocktail (Roche, Nutley, NJ, USA). MMPs (MMP-2, MMP-3, MMP-8, MMP-9, and MMP-12) and cytokines/chemokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, G-CSF, GM-CSF, IFNγ, TNFα, CXCL1, and RANTES) were quantitated using MILLIPLEX MAP Mouse MMP Magnetic Bead Panel 3 and Cytokine/Chemokine Panel Immunology Multiplex Assays (EMD Millipore, San Diego, CA, USA), per manufacturer’s instructions. Total protein concentrations were determined by Direct Detect Assay Kit (EMD Millipore) and 10 µg of total protein was loaded per well in duplicate.

RNA Isolation and Quantitative Real-Time PCR

For RNA isolation, corneal epithelial cells were removed by scraping, as previously reported.13 Corneas from three mice from the same genotype were pooled for each sample. Total RNA was extracted with RNeasy kits including DNase I treatment (Qiagen, Valencia, CA, USA) and complementary (c) DNA obtained with the AffinityScript cDNA synthesis kit (Agilent Technologies, Santa Clara, CA, USA). Quantitative (q) PCR was performed using intron-spanning primers and the Brilliant II SYBR Green QPCR system (Agilent Technologies). Primer sequences were: CXXLI (forward: TGGCAAGGAGCAGGTCG ACCAA, reverse: GTCCAGAGCAGGAGGTCACAC; IL1A (forward: CAGGCGAGAGGAGGTAGC; reverse: CAGGAA CTTTGCCCATCTTGTACAT, TNAF (forward: ACTGAAT TCGGGGTGTACG; reverse: TATCTGAGTGTGAGGCT TGG), IL1B (forward: GCACTTGCTCTCAGCTGAACAT, reverse: CCGAAACGCGACC TACTA, and MBD3 (forward: GGAATCATACCCCTCTTGT TG, reverse: ATTTYGGAAAAGGAACCCIAC). All samples were normalized to the housekeeping gene, RPII (forward: CTACACCACTACAGCCCTAC; reverse: TTTCATGGAGGTC CATGAGGAG), for analysis using the 2−ΔΔCT method.

mBD3 Immunostaining

Following either non-EDE or EDE conditions, whole eyes were collected, snap frozen in liquid nitrogen, and stored at −80°C until use. Tissue sections (10 µm) were cut, fixed in cold acetone for 5 minutes, and blocked for 2 hours with 5% BSA, 10% goat serum (Abcam, Cambridge, MA, USA), and 0.2% Triton X-100 (Sigma-Aldrich) in PBS. Slides were then incubated overnight at 4°C with primary antibody, rabbit anti-mouse mBD3 (Santa Cruz Biotechnology, Dallas, TX, USA), washed with PBS, and further incubated for 1 hour with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; Invitrogen, Carlsbad, CA, USA). After washing, slides were mounted with Airvol mounting media (courtesy of Alan Burns, UHCO) and slides imaged with the DeltaVision Imaging System (GE Healthcare, Issaquah, WA, USA).

Goblet Cell Counts

Following experimental conditions, whole eyes were enculeated and placed in 10% neutral buffered formalin, as previously described.13 After dehydration with ethanol and xylene incubation, tissue was embedded in paraffin and 10-µm sections were cut using a Leica 2235 Rotary Microtome (Leica Biosystems, Buffalo Grove, IL, USA). To visualize goblet cells, sections were stained with periodic acid and Schiff’s reagent (PAS) and hematoxylin counterstain. Total goblet cell counts were obtained from upper and lower conjunctiva of...
WT, IL-1R /-/ and MyD88 /-/ mice housed in either non-EDE control conditions or exposed to EDE. Upper and lower eyelid goblet cell counts were analyzed separately.

Meibography and Meibomian Gland Measurements

Following 5 days of EDE or non-EDE conditions, WT (C57; n = 7) and age-matched MyD88 /-/ (n = 6) mice, 12-weeks old, were euthanized and the eyelids were removed for Meibomian gland imaging using the Keratograph 5M infrared camera (OCULUS, Arlington, WA, USA). A region of interest from the center of the eyelid containing the superior (1.46 \times 3.15 \text{mm}), inferior (1.26 \times 3.15 \text{mm}), and combined Meibomian glands (2.60 \times 3.15 \text{mm}), was selected from each of the images and processed using a custom designed software developed in MATLAB (Kunnen CME. IOVS 2017;58:ARVO E-Abstract 2239). The proportion of Meibomian gland coverage (mm²) and average gland length (mm) was compared between WT and MyD88 /-/ mice and treatment condition for the superior and inferior eyelids separately.

