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ARTICLE

Alteration in nerves and neurotransmitter stimulation of lacrimal gland secretion in the TSP-1$^{-/-}$ mouse model of aqueous deficiency dry eye

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The purpose of this study is to determine neural, vascular, protein secretion, and cellular signaling changes with disease progression in lacrimal glands of the thrombospondin-1$^{-/-}$ (TSP-1$^{-/-}$) mouse model of dry eye compared to C57BL/6 wild-type (WT) mice. Neural innervation was reduced in TSP-1$^{-/-}$ lacrimal glands compared to WT controls, whereas the number of blood vessels was increased. Intracellular Ca^{2+} stores and the amount of lysosomes, mitochondria, and secretory granules, but not the endoplasmic reticulum, were reduced in TSP-1$^{-/-}$ compared to WT acini at 12 weeks of age. Ex vivo high KCl-evoked secretion was decreased in TSP-1$^{-/-}$ compared to WT lacrimal gland tissue pieces. The α_{1D}-adrenergic agonist-stimulated response was increased in TSP-1$^{-/-}$ at 4 and 24 weeks but decreased at 12 weeks, and the ATP and MeSATP-stimulated peak $[Ca^{2+}]_i$ responses were decreased at 24 weeks. These changes were observed prior to the appearance of mononuclear infiltrates. We conclude that in the lacrimal gland the absence of TSP-1: injures peripheral nerves; blocks efferent nerve activation; decreases protein secretion; and alters intracellular Ca^{2+} stores. Through these effects the absence of TSP-1 leads to disruption of ocular surface homeostasis and development of dry eye.

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INTRODUCTION

Dry eye is a multifactorial disease, which affects over 40 million Americans. Most of the currently available treatments and therapies for dry eye are restricted to palliative care and are limited in number.[1–3] Previous literature has implicated lacrimal gland dysfunction as the primary cause of aqueous deficiency dry eye (ADDE).[4–6] One form of ADDE is Sjögren's syndrome, an autoimmune disease that occurs predominantly in females that represent 90% of Sjögren's syndrome patients.[7] The dysfunction in ADDE includes an alteration in protein, electrolyte and water secretion from lacrimal glands and has also been linked to ocular surface inflammation commonly diagnosed in dry eye patients.[8] In this study, we characterized lacrimal gland structural and functional changes in a novel mouse model of ADDE, the thrombospondin-1 null (TSP-1$^{-/-}$) mouse, which was previously reported to mimic Sjögren's-associated chronic dry eye progression as seen in humans with Sjögren's-associated ADDE.[9,10]

Lacrimal gland secretion is regulated predominantly by a neural reflex in which stimuli on the ocular surface activate afferent sensory nerves. These activated nerves by a complex neural reflex stimulate both efferent parasympathetic and sympathetic nerves. The efferent nerves release their neurotransmitters acetylcholine, vasoactive intestinal peptide (VIP), and norepinephrine that interact with their receptors, muscarinic 3 acetylcholine receptors (M_3AchR), VIP receptors (VPACR), and α_{1D} adrenergic receptor (α_{1D}AR), respectively, on acinar and duct cells. Activation of these receptors stimulates a variety of calcium handling mechanisms and other signaling pathways to work in a tightly regulated fashion to produce lacrimal gland protein secretion.[11] These proteins produced along with isotonic electrolyte and water secretion constitute the lacrimal gland primary fluid,[12] which undergoes further modification by ductal cell secretion before being released onto the cornea and conjunctiva to help form the aqueous component of tears.[13]

The two major signaling pathways that regulate protein secretion by the lacrimal gland are the cholinergic (via muscarinic M_3AchR) and adrenergic (via α_{1D}-ADR) pathways.[5] Acetylcholine activates M_3AchRs, which are coupled to phospholipase Cβ (PLCβ). PLCβ activation produces the protein kinase C (PKC) activator diacylglycerol and inositol 1,3,5-trisphosphate (InsP3).[14] InsP3 increases the intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) using thapsigargin sensitive intracellular Ca^{2+} stores that, along with the activation of PKCα, −δ, and −ε, stimulate the secretion of protein stored in preformed secretory granules.[14,15] Stimulation of M_3AchRs also activates extracellular-regulated kinase (ERK) 1/2 and phospholipase D, which attenuate secretion.[16,17] Norepinephrine activates α_{1D}-AR, which causes an increase in $[Ca^{2+}]_i$ by a mechanism that is not yet determined but does not involve generation of InsP3 or use of thapsigargin-dependent cellular Ca^{2+} stores.[18] α_{1D}-ARs also transactivate the epidermal growth factor (EGF) receptor to increase ERK1/2 activity, which attenuates secretion.[19,20] VIP interacts with VPAC1 to stimulate the secretion by increasing cellular levels of cAMP and increasing $[Ca^{2+}]_i$.[21] Increasing $[Ca^{2+}]_i$ is a signaling mechanism in common to the activation of these three

