Inhibition of Cathepsin S in Autoimmune CD25KO Mouse Improves Sjögren Disease–Like Lacrimal Gland Pathology

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Purpose. CD25KO mice are a model of Sjögren disease (SjD) driven by autoreactive T cells. Cathepsin S (CTSS) is a protease crucial for major histocompatibility complex class II presentation that primes T cells. We investigated if a diet containing CTSS inhibitor would improve autoimmune signs in CD25KO mice.

Methods. Four-week female CD25KO mice were randomly chosen to receive chow containing a CTSS inhibitor (R05461111, 262.5 mg/kg chow) or standard chow for 4 weeks. Cornea sensitivity was measured. Inflammatory score was assessed in lacrimal gland (LG) histologic sections. Flow cytometry of LG and ocular draining lymph nodes (dLNs) investigated expression of Th1 and Th17 cells. Expression of inflammatory, T- and B-cell, and apoptotic markers in the LG were assessed with quantitative PCR. The life span of mice receiving CTSS inhibitor or standard chow was compared. $CD4^+$ T cells from both groups were isolated from spleens and adoptively transferred into RAG1KO female recipients.

Results. Mice receiving CTSS inhibitor had better cornea sensitivity and improved LG inflammatory scores. There was a significant decrease in the frequency of $CD4^+$ immune cells and a significant increase in the frequency of $CD8^+$ immune cells in the dLNs of CTSS inhibitor mice. There was a significant decrease in Th1 and Th17 cells in CTSS inhibitor mice in both LGs and dLNs. *Ifng*, *Ciita*, and *Casp8* mRNA in CTSS inhibitor mice decreased. Mice that received the CTSS inhibitor lived 30% longer. Adoptive transfer recipients with CTSS inhibitor-treated $CD4^+$ T cells had improved cornea sensitivity and lower inflammation scores.

Conclusions. Inhibiting CTSS could be a potential venue for the treatment of SjD in the eye and LG.

Keywords: cathepsin S, Sjögren disease, MHC II presentation, CTSS, autoimmunity

Sjögren disease (SjD) is an autoimmune disease characterized by dysfunction of secretory glands, such as the lacrimal gland (LG) and salivary gland (SG).[1] Previously, SjD was identified as Sjögren Syndrome, but advances in defining the etiology and pathology of the disease, in addition to patient advocacy, have made the term *disease* preferable.[2,3] The prevalence of the disease varies due to a lack of universally accepted criteria and unique biomarkers.[4] Therefore, estimates for the prevalence can vary from 0.2% to 14.2%.[5,6] SjD is characterized by severe lymphocytic infiltration in the SG and LG, and it is composed primarily of T cells and B cells.[7] Autoreactive B cells produce autoantibodies to Ro and La proteins and are present in the inflamed exocrine tissue.[8] Helper T cells (Th) activate B cells to generate this humoral response; in SjD, it has been found that there is a strong proinflammatory feedback loop between $CD4^+$ T cells and B cells, resulting in autoimmunity.[9] Consequently, biopsy specimens of SGs and LGs from patients with SjD have identified higher localization of Th cells in these tissues than in controls.[10,11] Addressing this feedback loop may be a therapeutic approach for SjD.

Cathepsin S (CTSS) is a protease that is important to major histocompatibility complex (MHC) class II peptide presentation. It degrades the last of the Ii-chain variants, allowing transport of the MHC II receptor to the surface for antigen presentation.[12] Increased CTSS levels have been associated with increased MHC II presentation and generation of autoreactive T cells.[13] One clinical marker proposed for better identification of SjD is protein activity measurement of CTSS, which is elevated in the tears of patients and is negatively correlated with tear and saliva production.[14] Furthermore, CTSS inhibition has promise as a treatment for SjD. Peripheral blood mononuclear cells (PBMCs) isolated from patients with primary SjD when stimulated in vitro using SS-A and SS-B antigens and a CTSS inhibitor showed decreased T-cell proliferation and decreased production of

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cytokines.[15] In the nonobese diabetic (NOD) mouse, CTSS inhibition resulted in reduced infiltration of lymphocytes into the LG and increased stimulated tear secretion.[16] While these indicate strong promise for the use of CTSS inhibition to decrease pathogenic T cells in SjD, further work in other animal models of SjD is needed.

The CD25KO mouse is a severe autoimmune model with long-lived autoreactive T cells. CD25 is the α chain of the IL-2 receptor. It is the binding arm of the heterodimeric receptor and critical for IL-2 signaling.[17] Among its key roles in T-cell homeostasis, IL-2 signaling results in differentiation and maintenance of $FOXP3^{+}CD4^{+}CD25^{+}$ T regulatory cells (Tregs).[18] Without IL-2 signaling, CD25KO mice develop a strong autoimmune response due to a lack of Tregs, spontaneously activated T cells, and lack of activation-induced T-cell death, all resulting in enlargement of lymphoid tissues, destruction and fibrosis of organs, and eventual fatal autoimmune complications.[19] Previously, we and others have shown that CD25KO mice develop spontaneous dacryoadenitis, sialadenitis, and lymphocytic infiltration in the exocrine glands.[20,21] By 16 weeks of age, LGs and SG are severely atrophied and present almost complete infiltration by lymphocytes, resulting in a diminished gland size.[22] However, it is unknown if this severe autoimmune phenotype can be stymied by addition of a CTSS inhibitor to standard chow. Herein, we investigated the use of CTSS inhibition for 4 weeks in CD25KO mice as a mouse model of SjD.[20,22]

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at Baylor College of Medicine approved all animal experiments (AN-6491). In addition, all studies adhered to the ARVO Statement 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.[23] The experiments were performed at the Ocular Surface Center, Department of Ophthalmology, Baylor College of Medicine, Houston, Texas.

Heterozygous breeder pairs of $CD25^{+/-}$ mice on a C57BL/6 background (B6.129S4-*Il2ra*^{tm1Dw}/J) and breeder pairs of RAG1KO mice (recombination activating gene 1; B6.129S7-*Rag1*^{tm1Mom}/J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) for establishing breeder colonies. Mice were housed in specific pathogen-free facilities at Baylor College of Medicine and kept on diurnal cycles of 12 hours/light and 12 hours/dark with ad libitum access to food, water, and environmental enrichment. The genotype of CD25KO mice was confirmed using a commercial vendor (Transnetx, Cordova, TN, USA). Wild-type (WT) mice were littermates from our in-house colony. All mice used in this study were female, because SjD disproportionately affects women compared to men at a ratio of 9:1,[24] and our previous work with CD25KO mice found no sex differences in LG inflammation.[25] Forty CD25KO mice, 5 WT mice, and 35 RAG1KO mice were used. Efforts were made to use the same mouse in multiple endpoints to decrease the sample size. The final sample size per endpoint can be found in figure legends.