Statistical Analyses

Statistical analyses were performed using unpaired, two-tailed, Student’s t-tests in experiments comparing two samples and 1-way or 2-way ANOVA with Bonferroni’s test for multiple comparisons. Data are representative of a minimum of three independent experiments with P ≤ 0.05 considered statistically significant. Analyses were performed using Prism 6.0 GraphPad software (GraphPad Software Incorporation, San Diego, CA, USA).

RESULTS

MyD88-Deficient Mice are Protected From EDE-Induced Ocular Surface Damage

In the mouse model of dry eye, like human aqueous-deficient DED, tear volume is diminished, leading to desiccation and damage, an important parameter in dry eye development. Scopolamine is an antimuscarinic drug that prevents tear and salivary secretions. Therefore, we evaluated tear production using the phenol red thread test following 5 days of desiccating stress and scopolamine administration. As expected, EDE conditions resulted in decreased tear production in all groups, regardless of genotype, with WT control (C57) values significantly reduced from 3.58 \pm 0.39 mm/15 sec to 0.33 \pm 0.13 mm/15 sec (P < 0.0001; n = 12; Fig. 1A).

Another hallmark of EDE, along with a decrease in tear production, is ocular surface damage or defects, visualized by corneal fluorescein staining. After 5 days of EDE or control conditions (non-EDE), mice were imaged for fluorescein staining using SD-OCT. WT C57 mice exposed to EDE had extensive ocular surface staining, indicated by diffuse punctate fluorescein staining on the cornea, with a significant increase in staining compared with control conditions (Fig. 1B). However, MyD88 /-/ mice were protected from ocular surface damage induced by EDE and had significantly lower fluorescein staining compared with EDE-treated C57 mice (P < 0.01). Importantly, MyD88 /-/ animals did not have an increase in fluorescein staining with dry eye conditions.
MMPs are important mediators of tissue remodeling and degrade extracellular matrix components during injury, infection, and inflammation, where they have increased activity and expression. MMPs also propagate inflammatory signaling through activation of cytokines and are increased by TLR ligands. Both in human dry eye patients and in mice exposed to EDE, MMP levels are elevated at the ocular surface and contribute to increased corneal permeability and impaired barrier function. Therefore, as MyD88 mice did not have ocular surface damage during EDE, we evaluated the expression of MMPs following EDE. The corneal epithelium was removed and lysates used for Luminex determination of MMP expression. MMP-2, -3, and -8 protein concentrations significantly increased in C57 mice exposed to EDE relative to non-EDE conditions, whereas no MMPs were upregulated in EDE-treated MyD88 animals (Fig. 2).

**MyD88 Mice are Protected From Increased MMP Expression During EDE**

MMPs are important mediators of tissue remodeling and degrade extracellular matrix components during injury, infection, and inflammation, where they have increased activity and expression. MMPs also propagate inflammatory signaling through activation of cytokines and are increased by TLR ligands. Both in human dry eye patients and in mice exposed to EDE, MMP levels are elevated at the ocular surface and contribute to increased corneal permeability and impaired barrier function. Therefore, as MyD88 mice did not have ocular surface damage during EDE, we evaluated the expression of MMPs following EDE. The corneal epithelium was removed and lysates used for Luminex determination of MMP expression. MMP-2, -3, and -8 protein concentrations significantly increased in C57 mice exposed to EDE relative to non-EDE conditions, whereas no MMPs were upregulated in EDE-treated MyD88 animals (Fig. 2).

**MyD88 Mice Have Decreased Expression of Proinflammatory Cytokines at the Ocular Surface During EDE**

Cytokines are also important mediators of inflammation and are elevated in both tears and ocular surface tissues of human dry eye subjects as well as mice exposed to desiccation. MyD88 signaling results in increased production of proinflammatory cytokines. Therefore, cytokine and chemokine expression were evaluated in MyD88 EDE and non-EDE-treated mice and compared with WT C57 and IL-1R animals. IL-1α, IL-9, IL-2, and CXCL1 gene expression were also reduced in MyD88 corneal and conjunctival tissue in both normal and EDE conditions (Fig. 4 and Supplementary Fig. S1). Unfortunately, IL-9 expression was not detected by qPCR.

