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Cathepsin S is a novel target for age-related dry eye

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Abstract

Cathepsin S (CTSS) is a protease that is proinflammatory on epithelial cells. The purpose of this study was to investigate the role of CTSS in age-related dry eye disease. *Ctss*$^{-/-}$ mice [in a C57BL/6 (B6) background] of different ages were compared to B6 mice. CTSS activity in tears and lacrimal gland (LG) lysates was measured. The corneal barrier function was investigated in naïve mice or after topical administration of CTSS eye drops 5X/day for two days. Eyes were collected, and conjunctival goblet cell density was measured in PAS-stained sections. Immunoreactivity of the tight junction proteins, ZO-1 and occludin, was investigated in primary human cultured corneal pithelial cells (HCEC) without or with CTSS with or without a CTSS inhibitor. A significant increase in CTSS activity was observed in the tears and LG lysates in aged B6 compared to young mice. This was accompanied by higher *Ctss* transcripts and protein expression in LG and spleen. Compared to B6, 12 and 24-month-old *Ctss*$^{-/-}$ mice did not display age-related corneal barrier disruption and goblet cell loss. Treatment of HCEC with CTSS for 48 hours disrupted occludin and ZO-1 immunoreactivity compared to control cells. This was prevented by the CTSS inhibitor LY3000328 or CTSS-heat inactivation. Topical reconstitution of CTSS in *Ctss*$^{-/-}$ mice for two days disrupted corneal barrier function. Aging on the ocular surface is accompanied by increased expression and activity of the protease CTSS. Our results suggest that CTSS modulation might be a novel target for age-related dry eye disease.

Keywords

Cathepsin S; dry eye; aging; inflammaging; corneal barrier; goblet cells; tight junction proteins

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1. Introduction

Aging is a complex biologic process that is accompanied by many chronic diseases. A growing body of evidence has shown that inflammaging, i.e., the inflammation that accompanies aging, is deleterious to many tissues (Franceschi and BonaFe, 2003; Franceschi and Campisi, 2014). Serum levels of biomarkers such as IL-6 and TNF-α correlate with increased mortality while TGF-β levels correlate with health in centenarians (Bruunsgaard et al., 2003a; Bruunsgaard et al., 2003b; Ferrucci et al., 1999). Studies have also shown that increased plasma levels of cathepsin S correlates with an increased risk of losing mobility and increased mortality in older adults (Jobs et al., 2011; Osawa et al., 2020).

Cathepsins are a group of enzymes with distinct functions at different locations inside and outside of the cells. Intracellular cathepsin S participates both in the physiological innate and adaptive immune responses. It plays a significant role in MHC II late presentation through degradation of the invariant li peptide into the class II-associated leupeptin-induced peptide (CLIP) in antigen-presenting cells (APCs), leaving the groove of MHC II free to present antigens (Bania et al., 2003; Beers et al., 2005; Riese et al., 1998; Saegusa et al., 2002a).

The role of cathepsin S in autoimmunity has been explored, as higher levels of cathepsin S correlates with the generation of auto-reactive cells by increased MHC II presentation time. (Saegusa et al., 2002b; Tato et al., 2017; Thanei et al., 2017). Similarly, cathepsin S inhibition or genetic deletion in mice can modify autoimmune manifestations such as the appearance of exocrinopathy and type 1 diabetes in the thymectomized and the nonobese diabetic (NOD) models (Hsing et al., 2010; Saegusa et al., 2002b). Cathepsin $S^{-/-}$ mice are resistant to experimental autoimmune encephalomyelitis and collagen-induced arthritis (Nakagawa et al., 1999; Yang et al., 2005). We have shown that aged C57BL/6 (B6) mice spontaneously develop dry eye disease, inclusive of increased corneal barrier disruption, goblet cell loss, $CD4^+$T cell infiltration in the conjunctiva, and loss of axonal nerves (McClellan et al., 2014; Stepp et al., 2018; Volpe et al., 2016), recapitulating many findings seen in human dry eye patients (Dohlman et al., 1976; Ralph, 1975; Yokoi and Kinoshita, 1995; Yokoi et al., 1997). We also reported that the adoptive transfer of $CD4^+$ T cells isolated from aged mice can induce goblet cell loss and lacrimal gland infiltration in young immunodeficient mice (Coursey et al., 2017; McClellan et al., 2014). Interestingly, age-related goblet cell loss in the B6 background is partially mediated by IFN-γ, as B6.*Ifn*$^{-/-}$ mice had decreased age-related goblet cell loss relative to wild-type B6 mice (Volpe et al., 2016). It is possible that age-related cathepsin S participates in the generation of autoreactive $CD4^+$ T cells during aging.

Besides its increase in serum, cathepsin S increases with aging in the retina and brain of aged mice (Ogawa et al., 2005; Park et al., 2009; Stichel and Luebbert, 2007; Wendt et al., 2008). Increased cathepsin S activity levels have also been observed in tears of dry eye subjects (Edman et al., 2018; Hamm-Alvarez et al., 2014; Hargreaves et al., 2019), including tears from patients with Sjögren Syndrome (an autoimmune form of dry eye). Dry eye is widespread in the elderly, and its prevalence increases with each decade after 40 (Schaumberg et al., 2009; Schaumberg et al., 2003). In addition, several studies have reported that the ocular surface and lacrimal glands develop pathological changes with aging

that can alter their function (de Souza et al., 2019; de Souza et al., 2020; Ebersole et al., 1988; Galletti and de Paiva, 2021a, b; McClellan et al., 2014; Nien et al., 2011; Parfitt et al., 2013; Segerberg-Konttinen, 1988), however, how aging specifically predisposes to dry eye remains to be established. In mice, features of dry eye are already evident in middle-age, coinciding with the loss of reproductive ability (McClellan et al., 2014; Volpe et al., 2016).

Histopathological alterations of the human and murine lacrimal glands have been described with aging; some of these anatomical changes are similar to the ones observed in Sjögren Syndrome (Batista et al., 2012; Bian et al., 2019; Coursey et al., 2015; Damato et al., 1984; Draper et al., 1999; Rocha et al., 2008; Yoon, 2020). Inhibition of cathepsin S reduces lacrimal gland inflammation and increases tear flow in a mouse model of Sjögren Syndrome (Klinngam et al., 2019), suggesting that modulation of cathepsin S might be a novel approach to improve dry eye-related changes.

