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.1 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.2 Hypersomolar stress (HOS) and tear film defects or deficiency lead to elevated tear osmolality in dry eye patients.2,3 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.4,5 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).6,7 Upon engagement, TLRs initiate inflammatory signaling pathways, leading to nuclear factor kappa B (NF-kB) and interferon regulatory factor (IRF) transcription factor activation and cytokine production.8–11 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.12–17

Myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein that is essential in coupling TLR-ligand binding to initiation of intracellular signaling.18 While there are also MyD88-independent TLR signaling pathways, MyD88 is the primary pathway culminating in NF-kB 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
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Bethesda, MD, USA).

was measured in a 1.5-mm circular area in the central cornea illumination. For corneal staining image analysis, pixel intensity
removed pooled fluorescein and debris. Eyes were immediately
fluorescein (Sigma-Aldrich, Springfield, MO, USA) was instilled
Vedco, Inc., St. Joseph, MO, USA) and 1.5
injection of ketamine/xylazine (75 mg/7.5 mg/kg body weight;
was determined by measuring the length of red-colored thread.
determined the amount of tear wicking.
by the scruff of the neck to prevent blinking and the end of a
phenol red thread was placed on the palpebral conjunctiva for
measure using phenol red impregnated cotton threads
by EDE conditions as previously
to study DED in vivo, C57BL/6 (WT), IL-1R
Animals
Eight- to 12-week old C57BL/6 (WT), MyD88+/−, and IL-1R−/− mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in a temperature and humidity-controlled environment maintained at 72°F and 55% to 65% relative humidity (non-EDE conditions). 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 Visual Research.

Mouse Model of Experimental Dry Eye
To study DED in vivo, C57BL/6 (WT), IL-1R−/−, 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.15 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-13, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-15, IL-17, IP-10, MKC, 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.15 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: CXCLI (forward: TGCAGAAGCGAGGTTCACC), IL1A (forward: CAGGCGCAGAGGGAGTCAC; reverse: CAGGAACTTGGTGCCATCTTGTAG), TNFA (forward: ACTGAACTTCGGGGGTGATCG; reverse: TGAATCGTGTTGAGGGTCTTGG), IL1B (forward: GCAATGGTCTCGAATCACT; reverse: ATCTTCTTGGGTTCCGTCACT), CRAMP (forward: GCGGCTGATTTTCTTGACAT; reverse: GCCAAGGGCCGCTAATCT), and MBDS (forward: GGAATCCATACCTCTGTGTTG; reverse: ATTTYGAGGAAGGAAACCCAC). All samples were normalized to the housekeeping gene, RPII (forward: CTACACCCACCTACAGGCTAC; reverse: TTCAGATGAGGTCGTTAGG), 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 enucleated and placed in 10% neutral buffered formalin, as previously described.15 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 × 3.15 mm), inferior (1.26 × 3.15 mm), and combined Meibomian glands (2.60 × 3.15 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 ± 0.39 mm/15 sec to 0.33 ± 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.
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.16 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.20–22 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.23–25 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−/− levels. 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 qPCR (Fig. 5A). MBD3 protein expression was also lower in MyD88−/− corneas, as determined by immunostaining (Fig. 5B).

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.26,27 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 assess 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup>, and MyD88<sup>−/−</sup> 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/C0/C0 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/C0/C0 mice had decreased baseline levels of goblet cells compared with IL-1R/C0/C0 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/C0/C0 mice to determine if knockout animals have baseline differences in gland area and length. The Meibomian glands are modified sebaceous glands that secrete...
The superior and inferior eyelids from WT (C57) and MyD88–/– mice were isolated and imaged with a Keratograph 5M infrared camera. A region of interest from the center of the eyelid containing the superior (upper) and inferior (lower) Meibomian glands was selected and determination of area (mm²) and length (mm) was performed using a custom designed MATLAB software program. Graph represents mean ± SEM with analysis by ANOVA and Bonferroni’s test for multiple comparisons, with no significance found between C57 and MyD88–/– mice.

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. In our study, during EDE, 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. MMPs, are increased in dry eye patients as well as during EDE. 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
the production of MMPs and cytokines in the context of ocular surface inflammation can be harmful and contribute to epithelial cell damage. TLR-induced expression of antimicrobial peptides are beneficial and could serve to protect the ocular surface from infection and invading pathogens during dry eye conditions, enhancing the readiness of the cornea to defend itself. Indeed, TLR signaling is critical for clearance of keratitis-related pathogens. 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.

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. TLRs have been shown to play a role in goblet cell development in the intestinal epithelium. Interestingly, TLR4-deficient mice exhibited increased epithelial to goblet cell differentiation in the small intestine and in vitro studies also showed an increase in goblet cells in TLR4−/− cell cultures. Therefore, we examined goblet cell numbers in the mice lacking MyD88-dependent TLR signaling. Contrary to what we hypothesized, MyD88-deficient mice had lower average goblet cell numbers under both non-EDE and EDE conditions, with a significant difference between WT C57 and MyD88−/− cells following EDE. This suggests that in addition to TLR-mediated protection through antimicrobial peptide production, MyD88-dependent TLR signaling is also beneficial for maintaining goblet cell numbers. Interestingly, in contrast to MyD88−/− mice, IL-1R−/− mice had increased numbers of goblet cells compared with WT in a normal, non-EDE environment. This result also points to the importance of TLR signaling, not just global MyD88 activation, in maintaining goblet cell numbers in the conjunctiva.

We also examined Meibomian gland morphology in the MyD88−/− mice to determine if MyD88-dependent TLR signaling contributes to normal gland development, as gland atrophy, blockage, and dropout are major causes of dry eye disease. No differences were observed in gland area or length between knockouts and WT animals, indicating that the protection from ocular surface damage seen in the MyD88−/− animals was not attributable to baseline Meibomian gland anatomic differences. However, after exposure to EDE conditions, there was a small but significant increase in Meibomian gland area, which was attributed to inferior gland enlargement. This increase appeared to be a result of widening, suggesting that a desiccating environment may induce morphologic changes in Meibomian glands that lead to early signs of gland dysfunction. It is theorized that gland widening is an initial sign of dysfunction, which then leads to atrophy, a leading cause of DED. Although the noticed change in area was small, if this early sign of dysfunction progressed to gland dropout, tear homeostasis would be further disrupted, propitiating more ocular surface damage in this model. Therefore, we are continuing to study Meibomian gland morphology, including contributions from TLR and IL-1R signaling during EDE.

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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