CTSS Inhibitor Regimen

Two groups of 4-week-old female CD25KO mice were randomized to receive either a standard chow or a customized chow with the CTSS inhibitor (R05461111, 262.5 mg/kg chow; Roche, Basel, Switzerland). Pharmacokinetic data indicate this dose results in an exposure dose of 30 mg/kg and stable plasma levels of 400 to 600 ng/mL for 8 weeks.[13] The medicated chow was commercially prepared by LabDiet (San Antonio, TX, USA). Mice were weighed weekly. All endpoints were tested at euthanasia at 8 weeks of age.

Survival Curve

CD25KO mice were given the CTSS inhibition chow ad libitum and evaluated three times a week for body condition. Endpoint days reflect either the natural death of the animal or the day of euthanasia. Criteria for euthanasia included loss of 20% or more of body weight and decreased body condition, rectal prolapse, or extreme moribund status as defined by hunched posture and minimal movement.

Corneal Mechanical Sensitivity

Corneal sensitivity was measured with the Luneau Cochet–Bonnet instrument (Western Ophthalmics, Lynnwood, WA, USA), as described previously.[26] Briefly, a nylon filament was applied to the central cornea, initially at a length of 6 cm (range, 0.5–6 cm). The step technique was used; if a specific length exhibited no response, the next lower step (meaning higher pressure) was tested until a positive response was obtained. A positive response was indicated by a clear stimulus-evoked blink or retraction of the eye into the ocular orbit. The response was considered negative when the monofilament touch elicited no blink. One eye per mouse was measured.

LG Inflammation Score

LGs were excised, fixed in 10% formalin, paraffin-embedded, and cut into 5-µm sections using a microtome (Microm HM 340E; ThermoFisher, Waltham, MA, USA), and stained with hematoxylin and eosin. Sections were graded by two masked independent investigators using a modified score described by White and Casarett,[27] changing the assigned score from 0 to 5 as done previously.[28]

RNA Isolation and Quantitative PCR

CD25KO LGs were excised, and total RNA was extracted using a QIAGEN RNeasy Plus Micro RNA isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized using the Ready-To-Go You-Prime kit (GE Healthcare, Chicago, IL, USA). Quantitative PCR (qPCR) was performed with specific minor groove binder probes for TNF (*Tnf*, Mm99999068), IL-1β (*Il1b*, Mm00434228), IFN-γ (*Ifng*, Mm00801778), CD4 (*Cd4*, Mm00442754), MHC II (*Ciita*, Mm00482914), caspase 3 (*Casp3*, Mm01195085), caspase 8 (*Casp8*, Mm00802247), caspase 9 (*Casp9*, Mm00516563), and hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*, Mm00446968). The HPRT1 gene was used as an endogenous reference. The results were analyzed by the comparative CT method and normalized by the CT value of HPRT1.

Flow Cytometry

LGs and cervical draining lymph nodes were excised and prepared as we have done previously.[28] They were stained with the antibodies anti-CD45_BV510 (clone 30-F11;

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BioLegend, San Diego, California, USA), anti-CD4_FITC (clone RM4-5; Invitrogen ThermoFisher, Waltham, Massachusettes, USA), anti-CD8a_APC (clone 53-6.7, BioLegend), anti–IL-17a_PE (clone eBio1787; Invitrogen ThermoFisher), and anti–IFN-γ_Pacific Blue (clone XMG1.2, BioLegend). Afterward, cells were incubated with an infrared fluorescent reactive live/dead dye diluted 1:32 (ThermoFisher).

The following gating strategy was used in this study: cells were identified by forward scatter area versus side scatter area; doublets were discriminated by forward scatter height versus forward scatter area (singlet 1), followed by side scatter height versus side scatter area (singlet 2); thereafter, dead cells were excluded by gating live fixable infrared dye versus side scatter; and subsequently, $CD45^+$ cells were gated and then either $CD4^+$ or $CD8^+$ cells were selected to calculate the frequency of intracellular markers IFN-γ^+, IL-17^+, or $FoxP3^+$ as described in each figure.

Negative controls consisted of fluorescence minus one combined cell suspensions from all groups. Cells were acquired with the BD Canto II Benchtop cytometer with BD Diva software version 6.7 (BD Biosciences, Franklin Lakes, New Jersey, USA). Final data were analyzed using FlowJo software version 10 (Tree Star, Ashland, OR, USA).

$CD4^+$ T-Cell Isolation and Adoptive Transfer

Single-cell suspensions were prepared from spleens and draining lymph nodes and $CD4^+$ T cells were isolated using a CD4 isolation magnetic kit (MACS system; Miltenyi Biotec, Bergisch Gladbach, Germany). Then, 2×10^6 cells were injected intraperitoneally into sex-matched 12-week-old RAG1KO mice. Five weeks posttransfer, their tissues were collected and used for histology and qPCR.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism (version 10; GraphPad Software, La Jolla, CA, USA). Nonparametric Mann–Whitney *U* tests were used to compare controls and CTSS-inhibited mice. Kaplan–Meier survival analysis was performed with the Mantel–Cox log-rank test. A $P < 0.05$ was considered significant.

Results

CTSS Inhibition Improves Cornea Sensitivity and Dacryoadenitis

We investigated if CTSS inhibition could improve signs of dry eye in CD25KO mice. Mice aged 4 weeks[29] were randomized to receive either a customized chow with CTSS inhibitor or standard chow and were evaluated at 8 weeks (Fig. 1A). We tracked the body weights of both cohorts during the diet regimen and found no significant differences in weights between groups (Supplementary Fig. S1). Prior to euthanasia, we assessed cornea sensitivity using the Cochet–Bonnet aesthesiometer. CD25KO mice receiving the CTSS inhibition diet had significantly improved sensitivity, like wild-type littermates (represented by dotted line; Fig. 1B). There was no difference in body mass between control mice and mice receiving CTSS inhibition (Fig. 1C). Mice that received the CTSS inhibitor had improved LG to body mass ratio compared to CD25KO mice on standard chow (Fig. 1D).