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receptors to stimulate protein exocytosis and secretion. Both increase in $[Ca^{2+}]_i$ and protein secretion can serve as markers of lacrimal gland function. Dysfunction of these cellular signaling pathways potentially can disrupt secretory function of lacrimal gland and contribute to the development of dry eye disease.

In addition, protein secretion into the tears by the lacrimal gland can also occur by a non-adrenergic non-cholinergic pathway (NANC). VIP, Substance P (Sub P) and purinergic receptors (P2X and P2Y) constitute some of the NANC candidates.[22] In the lacrimal gland, activation of P2X3 and P2X7 receptors increases $[Ca^{2+}]_i$ and stimulates protein secretion.[23] P2Y receptors play a role in the function of myoepithelial cells that surround the acinar cells.[24] Thus, abnormalities in protein secretion activated by the stimulation of non-canonical receptors in the lacrimal gland could also be involved in dry eye disease progression.

TSP-1 is a matricellular protein that activates latent transforming growth factor.[25] TSP-1 regulates extracellular and intracellular signaling complexes as it interacts with cell surface receptors, growth factors, cytokines, and extracellular matrix proteins. This activates many functions within the cell including cell migration, proliferation, and cell death.[26] The use of TSP-$1^{-/-}$ mice as a model for ADDE was recently validated by Turpie et al.[10] in male mice. In TSP-$1^{-/-}$ mice, they detected progressively exacerbated infiltration of the lacrimal glands, with inflammatory infiltrates containing predominantly CD4 T cells, as typically reported in autoimmune Sjögren's syndrome. Alterations in lacrimal gland protein secretion were observed as early as 8 weeks of age in TSP-$1^{-/-}$ mice, whereas mononuclear infiltrates were not seen in the lacrimal glands until these mice reached 24 weeks of age.[10] Therefore, unlike other mouse models where inflammation is indistinguishable as a cause or effect, our murine model exhibits destructive inflammatory responses preceded by distinct glandular dysfunction. We previously found that female TSP-$1^{-/-}$ mouse lacrimal glands also have minimal disease at 4 weeks of age, but extensive disease at 12 and 24 weeks of age.[27] Inflammation in female mouse lacrimal glands is similar to that in males. In addition, female TSP-$1^{-/-}$ mice have altered the expression of cytokines interleukin (IL)-1β, IL-6, Th17, and IFNγ, cellular morphology, progenitor cell markers and a decrease in cell proliferation.[27]

In the present study, we investigated potential aberrations in signaling pathways associated with lacrimal gland function at multiple levels in female TSP-$1^{-/-}$ compared to wild-type (WT) mice. We found that in the TSP$^{-/-}$ lacrimal gland peripheral nerves are damaged and efferent nerve activation is blocked leading to decreased protein secretion. Through these changes the absence of TSP-1 leads to disruption of ocular surface homeostasis and development of dry eye.

RESULTS

Neural innervation (parasympathetic, sympathetic, and sensory) is decreased in TSP-1-deficient lacrimal glands compared to WT glands

Lacrimal glands are innervated by sensory (Sub P and CGRP containing), parasympathetic (acetylcholine and VIP containing), and sympathetic (norepinephrine containing) nerves. To determine whether the secretory defect detected in *TSP-1*$^{-/-}$ glands is related to their innervation, we used confocal microscopy to visualize innervation patterns in TSP-1-deficient lacrimal glands prior to (4 weeks), during (12 weeks), and after the development of inflammation (24 weeks) compared to lacrimal glands from age-matched WT mice. The results obtained from analysis of TUJ-1 (a neural marker that indicates all nerve types) expression show substantially decreased innervation in 4-week-old, 12-week-old, and 24-week-old TSP-$1^{-/-}$ mice when compared to age-matched WT acini (Fig. 1).