Similar to Sjögren Syndrome, aged tears contain several inflammatory mediators (de Souza et al., 2020; Di Zazzo et al., 2019; Micera et al., 2018), but the presence of cathepsin S in aged tears and its effects on the aged ocular surface have not yet been investigated. Because extracellular cathepsin S has been implicated in matrix remodeling (Watari et al., 2000; Ziegler et al., 2018), we hypothesize that increased cathepsin S in tears might have a delerious effect on the aged ocular surface. This study shows for the first time that cathepsin S is increased in tears, lacrimal glands, and spleen of aged mice. We also demonstrate that aged *Ctss*$^{-/-}$ mice are resistant to age-related dry eye, with decreased levels of corneal barrier disruption and increased levels of conjunctival goblet cells. Furthermore, cathepsin S disrupts the tight junction proteins ZO-1 and occludin *in vitro*. And lastly, topical administration of cathepsin S to *Ctss*$^{-/-}$ mice is sufficient to cause corneal barrier dysfunction. Taken together, our results suggest that cathepsin S is a novel target for age-related dry eye.

2. Materials and methods

The Institutional Animal Care and Use Committees at Baylor College of Medicine approved all animal experiments. All studies adhered to the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the and Use of Laboratory, 2011). The experiments were performed at the Ocular Surface Center, Department of Ophthalmology, Baylor College of Medicine, Houston, Texas.

2.1 Animals

Young breeder pairs of C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) for establishing breeder colonies. One-hundred and sixty seven female B6 mice were maintained in specific-pathogen-free vivarium and were used at 2–3 (n = 71), 7 (n = 10), 10 (n = 10), 12 (n = 25), 15 (n = 8), or 22–24 months of age (n = 43).

Breeder pairs of cathepsin S (*Ctss*$^{-/-}$ mice, in a B6 background) were obtained from Dr. Thomas Reinheckel (Institute for Molecular Medicine and Cell Research University Medical

Center Albert-Ludwigs, University Freiburg, Freiburg, Germany), and a breeding colony was established. Seventy-eight *Ctss*$^{-/-}$ mice were used at 2–3 (n = 38), 12–13 (n = 18), 15–19 (n = 8) and 22–24 months of age (n = 14).

Breeder pairs of *Rab3d*$^{-/-}$ mice (in C57BL/6 background) were a kind gift of Dr. Sarah Hamm-Alvarez (USC, CA), and a breeding colony was established. *Rab3d*$^{-/-}$ mice (n = 17) were used at three months of age.

Mice were housed at specific pathogen-free Baylor College of Medicine facilities and were kept on daily cycles of 12 hours/light and 12 hours/dark with ad libitum access to food and water. Because dry eye is more frequent in women (Moss et al., 2000; Schaumberg et al., 2003), and aged male mice do not develop corneal barrier disruption (Volpe et al., 2016) (a hallmark of dry eye), only female mice were used. An effort was made to collect multiple tissues from each mouse. A final sample size per endpoint can be found in figure legends.

2.2 Cathepsin S activity assay in tears and lacrimal gland lysates.

One microliter of 1X PBS containing 0.1% BSA was placed on each eye of 2–3 and 24-month-old C57BL/6 mice, and then tear washings were collected using 1μl microcaps (Drummond, cat# 1–000-0010), as before (de Paiva et al., 2010). Tears from 4 eyes were pooled together as one sample into a tube containing 6μl of PBS + 0.1% BSA, centrifuged and stored at −80°C until the assay was performed. Cathepsin S activity was analyzed using Cathepsin S Activity Fluorometric Assay Kits (BioVision, Waltham, MA. Cat# K144–100) according to the manufacturer's protocol. Samples were read in a fluorometer instrument (Tecan Infinite M200, Männedorf, Switzerland). 50 μg of protein from lacrimal gland lysates was also used to assay cathepsin S activity as described above.

2.3 Preparation of lacrimal gland lysates

Extra-orbital lacrimal glands from young and 24-month-old B6 mice were harvested and lysed in RIPA lysis buffer (Thermo Fisher, Waltham, MA, Cat# 89900) plus a protease inhibitor cocktail (SIGMA, St. Louis, MO, Cat# P8340). Protein concentration was measured using a micro-BCA protein assay kit (Thermo Fisher, Waltham, MA, Cat# 23235).

2.4 RNA isolation and Real-time PCR

According to the manufacturer's protocol, total RNA from lacrimal glands was extracted using a QIAGEN RNeasy Plus Mini RNA isolation kit (Qiagen; Hilden, Germany). After isolation, RNA concentration was measured, and cDNA was synthesized using the Ready-To-Go You-Prime First-Strand kit (GE Healthcare, Chicago, IL). Next, real-time PCR was performed using specific MGB Taqman probes for Cathepsin S (*Ctss*, Mm00457902) and Taqman Universal PCR Master Mix AmpErase UNG in a commercial thermocycling system (StepOnePlus™ Real-Time PCR System, Applied Biosystems/Thermo Fisher Scientific; Foster City, CA), according to the manufacturer's recommendations. The hypoxanthine phosphoribosyltransferase 1 (*Hprt1,* Mm00446968) gene was used as an endogenous reference for each reaction. The quantitative PCR results were analyzed by the comparative

Ct method and were normalized by the Ct value of *Hprt1*(Coursey et al., 2014). The young group served as calibrators.

2.5 Western blotting

Lacrimal gland extracts (50μg) were resuspended in SDS sample buffer, boiled for 5 min, and analyzed on 4–15% mini-protean TGX stain-free gels (Bio-Rad, Hercules, CA, Cat# 4568084). The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Cat# 170–4157). The blots were probed with an anti-cathepsin S antibody (Santa Cruz, Cat# SC-6505) overnight at 4 °C. The blots were washed extensively with a solution containing 50 mM Tris, pH 8.0, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20. The antigen–antibodies complexes were detected by the ECL protocol (GE Healthcare, Chicago, IL, Cat# RPN2106) using horseradish peroxidase-conjugated goat anti-mouse IgG as a secondary antibody. Images were taken by ChemiDoc Touch Imaging Systems (Bio-Rad), and band densities were measured by Bio-Rad software (Bio-Rad, Hercules, CA, Image lab 6.0).