LGs were graded using a modified inflammation score.[27] Representative images of LG histologic sections are shown (Fig. 1E). CD25KO LGs from animals on standard chow had an average inflammation score of 4.25 (out of a total possible score of 5), indicating severe lymphocytic infiltration and destruction of healthy tissue. LGs from mice that had received the inhibitor had significantly lower inflammation scores (average 1.5) than controls (Figs. 1E, 1F).

CTSS Inhibition Reduces the Frequency of $CD4^+$ Th1 and Th17 Cells and Inflammatory Marker Expression

We then quantified the infiltrating immune cells in the LGs by flow cytometry, emphasizing T cells because $CD4^+$ Th1 and Th17 cells have been implicated in SjD.[30,31] LG suspensions were stained for CD45, CD4, CD8, IL-17, and IFN-γ. Representative dot plots are shown in Figures 2A and 2B. In mice who received standard chow, an average of 50% of alive cells in the LG were $CD45^+$, 20% of which were $CD4^+$ T cells (Fig. 2C). By contrast, mice receiving the CTSS inhibitor had significantly decreased $CD4^+$ T cells (12%) in their LGs (Fig. 2C). This decrease became starker when we investigated specific subsets of $CD4^+$ T cells. Both IFN-γ^+CD4^+ T cells (Th1) and IL-17^+CD4^+ T cells (Th17) were significantly decreased in the LGs of mice receiving the CTSS inhibitor compared to mice on standard chow (Fig. 2C). A similar decrease was observed in the eye and LG-draining cervical lymph nodes (dLNs) of the same mice (Fig. 2D).

While there was no difference between the frequency of $CD8^+$ T cells between groups, mice on CTSS inhibition had a significant decrease in IFN-γ^+CD8^+ T cells (Fig. 2C). In the dLNs, there was also a significant increase of total $CD8^+$ T cells in mice that received CTSS inhibition compared to mice on standard chow. $CD8^+$ T cells have nearly twice as much IL-2Rβ and IL-2Rγ as $CD4^+$ T cells,[32] and it has been shown previously that $CD8^+$ T cells proliferate in CD25KO mice,[33] likely due to increased exposure to IL-15.[34]

We validated these findings with qPCR in whole LG lysates. First, we verified our previously reported results investigating changes in CD25KO LGs compared to wild-type littermates. As before,[22] we found increased expression of *Ifng* and *Il1b*, in addition to increased *Tnf*, increased T-cell marker *Cd4*, and increased antigen-presenting cell marker *Ciita* (Supplementary Figs. S2A–S2C). CD25KO mice did not have significant changes in the transcripts of apoptotic markers *Casp3*, *Casp8*, or *Casp9*.

In CTSS-inhibited mice, there were no changes in the inflammatory markers *Il1b* and *Tnf* but significantly decreased expression of the inflammatory *Ifng* transcript (Figs. 3A, 3B). This is promising because exogenous treatment with IFN-γ can cause acinar cell death, and in vivo deletion of IFN-γ in the NOD and CD25KO mice ameliorates the SjD phenotype.[29,35] There was significantly decreased expression of the gene *Ciita* in CTSS-inhibited mice (Fig. 3B), suggesting that CTSS inhibition reduced MHC II presentation time. We found a significant decrease in *Casp8* in the CD25KO mice that received the inhibitor chow (Fig. 3C). *Casp8* is the marker for the first caspase in the extrinsic apoptosis pathway,[36] suggesting there is less cell death mediated through this pathway.

CTSS Inhibition Improves the Longevity of Mice

CD25KO mice often face early mortality due to systemic autoimmunity and have an average life span of 12 to 20 weeks.[37] We investigated whether systemic CTSS

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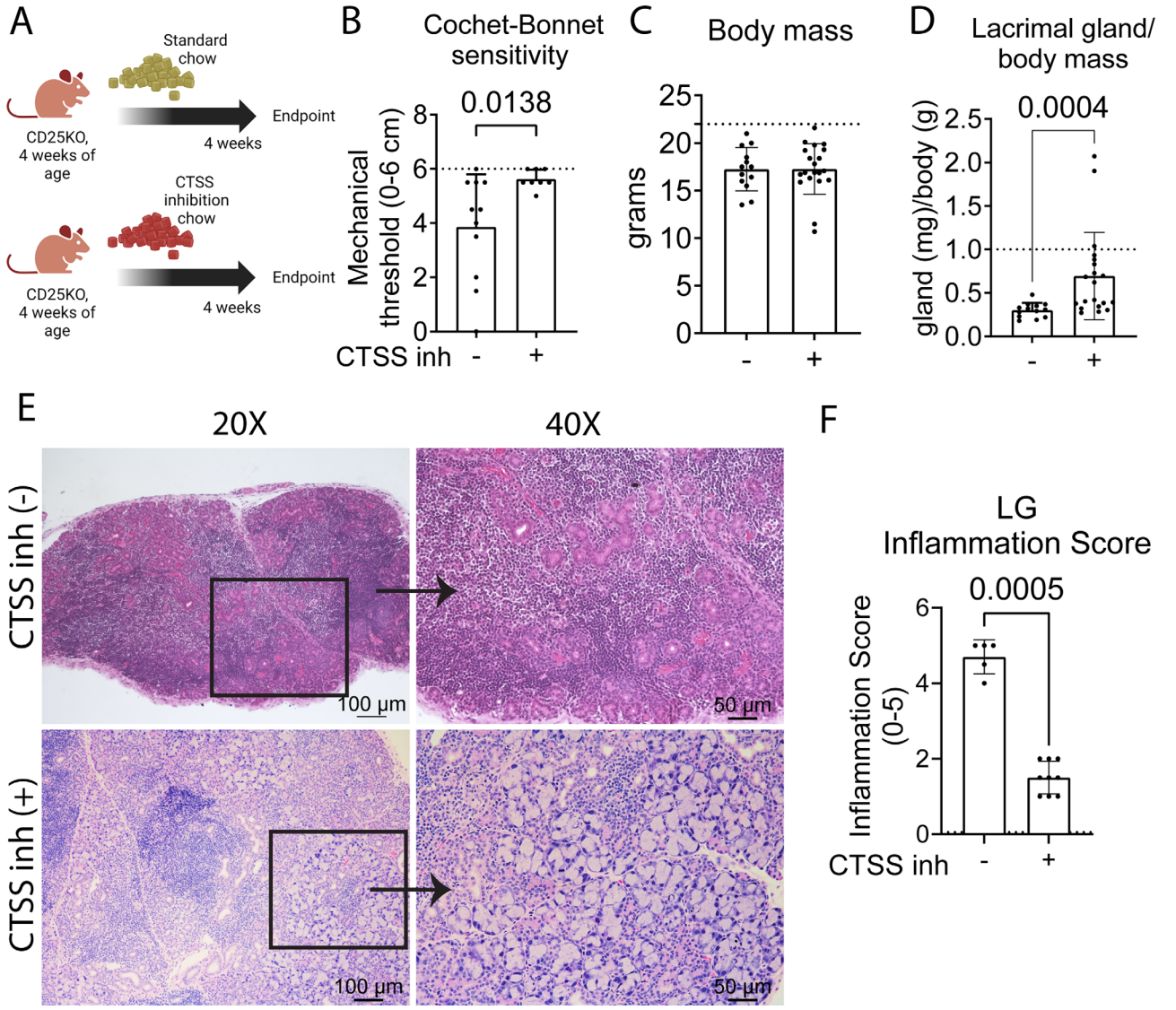