To specifically determine the effect of TSP-1 deficiency on the extent of parasympathetic innervation, we used anti-VIP antibodies

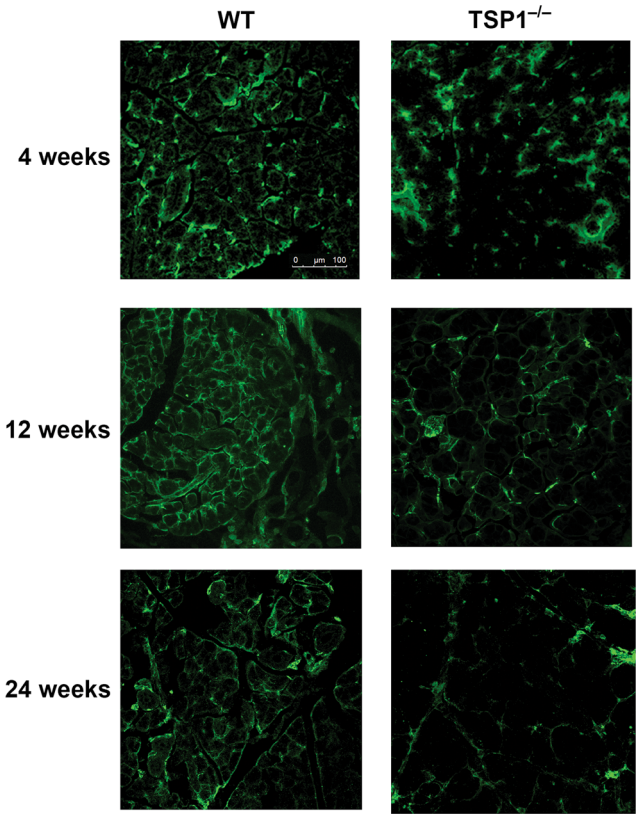

Fig. 1 Innervation is decreased with age in TSP-$1^{-/-}$ compared to WT mice. Lacrimal glands of 4-week-old, 12-week-old, and 24-week-old female WT (left) and TSP-$1^{-/-}$ (right) mice were fixed, sectioned, and stained with anti-TUJ-1 antibody (green) that indicates sympathetic, parasympathetic, and sensory nerves. Representative micrographs taken with confocal microscopy are shown from three independent animals of each age.

in the following set of experiments. As shown in Fig. 2, a decrease in parasympathetic nerves was detected in 4-week-old, 12-week-old, and 24-week-old TSP-$1^{-/-}$ lacrimal acini when compared to WT controls. As parasympathetic nerves are the major nerves in the lacrimal gland, it is not surprising that a similar change was detected in the staining by both anti-TUJ-1 and VIP antibodies.

These findings suggest that TSP-1 is important for nerve development or maintenance, and the disruption of neural connections from an early age prior to development of inflammation might be a contributing factor in the development of dry eye in TSP-1 deficient mice.

Increased blood vessels are detected in TSP-1-deficient lacrimal glands compared to WT glands

As lacrimal gland morphological changes were seen in 12-week-old TSP-$1^{-/-}$ mice,[27] the total area of blood vessels in lacrimal glands from 12-week-old mice was determined. Blood vessels were detected with anti-VE-cadherin (Fig. 3a) or anti-PECAM (Fig. 3b) antibody. When VE-cadherin was used, the amount of blood vessels was 174,159 ± 6062 μm^2 in TSP-$1^{-/-}$ mouse glands, a value significantly ($p = 0.00004$) increased from that in WT glands, which was 88,386 ± 3444 μm^2 (Fig. 3c). Similarly, when PECAM was used, the amount of blood vessels was 221,985 ± 8001 μm^2 in TSP-$1^{-/-}$ mouse glands, a value significantly ($p = 0.014$) increased from that in WT glands, which was 173,452 ± 5287 μm^2 (Fig. 3d). These results support the detection of significantly increased blood vessels in TSP-1-deficient lacrimal glands, which is in accordance with the antiangiogenic activity of TSP-1 previously described in the literature and serves as a positive control.[28]