2.6 Measurement of corneal barrier function

Corneal barrier function was assessed by quantifying corneal epithelial permeability to 70-kDa Oregon-Green-Dextran-AlexaFluor_488 (OGD; Invitrogen, Carlsbad, CA) according to a previously published protocol (de Paiva et al., 2011), with modifications. Briefly, one microliter of a 50mg/ml solution of OGD was instilled onto the ocular surface one minute before euthanasia. Corneas were rinsed with 2 mL of PBS and photographed with a stereoscopic zoom microscope (model SMZ 1500; Nikon) under fluorescence excitation at 470nm. OGD staining intensity was graded in digital images by measuring the mean fluorescence intensity within a 2-mm diameter circle placed on the central cornea using NIS Elements software (version AR, 5.20.02) by two masked observers. The mean intensity of the right and left eyes was averaged, and the mean average from biological replicates was calculated and analyzed.

2.7 Goblet cell density using histology and PAS

Eyes and ocular adnexa were excised, fixed in 10% formalin, paraffin-embedded, and cut into 5-μm sections using a microtome (Microm HM 340E, Thermo Fisher Scientific Waltham, MA). Sections cut from paraffin-embedded globes and 2–3 nonconsecutive sections that were obtained from the center of the eye were stained with Periodic Acid Schiff (PAS) reagent per mouse. The goblet cell density was measured in the superior and inferior bulbar and tarsal conjunctiva using NIS-Elements software (AR, version 5.20.2; Nikon Melville, NY) as published (de Paiva et al., 2007). The results were averaged per mouse and multiple mice of the same group were used. Goblet cells are expressed as the number of positive cells per millimeter. A final sample size can be found in figure legends.

2.8 In vitro culture model for human corneal epithelial barrier disruption exposed to cathepsin S

Fresh human corneoscleral tissues (<72 h after death) from donors aged 35–57 years without allergic conditions were obtained from the Lions Eye Bank of Texas (Houston, TX). Human

corneal epithelial cells were cultured in eight-chamber glass slides using explants from corneal limbal rims in a supplemented hormonal epidermal medium (SHEM) containing 10% fetal bovine serum (FBS) using our previous methods (Hu et al., 2020; Li et al., 2005). Confluent corneal epithelial cultures were switched to serum-free SHEM and treated for 24 or 48 hours with increasing concentrations of cathepsin S (ProSpec Bio, East Brunswick, NJ). In addition, some wells were treated with cathepsin S and a cathepsin S inhibitor (LY3000328, Cayman Chemical, Ann Arbor, MI) or heat-inactivated cathepsin S. The cathepsin S inhibitor, LY3000328, was dissolved in DMSO to make the stock solution at 10mM, which was 1:200 diluted to be 50μM for the treatment. DMSO concentration is 0.5% when 50μM of LY3000328 used in culture. Our primary cells are routinely cultured in SHEM medium that contains 0.5% DMSO. To account for any added effect of DMSO, some wells were treated with LY inhibitor only.

Heat inactivation of cathepsin S was performed by incubating cathepsin S at 90°C for 30 minutes.

2.9 Immunofluorescence detection for tight junction proteins.

Cultured cells were fixed in acetone for 3 min at −20°C for staining. IF staining was performed as previously described (de Paiva et al., 2005; Hu et al., 2020). The primary antibodies used for this study included: rat rabbit anti-mouse ZO-1 (1:200, catalog #61–7300, Invitrogen-Thermo Fisher Scientific, Waltham, MA) and occludin (1:200, catalog #40–4700 Invitrogen-Thermo Fisher). Digital confocal images were captured with a laser scanning confocal microscope (Nikon A1 RMP, Nikon, Melville, NY, USA) wavelength 400–750 nm. The images were processed using NIS Elements 4.20 version (Nikon). Quantification of the percentage (%) area with tight-junction proteins was performed by a blinded examiner. A field was considered 100% and the area covered by tight junction proteins were manually drawn using the “polygon area” function of NIS Elements. At least six random digital images per condition per experiment were measured. At least three different experiments were performed, and results were averaged.

2.10 Exogenous administration of cathepsin S to *Ctss*$^{-/-}$ mice.

Young (2–3-month-old) *Ctss*$^{-/-}$ mice received bilateral topical eye drops of either vehicle (PBS) or cathepsin S (5μL, 400ng/ml, Prospec Protein Specialists, East Brunswick NJ 08816, Cat# ENZ-686) every 2 hours, five times/day for two days. On the morning of the 3rd day, mice were dosed once, and corneal barrier function was assessed in all mice at least one hour after the last dosing.

2.11 Statistical analysis

The sample size was calculated *a priori* with StatMate2 Software (GraphPad Software, San Diego, CA, version 2) based on pilot studies. Statistical analyses were performed with Graph Pad Prism software (GraphPad Software, San Diego, CA, version 9.1). Data were first evaluated for normality with the Kolmogorov-Smirnov normality test. Then, appropriate parametric (t-test) or non-parametric (Mann-Whitney) statistical tests were used to make comparisons between two age groups. Whenever adequate, one-way or two-way ANOVA or

Kruskal-Wallis followed by post hoc tests were used. All experiments were repeated at least once. The final sample per experiment is shown in the figure legends.

3. Results

3.1 Age-related increase of cathepsin S in tears, lacrimal glands, and spleen.

Cathepsin S in a lysosomal cysteine protease that is active even in neutral pH (Turk et al., 2012). Cathepsin S has been reported to increase in the aged population (Park et al., 2009). To investigate if aging in the eye and lacrimal gland is accompanied by changes in cathepsin S activity and expression, young and 24-month-old B6 tears and lacrimal gland lysates were collected and analyzed. Cathepsin S activity levels were increased in tears and lacrimal gland lysates of the aging mice (Fig. 1A). We also observed increased *Ctss* mRNA in lacrimal glands by quantitative real-time PCR (Fig. 1B). There was a significant increase in cathepsin S in lacrimal gland and spleen in aged (24-month-old) compared to young mice by Western blotting while no change in pro-cathepsin S was seen (Fig. 1C–1E). Similar to other cathepsins, cathepsin S is secreted as a pro-cathepsin but can rapidly be activated by changes in the pH or by autocatalytic activation (Turk et al., 2012). For example, negative charged molecules such as glycosaminoglycans and dextran have been shown to accelerate cathepsin S activation (Vasiljeva et al., 2005). Activation of pro-cathepsin can occur intracellularly as well as extracellularly, depending upon organelle pH and other factors which modulate activity. Taken together, increased cathepsin S activity, transcription and protein were observed systemically and also in ocular tissues in B6 as they aged.