Figure 1. CTSS inhibition improves signs of dry eye in CD25KO mice. (**A**) Schematic of treatment. Created with Biorender.com. (**B**) Cornea sensitivity of mice at endpoint. The *dotted line* represents the average cornea sensitivity of wild-type littermates. Mann–Whitney *U* test. Sample size: CD25KO = 11; CTSS-inh CD25KO = 8. (**C**) Weight of mice at endpoint. The *dotted line* represents the average weight of wild-type littermates. (**D**) Normalized LG to body mass ratio. The *dotted line* represents the average normalized LG mass of wild-type littermates. Mann–Whitney *U* test. Sample size: CD25KO = 13; CTSS-inh CD25KO = 20. (**E**) Representative hematoxylin and eosin–stained LG images of CD25KO mouse on either normal (CTSS inh (−)) or inhibited (CTSS inh (+)) chow. *Scale bar* at 20×: 100 µm. *Scale bar* at 40×: 50 µm. (**F**) LG pathology assessed with modified White and Casarett inflammation score[27] as done previously.[28] The *dotted line* represents the average inflammation score of wild-type littermates (average of 0). Mann–Whitney *U* test. CTSS inh = CTSS inhibition chow. Sample size: CD25KO = 5; CTSS-inh CD25KO = 9. Each *dot* represents an independent biological sample (either mouse or LG).

inhibition could improve the life span of our model. Control mice in our study had a median survival of 82 days. In comparison, mice on the inhibitor chow lived an average of 30% longer, reaching a median survival of 113 days (Fig. 4). These observations suggest that CTSS inhibition may also decrease the immune infiltration in other organs and delay the appearance of systemic autoimmunity that can lead to death.

Adoptive Transfer of CD4$^+$ T Cells From CTSS-Inhibited Donor Mice Shows Ameliorated Disease in Recipient Mice

We then investigated the functional ability of these inhibited CD4$^+$ T cells with an adoptive transfer experiment. Previously, we showed that adoptive transfer of CD25KO CD4$^+$ T cells can cause SjD-like disease in immunocompromised recipients.[28] We isolated CD4$^+$ T cells from the spleen and dLNs of mice that either received CTSS inhibitor or standard chow and adoptively transferred 2 × 10^6 cells to female RAG1KO mice (Fig. 5A). Cornea sensitivity was evaluated 2 days prior to euthanasia. Signs of LG pathology were evaluated 5 weeks posttransfer.

Cornea sensitivity was reduced in recipients of CD4$^+$ T cells from standard chow donor mice but rescued in recipients of CD4$^+$ T cells from CTSS inhibitor-fed donor mice (Fig. 5B). LG physiology was determined with inflammation score as before (Figs. 5C, 5D). Histologic evaluation of LGs of recipient mice of CD4$^+$ T cells without CTSS inhibition showed extensive immune infiltration, resulting in large foci of immune cells and acinar cell

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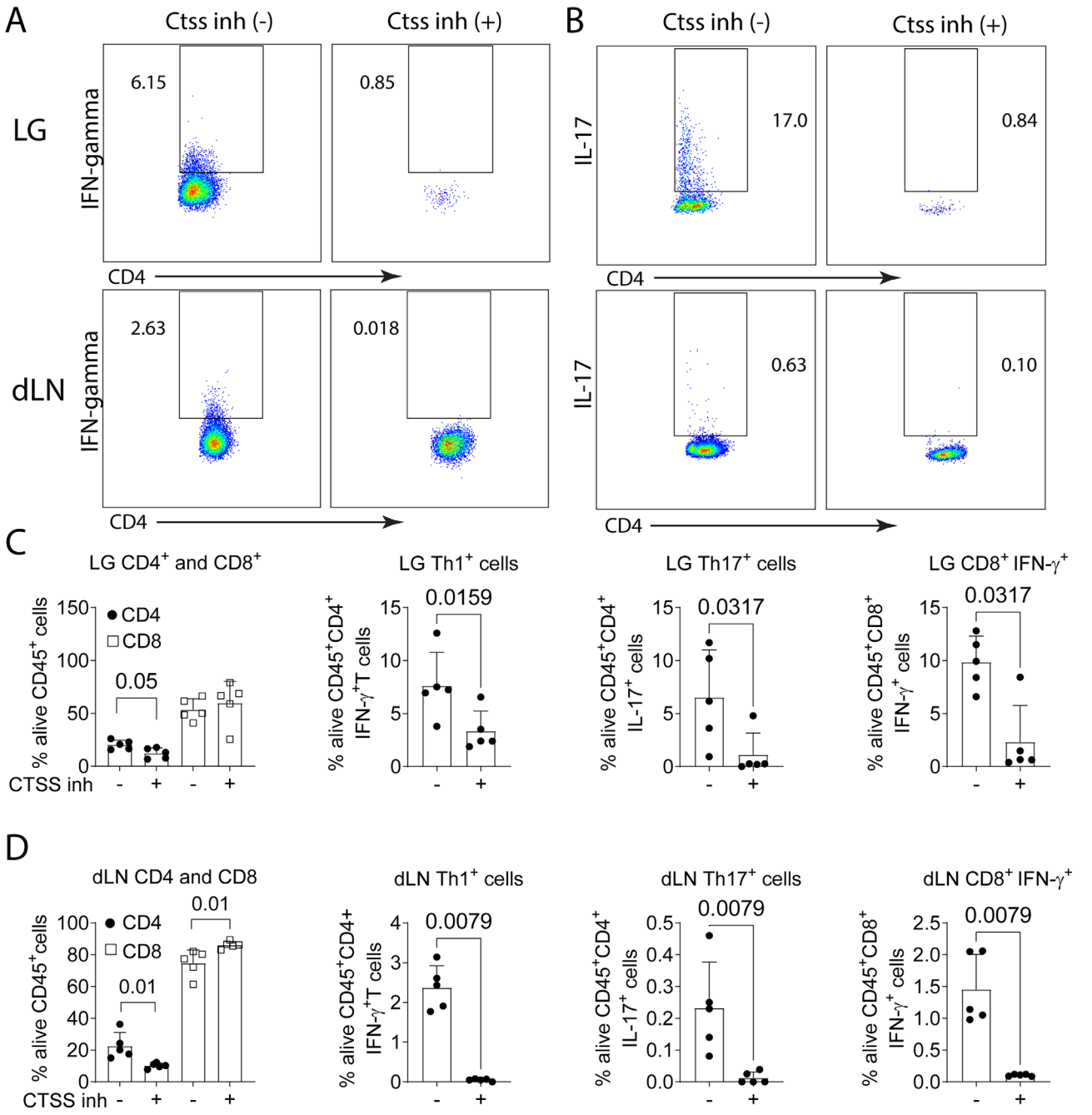