Interestingly, the antimicrobial peptide, mouse beta defensin-3 (mBD3) was also decreased by 50% in EDE-treated MyD88 corneas compared with WT and IL-1R, which remained lower following EDE, as determined by Luminex assay (Fig. 5A). There were also no increases in cytokine expression in the MyD88 corneas with EDE treatment. Similarly, MyD88 cytokine levels were lower in conjunctival tissue (Fig. 3B), with the exception of IL-9. Interestingly, although significantly lower in non-EDE normal conditions, following 5 days of desiccating stress (EDE) IL-9 expression was upregulated in the MyD88-deficient conjunctiva. Similar to protein levels, IL-1α, IL-2, and CXCL1 gene expression were also reduced in MyD88 corneal and conjunctival tissue in both normal and EDE conditions (Fig. 4 and Supplementary Fig. S1). Unfortunately, IL-9 expression was not detected by qPCR.

**MyD88 Mice Have Lower Numbers of Conjunctival Goblet Cells**

Goblet cells of the conjunctiva produce and secrete gel-forming mucins that are important protective components of healthy tears, acting to hold the tear film to the ocular surface, preventing desiccation and aiding in lubrication. As dry eye conditions have been shown to induce goblet cell loss, furthering ocular surface inflammation and disruption of the tear film, we evaluated goblet cell numbers to access the role of MyD88 during EDE. Goblet cells were identified and counted in both the upper and lower conjunctival
**FIGURE 3.** Ocular surface cytokine protein expression is decreased in MyD88−/− animals during EDE conditions. IL-1α, CXCL1, IL-9, and IL-2 expression were quantitated in corneal and conjunctival homogenates from WT (C57) and MyD88−/− mice after 5 days of EDE treatment or normal (non-EDE) conditions by Luminex multiplex assay. Data represent mean ± SEM of three independent experiments with each sample pooled from five mice. Analysis was by ANOVA with Bonferroni’s test for multiple comparisons, with comparison to C57 samples, **P < 0.01, ***P < 0.001.

**FIGURE 4.** MyD88-deficient mice have lower cytokine, chemokine, and antimicrobial peptide expression following EDE. Following EDE or normal (non-EDE) conditions, (A) IL-1α, IL-1β, TNFα, and CXCL1 expression were determined in corneal and conjunctival lysates of WT (C57), IL-1R−/−, and MyD88−/− mice by qPCR. Graphs represent mean ± SEM of three independent experiments with each sample pooled from three to four mice. Analysis was by (A) ANOVA with Bonferroni’s test for multiple comparisons, *P < 0.05, ****P < 0.0001 and (B) Student’s t-test, **P < 0.01.
epithelium by PAS staining (Fig. 6). Interestingly, MyD88−/− mice had significantly lower numbers of goblet cells in the upper lid following EDE (68.4 ± 6.9 goblet cells compared with WT 140.3 ± 24.4 cells). In addition, MyD88−/− mice had decreased baseline levels of goblet cells compared with IL-1R−/− mice (Fig. 6A), suggesting that MyD88-dependent TLR signaling is important for maintaining goblet cell numbers.

**Meibomian Gland Morphology is Similar in WT and MyD88-Deficient Mice; However, Relative Gland Area Increases With EDE**

In addition to evaluating goblet cell numbers in the conjunctiva, we also examined Meibomian gland morphology in WT C57 and MyD88−/− mice to determine if knockout animals have baseline differences in gland area and length. The Meibomian glands are modified sebaceous glands that secrete...
lipids, in the form of meibum, into the tear film, providing protection against tear evaporation and desiccation. Therefore, these glands are an integral part of maintaining ocular surface health.

To image and evaluate Meibomian glands, lids were removed from 12-week-old C57 and MyD88 \(-/-\) mice. Glands were imaged by meibography and the area and length of Meibomian glands in both the superior (upper) and inferior (lower) Meibomian glands were assessed. There were no significant differences in either gland area or length between C57 and MyD88-deficient animals (Fig. 7). Therefore, the difference in ocular surface damage between the genotypes does not appear to be a result of Meibomian gland morphology.

We also evaluated Meibomian gland area and length following EDE (Fig. 8). There was a significant increase in total gland area following desiccating conditions (Fig. 8B). This change was a reflection of increased area in the inferior glands of the EDE-treated mice compared with non-EDE controls, as there was no significant difference in the superior glands following treatment. In addition, there was no change in gland length in either inferior or superior glands in either group. These data suggest that dry eye conditions increase Meibomian gland area through widening of the glands, possibly leading to dysfunction and propagation of ocular surface disease.