3.2 Cathepsin $S^{-/-}$ mice are resistant to age-related dry eye

We have previously shown that B6 mice aged 6–9 and 15-month-old have spontaneously increased corneal barrier disruption and diminished goblet cell density (McClellan et al., 2014; Volpe et al., 2016). Here we analyzed these properties of the cornea and conjunctiva over time, confirming a significant age-related disruption in the corneal barrier as B6 mice aged up to 12 months (Fig. 2A–B). Furthermore, there was a positive and significant correlation (Fig. 2C, $R = 0.92$, $P < 0.0001$) between age and the intensity of Oregon-Green-Dextran (OGD) levels, the dye used to investigate corneal epithelial barrier in mice (de Paiva et al., 2006).

To further evaluate the effects of increased age-related cathepsin S on the eye, OGD staining in the cornea and goblet cells density in the conjunctiva were evaluated in young and aged wild-type B6 and *Ctss*$^{-/-}$ mice. No differences in corneal staining were found between young *Ctss*$^{-/-}$ and B6 mice; however, significantly decreased age-related corneal barrier disruption was observed in 12–13-month-old *Ctss*$^{-/-}$ mice than wild-type mice (Fig. 2D). Conjunctival goblet cell density was similar between young *Ctss*$^{-/-}$ and B6 mice, but it was significantly decreased as B6 mice aged, as we previously reported (McClellan et al., 2014; Volpe et al., 2016). However, conjunctival goblet cell density in *Ctss*$^{-/-}$ mice did not decrease during aging [ages 12–13, 15–19, and 22-month or older (Fig. 2E)].

These data indicated *Ctss*$^{-/-}$ mice have ameliorated corneal barrier disruption and do not lose goblet cells with aging, suggesting that cathepsin S participates in the age-related dry eye disease.

3.3 Cathepsin directly affects corneal barrier function by disrupting the tight junction proteins ZO-1 and occludin.

After showing that aged *Ctss*$^{-/-}$ mice have ameliorated corneal barrier function with aging, we hypothesize that cathepsin S can directly degrade tight junction proteins (Zonula occludens-1 [ZO-]) and occludin) that maintain the barrier integrity of several epithelia, including corneal epithelial cells (Beardsley et al., 2008; de Paiva et al., 2006; Hu et al., 2020; Pflugfelder et al., 2005). Corneal barrier disruption is a hallmark of dry eye. To test our hypothesis, human corneal epithelial cells (HCECs) were cultured in vitro with and without increasing concentrations of cathepsin (0.2μg/ml or 0.4μg/ml) for 24 and 48 hours. In some experiments, a cathepsin S inhibitor was added 1 hour before adding cathepsin S. In a separate experiment, cathepsin S was inactivated by heat (90°C) for 30 minutes before adding to the cultures. As published, the integrity of tight junction proteins was investigated using immunofluorescence for ZO-1 and occludin (Hu et al., 2020). In control HCECs, ZO-1 and occludin showed a net-like appearance characteristic of its expression around the periphery of corneal cells within the monolayer (Fig 3A, 3B). The addition of cathepsin S to in vitro cultures for 24 hours did not affect these tight junction proteins (data not shown). However, a profound effect was observed after 48 hours, as cathepsin S dramatically altered ZO-1 and occludin net-like distribution in vitro, with regions showing denuded or irregular and punctate expression, demonstrating loss of these proteins. Digital image analysis using image software showed that treatment of HCEC with cathepsin S significantly decreased the area covered by ZO-1 and occludin proteins by at least 50% (Fig. 3C). Specifically, for occludin, a doubling of cathepsin S concentration from 0.2 to 0.4ug/ml further decreased its area of staining, but it did not reach statistical significance.

A specific cathepsin S inhibitor was used to investigate if this effect was directly mediated by cathepsin S. Pre-treatment of HCECs with the cathepsin S inhibitor LY3000328 prevented cathepsin S-induced ZO-1 and occludin protein loss (Fig 3A, 3B). A dose-response to the inhibitor was not noted, but LY treatment did not induce any rupture in tight junction proteins (Fig 3B). To further investigate if an active cathepsin S was needed for cathepsin S-induced tight junction protein disruption, we repeated the experiment with heat-inactivated cathepsin S and stained labeled HCECs with occludin antibody. As shown in Fig 3B, 3C, heat inactivation of cathepsin S prevented cathepsin S-mediated tight junction protein loss.

These results demonstrate cathepsin S can directly disrupt the corneal barrier *in vitro,* and that its activity protein is needed for this effect.

3.4 Cathepsin S eye drops disrupt corneal barrier in vivo

Because tight junction proteins were disrupted *in vitro*, we asked if exogenous administration of cathepsin S in vivo would recapitulate the same results, considering that *Ctss*$^{-/-}$ mice had decreased age-related corneal barrier disruption compared to wild-type

mice (Fig. 2D). Disruption of tight junction proteins *in vivo* has been demonstrated in animal models of dry eye and aged corneas (Beardsley et al., 2008; Pflugfelder et al., 2005). Therefore, to investigate this, we dosed young *Ctss*$^{-/-}$ mice with cathepsin S eye drops (0.4ug/ml) 5x/day for two consecutive days. On the morning of the third day, mice were dosed again (for a total of 11 doses), and evaluation of corneal barrier was investigated one hour after the last dosing (Fig 4A). Age-matched *Ctss*$^{-/-}$ mice that received vehicle eye drops with the same frequency served as controls. Corneal barrier function was investigated using the OGD dye, as shown in Fig. 2A. Topical administration of cathepsin S in *Ctss*$^{-/-}$ mice was sufficient to induce corneal barrier disruption relative to vehicle-treated *Ctss*$^{-/-}$ mice (Fig. 4B), indicating the cathepsin S can also reduce tight junction proteins *in vivo*.

Accumulated evidence suggests that *RAB3D* is a positive modulator of exocytosis. We have previously reported *Rab3d*$^{-/-}$ mice exhibit increased cathepsin S secretion levels in tears (Fu et al., 2021; Meng et al., 2016). Therefore, we hypothesize that naïve young *Rab3d*$^{-/-}$ mice would have increased corneal barrier disruption. Evaluation of the ocular surface of young *Rab3d*$^{-/-}$ mice using the OGD dye showed increased uptake of this dye compared to the wild-type B6 group, demonstrating that the corneal barrier is spontaneously compromised in the *Rab3d*$^{-/-}$ mice (Fig. 4C).

4. Discussion

Aging is a complex biological process. The incidence and prevalence of chronic diseases increase with aging, and there is a great interest in modulating age-related diseases. Dry eye is one of the diseases that increases with aging. Our results show that cathepsin S is increased in ocular tissues with aging and that modulation of its expression or activity might be a novel target for age-related dry eye.