Figure 2. CTSS inhibition decreased Th1, Th17, and IFN-γ^+ $CD8^+$ T cells in the LG and ocular draining lymph nodes. (**A**) Representative gating scheme for LG and ocular dLN staining for IFN-γ in control (CTSS inh (−)) and inhibited (CTSS inh (+)) CD25KO mice. (**B**) Representative dot plots for LG and ocular draining lymph nodes staining for IL-17 in control (CTSS inh (−)) and inhibited (CTSS inh (+)) CD25KO mice. (**C**) Cumulative data of $CD4^+$, $CD8^+$, Th1, and Th17 cells in LGs of control (CTSS inh (−)) and inhibited (CTSS inh (+)) CD25KO mice. (**D**) Cumulative data of $CD4^+$, $CD8^+$, Th1, and Th17 cells in draining ocular lymph nodes of control (CTSS inh (−)) and inhibited (CTSS inh (+)) CD25KO mice. Mann–Whitney *U* test. KO = CD25KO. CTSS inh = CTSS inhibition chow. Sample size: $n = 5$/group.

death (Fig. 5C). However, recipients of T cells from CTSS-treated mice showed minimal mononuclear lymphocytic infiltration and a significantly lower inflammation score (Fig. 5C).

Since we observed improved LG physiology, we also investigated inflammatory marker expression with qPCR (Fig. 6). Recipients of untreated $CD4^+$ T cells had increased expression of *Il1b*, *Tnf*, *Ifng*, and *Ciita* compared to untouched RAG1KO mice (Supplementary Figs. S3A–S3C). While there were few changes, there was a significant decrease in the apoptotic marker caspase 8 and a significant increase in caspase 9 in CTSS inhibitor-treated recipient mice compared to recipients of untreated $CD4^+$ T cells (Fig. 6C).

Discussion

Patients with SjD have a reduced quality of life. Due to sicca symptoms, patients have blurry vision, difficulty swallowing,

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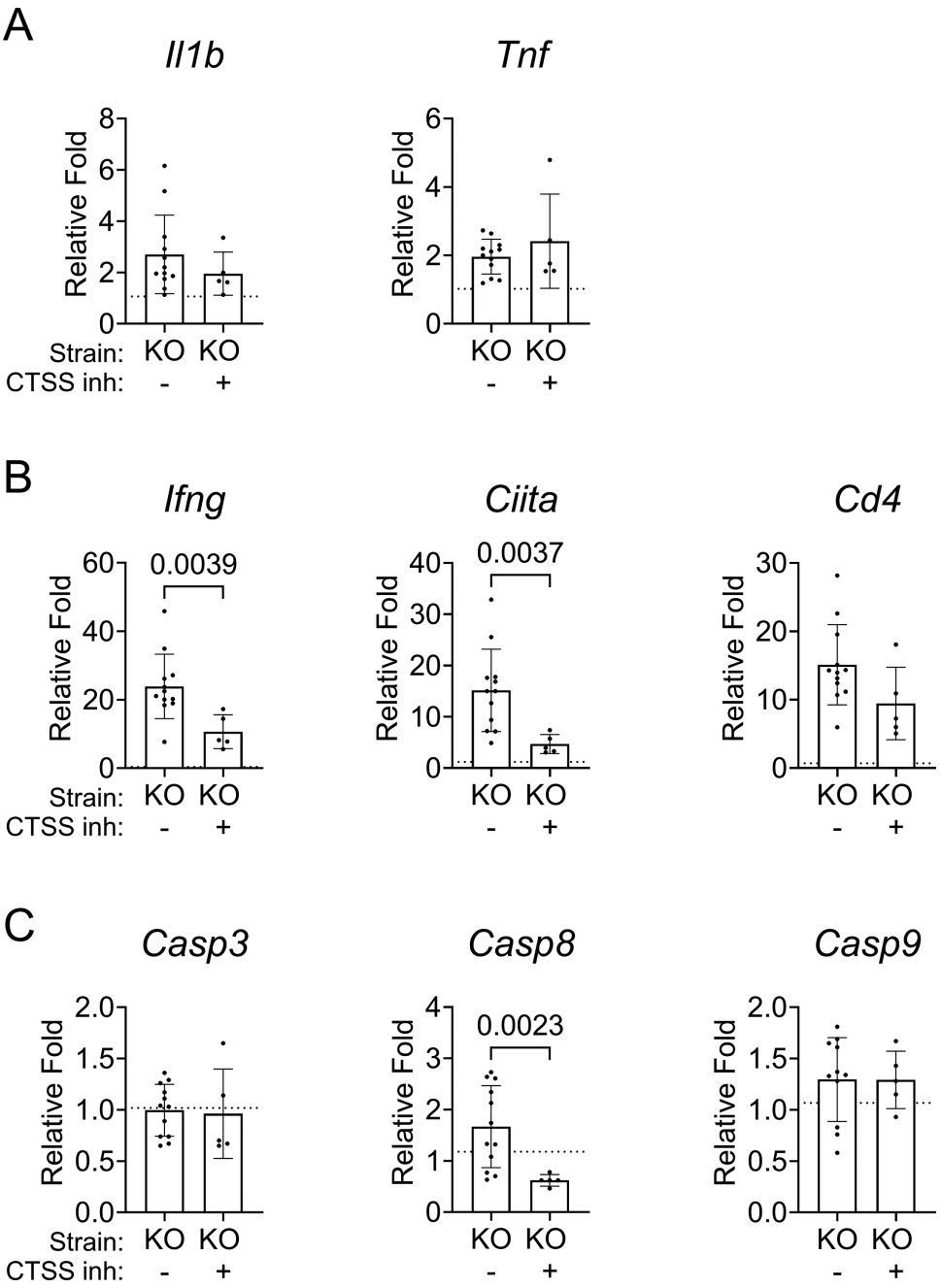