**DISCUSSION**

Inflammatory events play a major role in the pathogenesis of DED. Hyperosmolarity and tear film deficiencies or increased evaporation lead to production of proinflammatory cytokines and MMPs at the ocular surface and activation of immune cells, leading to a cycle of inflammation and damage to the lacrimal function unit. TLR signaling is known to induce proinflammatory mediator expression, largely through NF-κB transcriptional activity, in human corneal cells in response to ligand binding. TLRs have also been implicated in propagating dry eye-associated inflammation. In this study, a mouse model of EDE was used to demonstrate that lack of MyD88-dependent TLR signaling influences dry eye-induced ocular surface damage. While WT animals had an expected increase in corneal fluorescein staining following exposure to dry eye conditions, MyD88 \(-/-\) animals were protected from this increase in staining and damage.

We have previously demonstrated that MyD88-deficiency results in lower baseline levels of proinflammatory cytokines as well as MMPs. MMPs are proteases that are integral in the process of corneal epithelial turnover, wound healing, and inflammation, where they are released and activated to degrade extracellular matrix components, break down cell adhesion complexes, including tight junctions, and activate cytokines. MMP-9 expression and activity are increased in both dry eye patients and mouse models of experimental dry eye, in ocular surface cells as well as in the tear film, as well as during hyperosmolar stress in cultured corneal cells. MMP-9 is also used clinically as a marker for DED diagnosis. As TLR activation is known to enhance MMP expression and MMPs contribute to dry eye–induced ocular surface damage, we evaluated expression following EDE in the knockout and WT animals. Indeed, while MMP-2, -3, and -8 increased in the corneal epithelium during EDE in the C57 mice, no increase was observed in the MyD88 \(-/-\) mice. Therefore, we hypothesize that the protection from damage seen in the MyD88 \(-/-\) mice is a result of altered MMP expression. This hypothesis is supported by the findings that MMP-9 knockout mice are also protected from increased corneal epithelial permeability to fluorescein dye following EDE and that MMP-9 significantly contributes to corneal barrier function disruption during dry eye.

In addition to MMP expression, TLR activation also leads to the production and secretion of proinflammatory cytokines and chemokines in corneal cells. These mediators play an important role in dry eye–related inflammation and, like MMPs, are increased in dry eye patients as well as during EDE. Effective anti-inflammatory therapies that block these cytokines, such as TNFα and IL-1, have been used successfully for dry eye treatment, decreasing corneal fluorescein staining as well as further cytokine expression, and other inflammatory signs of dry eye. In our study, during EDE, MyD88-deficient mice had decreased levels of IL-1α, IL-1β, IL-2, IL-9, TNFα, and CXCL1 in ocular surface tissues. This demonstrates that lack of MyD88-dependent TLR signaling dampens inflammatory molecules during dry eye and points to a potential target for anti-inflammatory treatment of DED. In previous studies, we have also shown that mice exposed to EDE had increased ocular surface expression of TLR2, 4, and 9, as well as enhanced TLR5 expression in the lacrimal gland, providing further evidence that TLRs are involved in the pathogenesis of DED.

Interestingly, the expression of the antimicrobial peptide mouse beta defensin 3 (mBD3) was lower in MyD88 \(-/-\) mice compared with WT controls and IL-1R–deficient mice. While
catastrophic stress.

Conjunctival goblet cells produce mucins, important glycoproteins of the tear film and ocular surface epithelia glycocalyx. Spdef-deficient mice lack goblet cells and have a dry eye-like phenotype, with increased fluorescein staining and inflammation at the ocular surface, and are, in fact, used as a model of dry eye. Therefore, while TLR signaling can propagate inflammation, furthering damage during dry eye, it also serves to protect the cornea, a critical function of these pattern recognition receptors. Our results demonstrate the importance of MyD88-dependent TLR activation for antimicrobial peptide expression during desiccating stress.

In conclusion, the current study demonstrates an important role for MyD88-dependent TLR signaling during the development of dry eye, with protection against ocular surface damage and inflammatory mediator production when TLR/MyD88 signaling was abolished. These results provide value information in exploring anti-inflammatory therapies for the treatment of dry eye disease and warrant further studies to dissect the role of TLRs in ocular surface inflammation.

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