We found increased cathepsin S activity levels in tears, lacrimal gland lysates, and increased cathepsin S protein in the lacrimal gland and spleen. These results are in agreement with previous studies in animal models of Sjögren Syndrome (Janga et al., 2019; Klinngam et al., 2019; Li et al., 2010) and also in patients with dry eye and Sjögren Syndrome (Edman et al., 2018; Hamm-Alvarez et al., 2014) that also showed increased cathepsin S activity levels in tears. However, this is the first time increased cathepsin S levels have been found in an age-related dry eye model. This is important because increased levels of cathepsin S in other biofluids in the elderly are a biomarker for poor mobility and increased systemic inflammation (Jobs et al., 2011; Osawa et al., 2020; Park et al., 2009).

Interestingly, aged *Ctss*$^{-/-}$ mice were resistant to age-related corneal barrier disruption and loss of goblet cells. We and others have shown that aged B6 mice spontaneously develop dry eye disease, recapitulating many of the hallmarks of dry eye in humans, including corneal barrier disruption and goblet cell loss (Bian et al., 2019; de Souza et al., 2020; McClellan et al., 2014; Volpe et al., 2016; Yoon, 2020). This is important because there is a close relationship between IFN-γ and cathepsin S and IFN-γ participates in age-related goblet cell loss (Volpe et al., 2016). Inflammatory mediators such as LPS, IL-1, TNF-α, and IFN-γ stimulate expression and secretion of cathepsin S from macrophages, microglia, and epithelial cells (Klinngam et al., 2018; Li et al., 2020; Liuzzo et al., 1999). Treatment of

lacrimal gland acinar cells with IFN-γ increased cathepsin S and decreased the endogenous cathepsin S inhibitor, cystatin C transcript levels, while increasing MHC II related molecules (Meng et al., 2017). IFN-γ treatment was sufficient to increase cathepsin S and MHC II molecules in human corneal epithelial cells (Meng et al., 2017). Cathepsin S may likewise participate in the age-related goblet cell loss by promoting the generation of autoreactive $CD4^{+}IFN\text{-}\gamma^{+}$, which we have previously shown to infiltrate the eye and lacrimal gland in elderly mice (Bian et al., 2019). This hypothesis is currently under investigation.

Our collective results suggest that cathepsin S directly disrupts epithelial tight junctions *in vitro*. Cathepsin S addition to cultured corneal epithelial cells reduced expression of tight junction proteins in a process dependent on enzyme activity while administration of topical cathepsin S to *Ctss*$^{-/-}$ mice also was sufficient to increase fluorescent dye uptake compared to vehicle-dosed mice. Cathepsin S, as a protease, can elicit effects both directly and indirectly which could lead to such changes. Cathepsin S can degrade multiple proteins through direct cleavage; if any of these proteins, including occludin, ZO-1, or other proteins responsible for tight junctional scaffolding associated with these proteins, is a cathepsin S substrate. Multiple other proteases are secreted from cultured cells as part of the process of remodeling matrix, such as matrix metalloproteinases. Cleavage of other proteases present in extracellular fluid that then degrade tight junction proteins might have the same effect. Cathepsin S is known to evoke matrix remodeling in other tissues (Gautam et al., 2018; Liuzzo et al., 1999). Furthermore, cathepsin S degrades proteoglycans, a protein critically involved in lubricating the ocular surface (Regmi et al., 2017). These findings collectively suggest that cathepsin S activity may act directly on junctional complexes and/or their associated proteins

Cathepsin S exposure to cells also has profound effects on intracellular signaling through its ability to cleave the external domain of the protease-activated receptor 2 (PAR-2), creating a novel tethered ligand that promotes changes in intracellular signaling (Elmariah et al., 2014; Lieu et al., 2016; Zhao et al., 2014a). These changes in signaling in neurons have been linked to elevations in cAMP and activation of transient potential vanilloid receptor 4 (TRPV4) and induction of inflammatory pain (Zhao et al., 2014a; Zhao et al., 2014b). Treatment with recombinant cathepsin S in human corneal epithelial cells at similar concentration levels to those found in tears from Sjögren Syndrome patients can upregulate *Tnf*, *Il6*, *Il1b*, *Mmp9* mRNA levels (Klinngam et al., 2017) although the intracellular signaling pathways responsible have not been fully determined. The mechanism of PAR-2-mediated upregulation is likewise dependent on cathepsin S activity. Cytokines can have profound effects on tight junctions in various epithelia, so it is possible that cathepsin S activity may influence tight junctions through downstream changes in cytokines.

We observed increased corneal barrier disruption in *Rab3d*$^{-/-}$ mice. Hamm-Alvarez and colleagues have shown that *Rab3d*$^{-/-}$ mice have increased cathepsin S in tears (Fu et al., 2021; Meng et al., 2016). RAB3D is widely expressed in nonneuronal cells, including adipocytes, exocrine glands, and several hematopoietic cells. It localizes to secretory granules and vesicles and plays a key role during regulated exocytosis in exocrine secretion. *Rab3d* knockout increases cathepsin S secretion from lacrimal acini in a mouse model of Sjögren Syndrome (Meng et al., 2016).

Cathepsin S inhibition has been shown to improve signs of disease in animal models of Sjögren Syndrome, type 1 diabetes, experimental autoimmune encephalomyelitis, and collage-induced arthritis (Hsing et al., 2010; Klinngam et al., 2019; Nakagawa et al., 1999; Saegusa et al., 2002b; Yang et al., 2005). Our studies suggest that targeting cathepsin S might also be a viable approach to treat age-related dry eye disease. Further studies are needed to investigate the precise mechanisms that cathepsin S inhibition ameliorates age-related dry eye disease.

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Highlights

- Aged tears and aged lacrimal gland lysates have increased cathepsin S activity levels.
- Mature cathepsin S levels are present in the aged lacrimal gland and spleens.
- There is an age-related increase in corneal barrier disruption.
- Aged cathepsin S $^{-/-}$ mice are resistant to age-related corneal barrier disruption and do not show conjunctival goblet cell density loss.
- Exogenous administration of active cathepsin S to human culture epithelial cells disrupts tight junction proteins Zo-1 and occludin.
- Topical administration of cathepsin S as eye drops to cathepsin $S^{-/-}$ mice disrupts corneal barrier in vivo.