Figure 3. CTSS inhibition reduced expression of genetic markers *Ifng*, *Ciita*, and *Casp8* in the LG. (**A–C**) qPCR of broad inflammatory markers *Il1b* and *Tnf* (**A**); T-cell-related markers *Ifng*, *Ciita*, and *Cd4* (**B**); and apoptosis-related markers *Casp3*, *Casp8*, and *Casp9* (**C**). Mann–Whitney *U* test. The *dotted line* represents wild-type littermates' average expression. Wild-type littermates served as calibrators for each reaction. KO = CD25KO. CTSS inh = CTSS inhibition chow. Sample size: KO = 12; CTSS-inh KO = 5. Each *dot* represents one LG per mouse. *P* value as shown.

significant pain, fatigue, and increased rates of insomnia.[38] Therefore, it is important to find therapeutics for SjD that can address these symptoms. This study showed that CTSS inhibition reduced the pathogenic capacity of autoreactive T cells, resulting in improved cornea sensitivity, less lymphocytic infiltration to the LG, a reduction in the number of Th1 and Th17 cells, less production of inflammatory and apoptotic mRNA transcripts, and less disease progression in the cornea and LG of our adoptive transfer recipients. Finally, we showed an extension in the life of our model with the CTSS inhibitor. This study highlights one potential therapeutic for treating SjD and demonstrates its use in a systemic autoimmune mouse model.

CTSS inhibition improved cornea sensitivity in CD25KO mice. Patients with SjD have decreased cornea sensitivity despite increased ocular irritation symptoms.[39–41] One hypothesis to explain this incompatibility in signs and symptoms is that the corneal nerves become neuropathic.[42] We have shown that CD25KO mice have decreased corneal innervation and decreased mechanosensitivity.[25]

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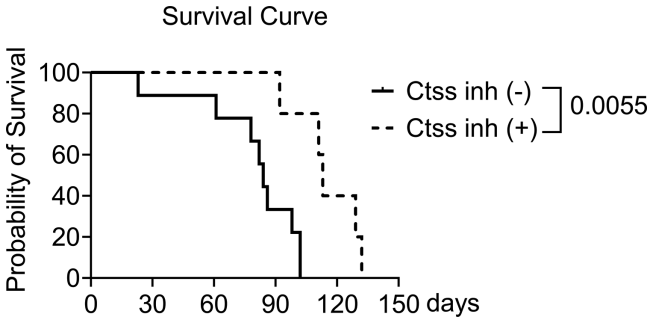

Figure 4. CTSS inhibition improves survival of CD25KO mice. Kaplan–Meier survival analysis, Mantel–Cox test. Ctss inh (−) = CD25KO mice receiving standard chow. Ctss inh (+) = CD25KO receiving CTSS inhibition chow. Sample size: standard chow CD25KO = 9; CTSS inhibition chow CD25KO = 5.

The improvement in cornea sensitivity suggests an improvement in the corneal nerve health of CTSS inhibitor-recipient mice. While to our knowledge, there have been no studies published yet that have described CTSS inhibition improving corneal nerves, there is some indirect evidence. A study investigating neuropathic orofacial pain in male rats found that a CTSS inhibitor improved signs of hyperalgesia.[43] Work from our laboratory has shown that exogenous administration of CTSS to corneal epithelial cells breaks tight junction proteins in vitro and breaks the corneal barrier in vivo,[44] which is important for preserving corneal nerves. Similarly, exogenous addition of CTSS to human corneal epithelial cell cultures resulted in increased expression of the protein protease-activated receptor 2, which can induce neurogenic inflammation and participate in pain signaling.[45,46]

Likewise, CTSS inhibition improved inflammation scores in the LGs of CD25KO mice. Lymphocytic infiltrates of the exocrine glands are a hallmark of SjD. They are found in patient's minor SG and LG biopsy specimens[10,11,47] and result in the sicca symptoms that cause patient discomfort. This infiltration to the exocrine glands is recapitulated in our murine model of SjD.[22,29] Here, we described how systemic administration of CTSS inhibition not only reduced lymphocytic infiltration but also resulted in improved health of the remaining LG tissue, likely by reducing the pathogenicity of the autoreactive $CD4^+$ T cells. Furthermore, the CTSS inhibitor chow reduced the number of Th1 and Th17 cells in the LG lymphocytic infiltrates. Th1 and Th17 cells are present in labial SG and LG biopsy specimens of patients with SjD and play a pathogenic role in the pathogenesis of the disease.[30] The presence of Th1 cells in labial SG biopsy specimens is associated with disease progression.[47] Furthermore, patients with SjD have unique Th1 and Th17

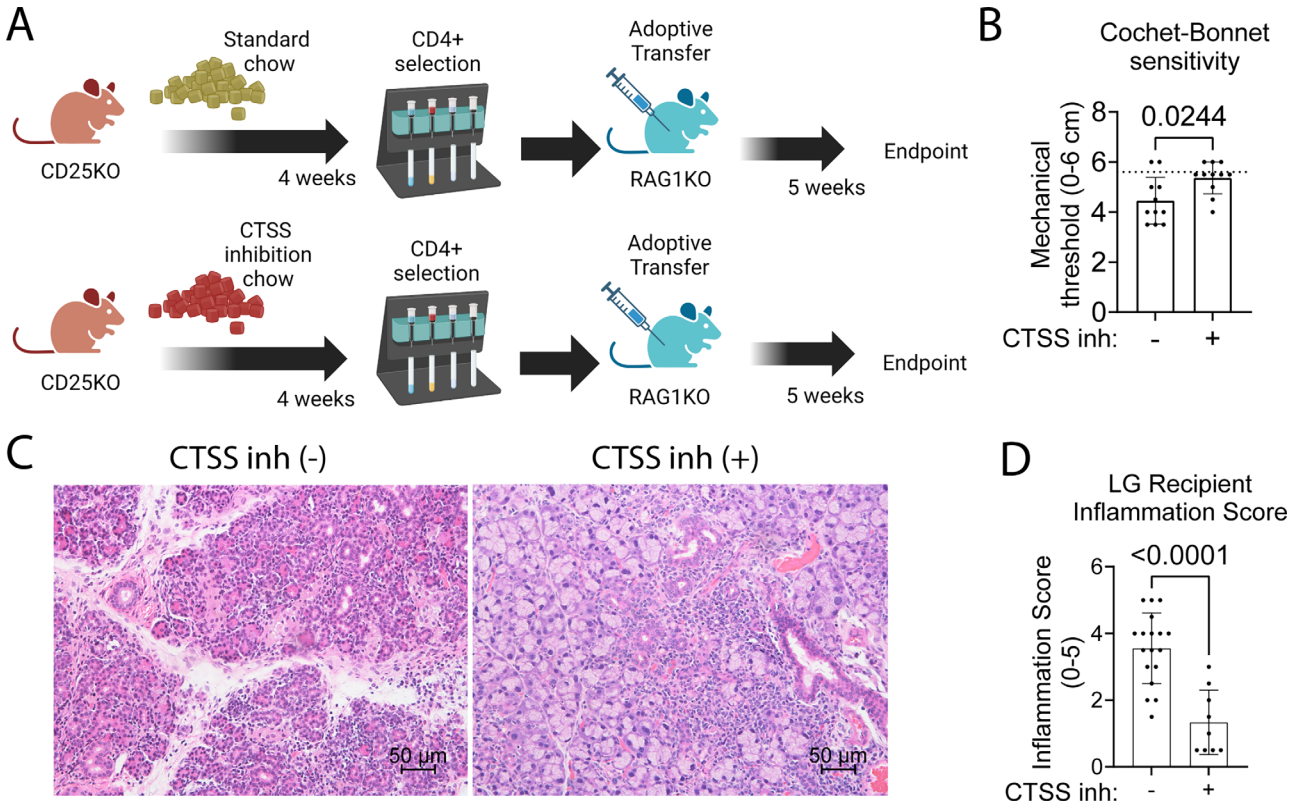

Figure 5. CTSS inhibition-treated $CD4^+$ T cells have reduced pathogenicity in RAG1KO adoptive transfer recipients. (**A**) Schematic of adoptive transfer experiment. Created with Biorender.com. (**B**) Cornea sensitivity of mice at endpoint. The *dotted line* represents average cornea sensitivity of wild-type littermates. Mann–Whitney *U* test. Sample size: Standard chow recipient LGs = 11, CTSS inhibition chow recipient LGs = 11. (**C**) Representative hematoxylin and eosin–stained LG images of RAG1KO mouse receiving adoptive transfer of normal CD25KO (CTSS inh (−)) or inhibited CD25KO (CTSS inh (+)) $CD4^+$ T cells. (**D**) RAG1KO mouse recipient LG pathology assessed with modified White and Casarett inflammation score[27] as done previously.[28] CTSS inh – = $CD4^+$ T cells from CD25KO mice on standard chow adoptively transferred to RAG1KO mice. CTSS inh + = $CD4^+$ T cells from CD25KO mice on CTSS inhibition chow adoptively transferred to RAG1KO mice. Mann–Whitney *U* test. Sample size: Standard chow recipient LGs = 18, CTSS inhibition chow recipient LGs = 9. Each *dot* represents one biological sample.