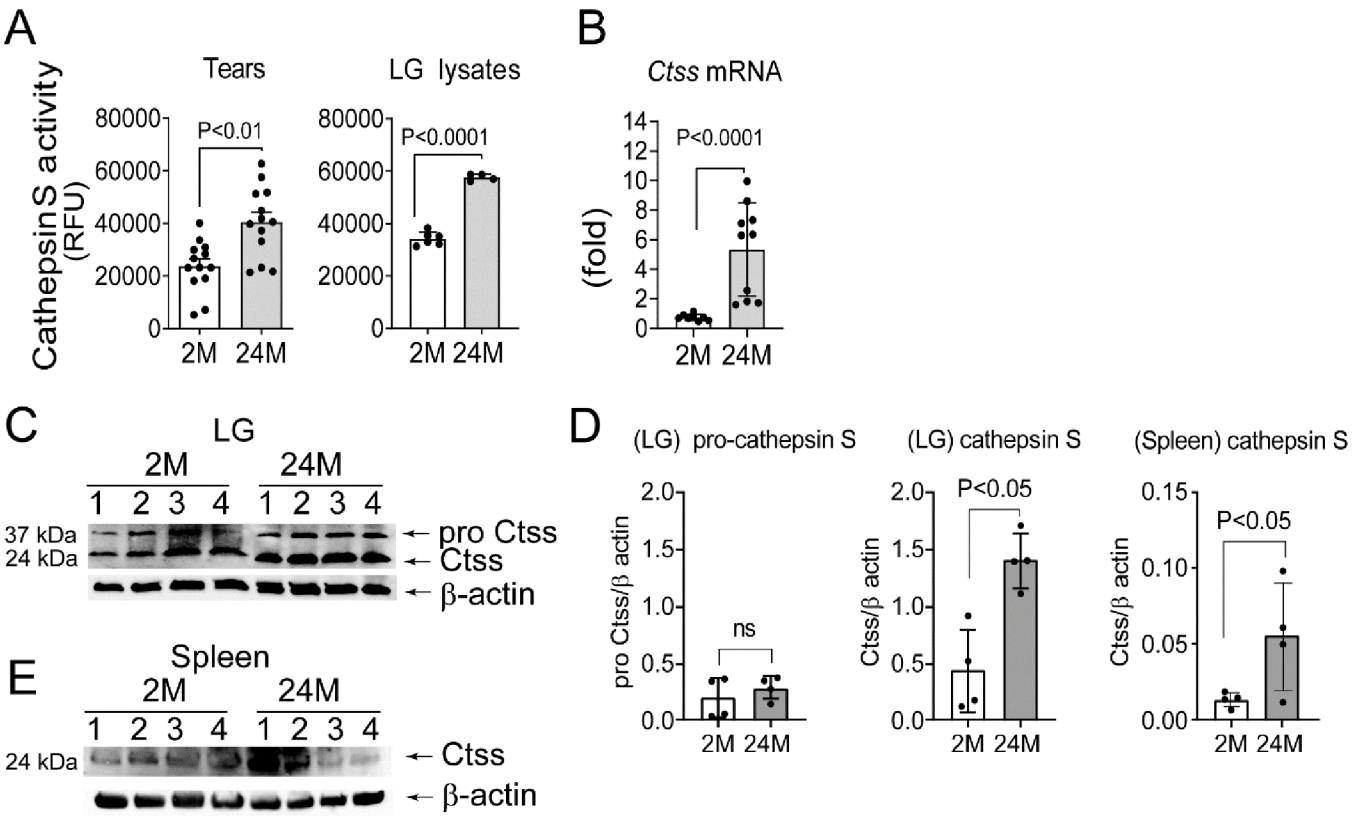

Fig. 1. Cathepsin S is increased in aged tears and lacrimal glands from B6 mice.

A. Cathepsin S activity in tear washings and lacrimal gland (LG) lysates of 2 and 24-month-old B6 mice (2M and 24M, respectively). Each dot represents pooled tear washings from 2 animals. Mann-Whitney U test.

B. Relative fold expression of cathepsin S (*Ctss)* mRNA in lacrimal gland. Each dot represents one right lacrimal gland, n = 8–10/age. Mann-Whitney U test.

C. Representative Western blot for cathepsin S and β-actin in 4 different biological samples from young and elderly lacrimal glands.

D. Densitometry showing cathepsin S/β-actin ratios, n = 4 biological samples. Mann-Whitney U test. NS = non-significant

E. Representative Western blot for cathepsin S and β-actin in 4 different biological samples from young and elderly and spleen lysates.

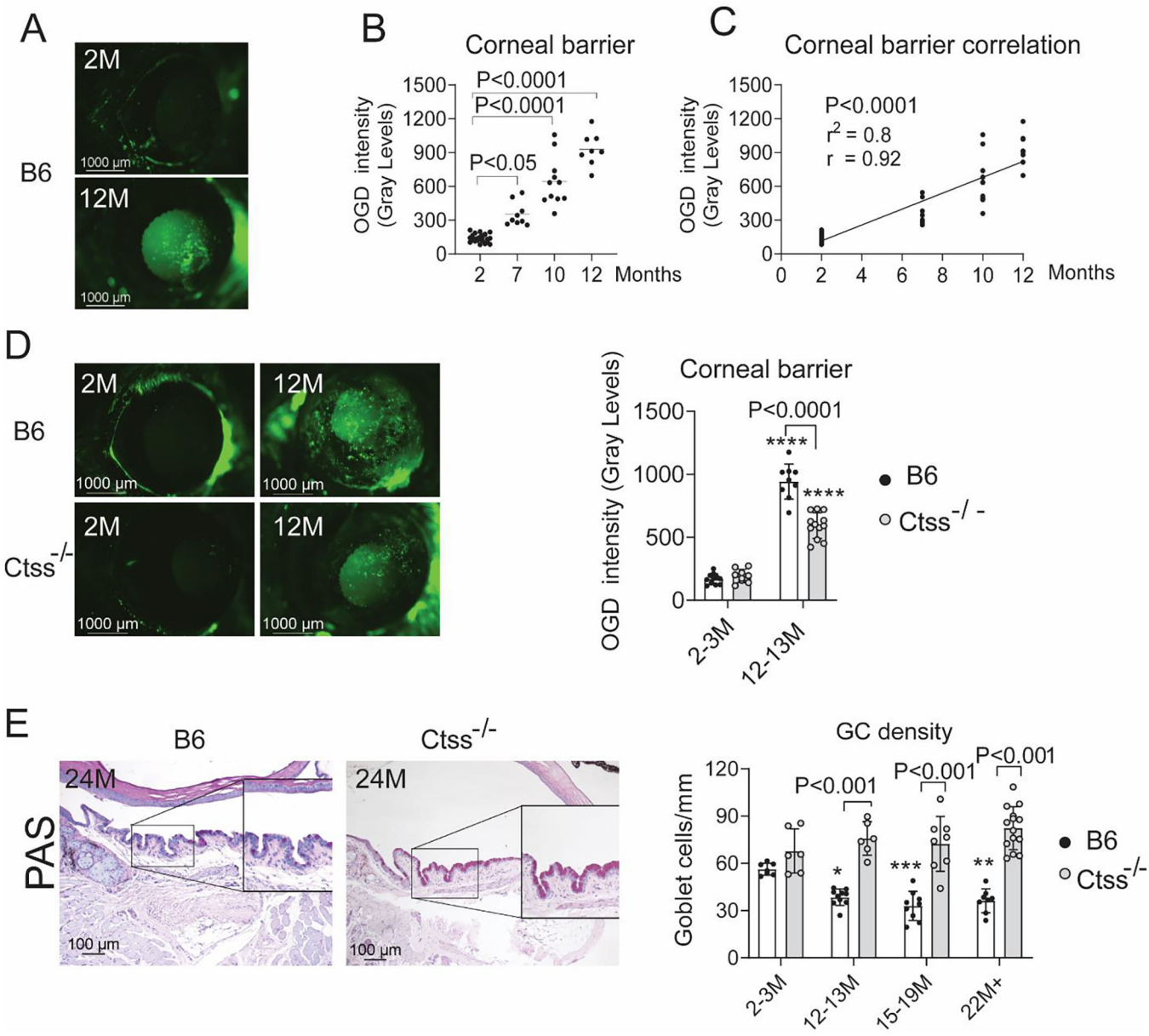

Fig. 2. Age-related dry eye is decreased in *Ctss*$^{-/-}$ mice.

A. Representative images of corneas stained with Oregon-Green Dextran (OGD) in young and 12-month (12M) B6 mice. Scale bar = 1000 μm. M = months.

B. Corneal barrier evaluation in B6 mice of different ages; each dot represents one animal (average of right and left eyes, n = 10–12/age). Non-parametric Kruskal-Wallis non-parametric test followed by Dunn's comparison.

C. Spearman's correlation of cornea barrier disruption scores and age. r^2 = coefficient of determination; r = coefficient of correlation.

D. Representative images of corneas stained with Oregon-Green Dextran (OGD) in young and middle-aged B6 mice and *Ctss* $^{-/-}$ mice. Scale bar = 1000 μm. Cumulative data on the graph; each dot represents one animal (average of right and left eyes, n = 8–10/

strain/age). Non-parametric Kruskal-Wallis followed by Dunn's multiple comparison test. ****P<0.0001 compared to young mice of the same strain.

E. Representative images of conjunctival sections of elderly mice (24-month, 24M) stained with PAS (purple-magenta) showing increased goblet cell density in the *Ctss*$^{-/-}$ mice. Insets are a higher magnification of the area on the left, which is demarcated by a square. Cumulative graph on the right, showing several age intervals. Non-parametric Kruskal-Wallis followed by Dunn's multiple comparison test. Scale bar = 100 μm. Each dot represents one animal, n = 6–14.

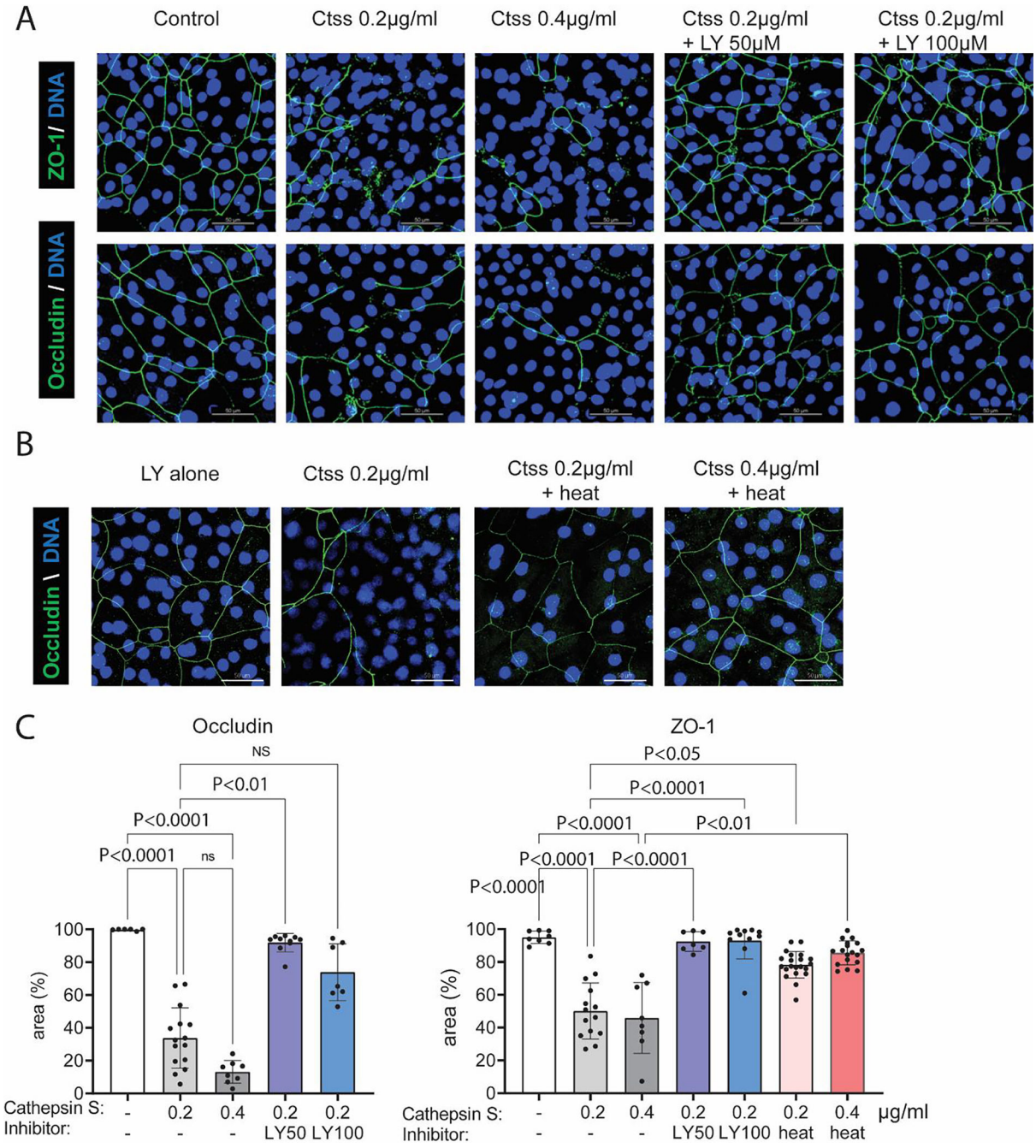

Fig. 3. Exogenous administration of Cathepsin S in culture disrupts tight junction proteins *in vitro*.