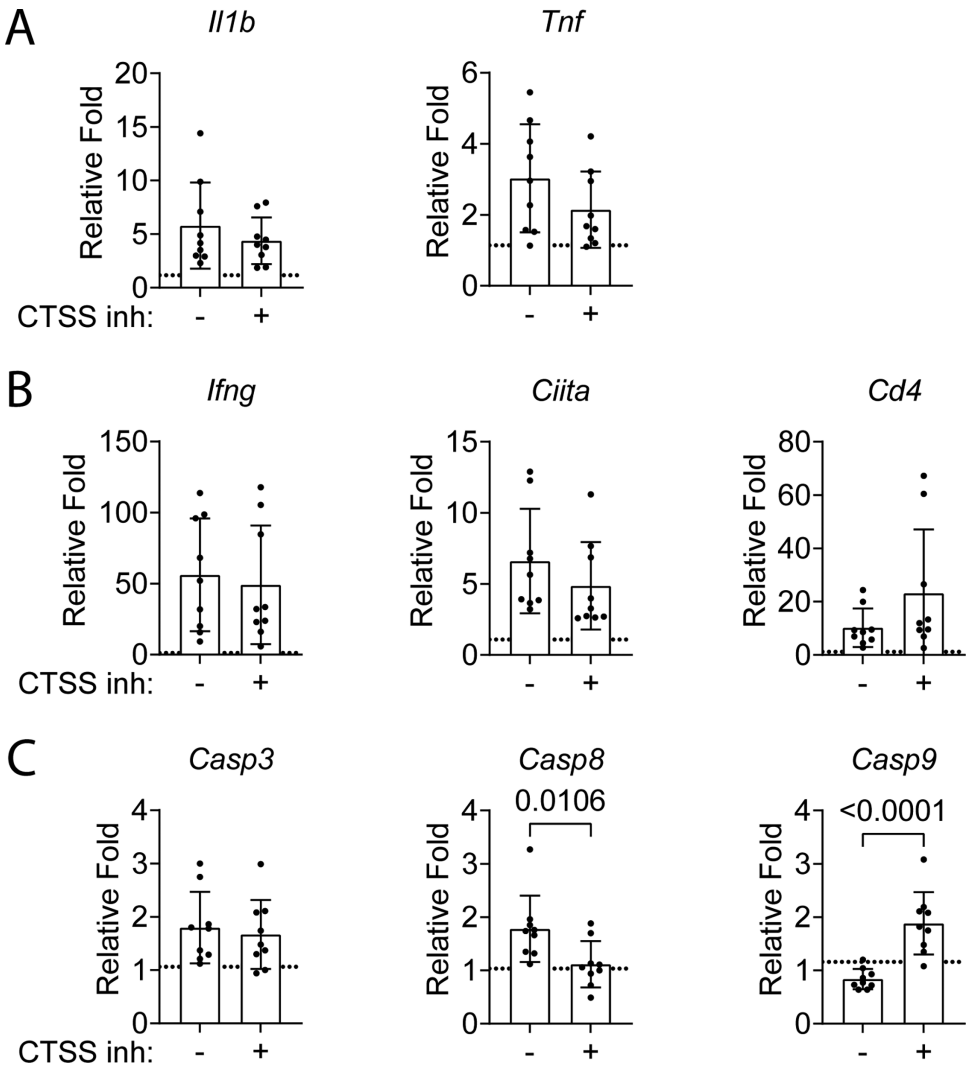

Figure 6. CTSS inhibition-treated CD4$^+$ T cells result in differential expression of apoptotic markers. qPCR of broad inflammatory markers (**A**) *Il1b* and *Tnf*; T-cell- and B-cell-related markers (**B**) *Ifng*, *Ciita*, and *Cd4*; and apoptosis-related markers (**C**) *Casp3*, *Casp8*, and *Casp9*. Mann–Whitney *U* test. Naive RAG1KO mice were used as calibrators for each qPCR reaction and the *dotted line* represents their average expression for each marker. CTSS inh = CTSS inhibition chow given to donor mice. Sample size: RAG1KO + adoptive transfer standard chow CD4$^+$ cells = 9; RAG1KO + adoptive transfer CTSS inhibition treated CD4$^+$ T cells = 9.

cell receptors and restricted clonal expansion,[48] implying a role of T-cell pathology in the disease. SjD-like symptoms in the NOD mouse LG were ablated after the removal of IFN-γ, inhibiting Th1 cells.[35] We also showed similar results in the CD25KO mouse after genetic deletion of IFN-γ.[29] Our findings that the CTSS inhibition significantly reduced the number of Th1 and Th17 cells agree with previous literature[15,26] and shed light on the role of T cells in SjD.

Treatment also reduced mRNA transcript expression of cytokines associated with Th1 cells, antigen-presenting cells, and apoptosis. Th1 cytokines have been associated with disease severity in tears and saliva of patients with SjD[49] and mice.[29] IFN-γ is can cause apoptosis in cultures of human SG cells and conjunctiva cells,[50,51] and treating LG acinar cells with IFN-γ in vitro resulted in increased genetic expression of CTSS and a disease-like phenotype.[52] The downregulation of *Ifng* seen in our model concurs with these findings. The downregulation of *Ciita* and *Casp8* could suggest a reduction in autoreactive T-cell-mediated apoptosis of self-tissues. Aberrant apoptosis of epithelial cells was found to induce SjD-like autoimmune disease,[53] and inhibition of apoptosis in a murine model of SjD was sufficient to reduce the presence of lymphocytic infiltration.[54] This suggests that excessive apoptosis furthers disease progression in SjD, and CTSS inhibition appears to help stem this excess. Taken together, our qPCR data helps validate our flow cytometry and LG histology data.

Of note is the ability of CTSS inhibition to extend the life of CD25KO mice. As reported previously, CD25KO mice have early mortality due to their systemic autoimmunity. Fifty percent of mice die by 9 weeks and the rest by 20 weeks.[37] We found that ad libitum administration of CTSS inhibitor to female CD25KO mice starting at 4 weeks of age dampened inflammation and autoreactivity enough to extend their life span by 30%. Females are more likely to develop autoimmune diseases than males; in SjD, in particular, the prevalence of the disease is greater in women than in men by a ratio of 9:1.[24] This underscores the impor-

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tance of finding therapeutics specifically working in female patients.

Interestingly, our results demonstrate that the number of infiltrating immune cells to the LG is not only reduced, but their capacity for autoreactivity is also diminished. In RAG1KO recipients, there was a decrease in inflammation score and caspase 8 mRNA transcript. This implies that CTSS inhibition-treated T cells are less pathological. Therefore, interfering with MHC II presentation decreases autoimmunity and may break the vicious cycle of inflammation driven by T cells.

Our results did not completely stem the autoimmunity of the mouse, suggesting there is compensation by the immune system. Likewise, a recent phase II clinical trial investigating a different CTSS inhibitor, RO5459072, did not identify improvement in EULAR Sjögren's Syndrome disease activity index (ESSDAI) scores, unstimulated tear production, or stimulated salivary flow in a cohort of patients with established primary SjD.[55] However, this does not necessarily invalidate all forms of CTSS inhibition for treatment, like the inhibitor we used, RO5461111. First, the parameters of the study investigated only one dosage for 12 weeks, and it may be that there is greater efficacy in humans at a different dosage or time. The study reported a nonsignificant decrease in B and T cells and less of a decline in unstimulated tear production in the treatment arm. Because SjD is a highly variable disease, it may be that the efficacy of CTSS inhibition is more useful in a subset of patients. The presence of Th1 and Th17 cells, for instance, in labial SG biopsy specimens is more prevalent in nongerminal centers in the tissue,[47] suggesting these cells may be more prominent in the earlier stages of SjD. We investigated mice from the ages of 4 to 8 weeks, which is when autoimmunity begins in the CD25KO mouse. By 8 weeks of age, the CD25KO LG is atrophied and largely devoid of healthy acinar cells. Our study thus investigated the prevention of the onset of disease and did not investigate if treatment with CTSS inhibition could reverse signs once already established. Therefore, treatment with CTSS inhibition may be more potent in earlier stages of SjD. Whether CTSS inhibition can reverse already established autoimmunity is an exciting avenue for future research.

A limitation of our study was that by administering the CTSS inhibitor through the diet, we could not ensure that each mouse received the same amount of drug. To address this, we tracked the weight of a subset of mice for the duration of the regimen and found no significant differences in weight between groups. This suggests that the mice were eating approximately the same amount of food and received a similar amount of the inhibitor. Furthermore, we did not investigate whether the improvement in LG pathology resulted in improving the quality of the tear film. This is a promising avenue for future research.

Overall, our findings suggest that CTSS inhibition is a tenable option for stemming autoreactivity in the eyes and LGs of patients with SjD.

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