Confluent human cultured corneal epithelial cells were switched to serum-free SHEM media and treated with two different concentrations of cathepsin S, with or without the cathepsin S inhibitor (LY3000328, LY) for 48 hours. Cathepsin S inhibitor was added 1 hour before adding cathepsin S. In some experiments (B), cathepsin S was heat-inactivated before being added to HCECs. (A-B) Representative merged images of laser scanning confocal microscope images of HCECs stained for either ZO-1 or occludin (green) with and

DAPI (blue, nuclei counterstaining) after treatment, as depicted. Scale bar = 50 μm. This experiment was repeated at least three times.

C. Accumulative graph shows the percentage of area covered by tight junctions in the different groups. Non-parametric Kruskal-Wallis followed by Dunn's multiple comparison test. Each dot is data from one randomly chosen image from 3 different experiments.

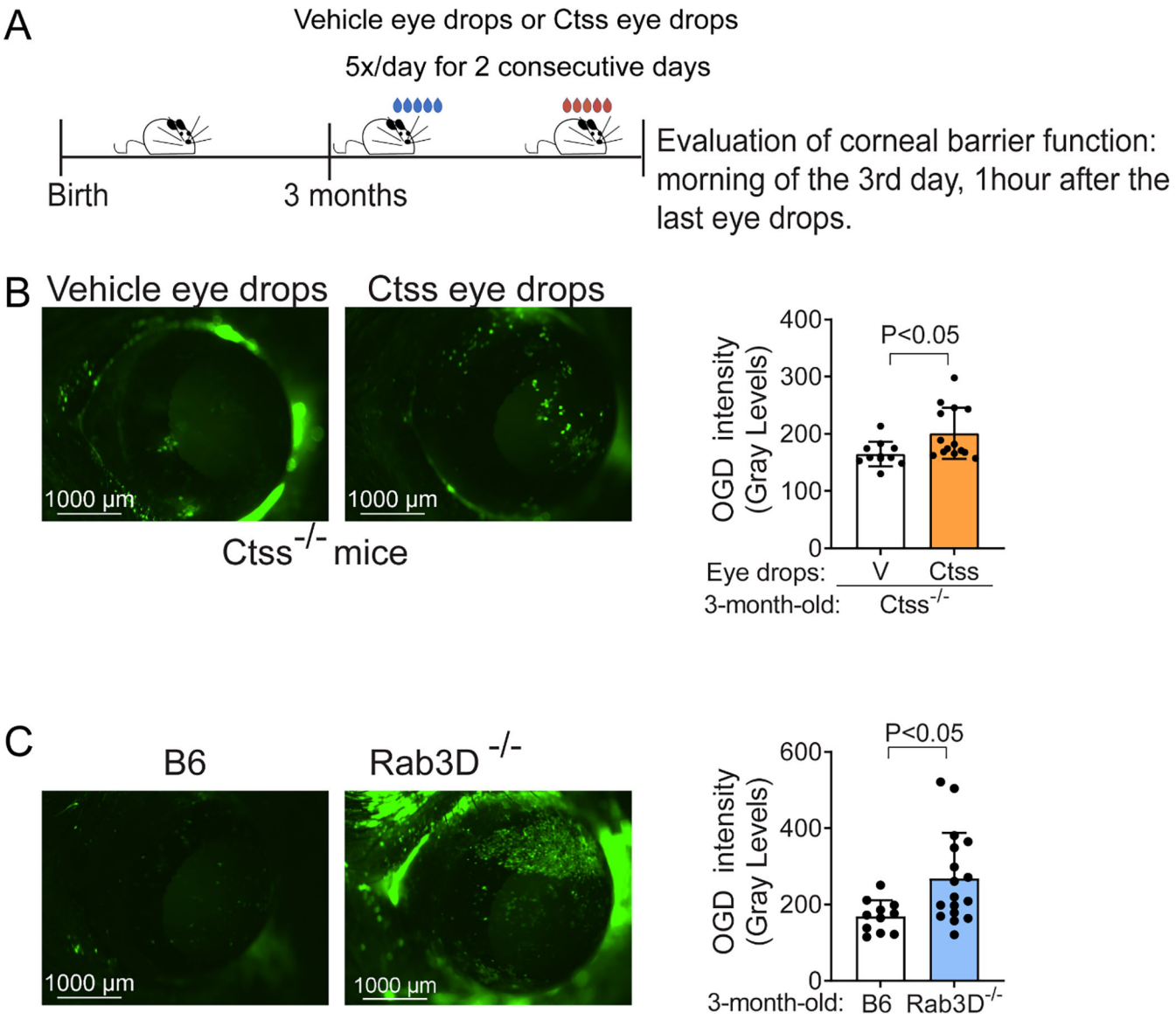

Fig. 4. Exogenous administration of cathepsin S disrupts corneal barrier in vivo.

A. Schematic showing how cathepsin S dosing was performed in 3-month-old *Ctss*$^{-/-}$ mice. These mice received eye drops of either cathepsin S or vehicle 5x/day for two consecutive days. Evaluation of corneal barrier function was performed on the morning of the 3rd day, at least one hour after the last dosing (11 doses in total).

B. Representative images of corneas stained with Oregon-Green-Dextran (OGD) in *Ctss*$^{-/-}$ mice after dosing with either cathepsin S or vehicle (V). Cumulative data on the graph; each dot represents one animal (average of right and left eyes, n = 10–12/group). Mann-Whitney U test. Scale bar = 1000 μm.

C. Representative images of corneas stained with Oregon-Green Dextran (OGD) in 3-month-old B6 compared to *Rab3d*$^{-/-}$ mice. Cumulative data on the graph; each dot represents one animal (average of right and left eyes, n = 11–17). Mann-Whitney U test. Scale bar = 1000 μm.