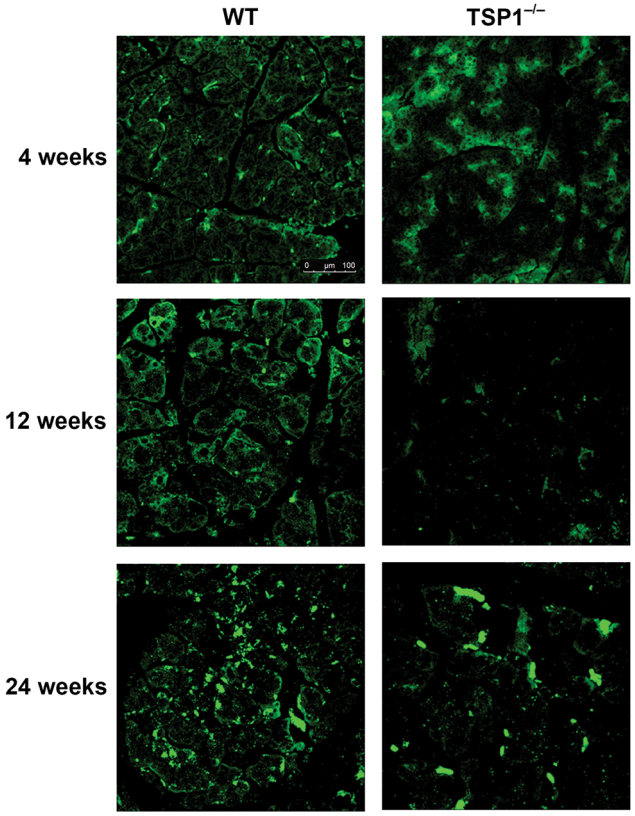

Fig. 2 Parasympathetic Innervation is Decreased with Age in Lacrimal Glands in TSP-1$^{-/-}$ Compared to WT Mice. Lacrimal glands of 4-week-old, 12-week-old, and 24-week-old female WT (left) and TSP-1$^{-/-}$ (right) mice were fixed, sectioned, and stained with anti-VIP antibody (green) that indicates parasympathetic nerves. Micrographs taken with confocal microscopy are representative of three independent animals of each age.

Neural stimulation of protein secretion is decreased in TSP-1-deficient lacrimal glands compared to WT glands

Upon depolarization, nerve endings release neurotransmitters which bind to the membrane surface receptors located on lacrimal gland acinar cells,[5] leading to initiation of signaling cascades resulting in a rise in $[Ca^{2+}]_i$ and protein secretion. We next determined whether disrupted neural connections in TSP-1-deficient lacrimal glands lead to abnormalities in their protein secretion in an ex vivo experiment. To depolarize nerves we incubated lacrimal gland tissue pieces that contained functioning efferent nerve endings in buffer containing 4 mM basal KCl or 75 mM high KCl, and measured protein secretion (Fig. 4a–c). As expected, high KCl-stimulated secretion was significantly increased over basal KCl in WT lacrimal gland tissues derived from both 12-week-old (Fig. 4b) and 24-week-old (Fig. 4c) mice. However, similar stimulation in lacrimal gland tissues derived from age-matched TSP-1$^{-/-}$ mice was significantly reduced 83.9% ($p = 0.026$) and 294.2% ($p = 0.0037$) as compared to WT lacrimal gland tissue. In addition, at 24 weeks, high KCl-stimulated secretion was significantly reduced from secretion with basal KCl ($p = 0.007$). Thus, in TSP-1$^{-/-}$ lacrimal glands a decrease in nerves or their function corresponds to a decrease in neural-stimulated protein secretion.

TSP-1 deficiency alters structure of lacrimal gland acinar cells

Consistent with their abnormal innervation, which was observed at 12 weeks of age, we also detected altered morphology of lacrimal gland acini in TSP-1-deficient lacrimal gland. Lacrimal gland sections derived from 12-week-old mice were stained with hematoxylin and eosin (H&E) and examined under higher-power magnification. As shown in Fig. 5, TSP-1$^{-/-}$ acini look visibly altered in that they appear coarser and contain more vacuoles compared to WT acini. This result suggests that an absence of TSP-1 could affect the expression of intracellular organelles thereby manifesting a distinct cellular morphology in TSP-1$^{-/-}$ mice.

To assess quantitative changes in intracellular organelles including endoplasmic reticulum (ER), lysosomes, mitochondria, and secretory granules in TSP-1$^{-/-}$ compared to WT lacrimal gland acini from 12-week-old mice, we performed western blot analysis (Fig. 6). The amount of ER, critical in protein synthesis and in regulation of the $[Ca^{2+}]_i$, was determined using anti-calnexin antibody (Fig. 6a). However, the amount of these organelles was not significantly different in TSP-1$^{-/-}$ compared to WT lacrimal glands. Lysosomes are responsible for degradation and recycling of proteins to maintain cell homeostasis. Lysosomes can play a role in regulation of $[Ca^{2+}]_i$ as they can take up Ca^{2+} released from the ER through multiple Ca^{2+} channels.[29,30] The amount of Lamp1 protein, a lysosomal marker, was significantly decreased by 74.3% in TSP-1$^{-/-}$ compared to WT lacrimal glands (Fig. 6b). Mitochondria play a role in regulating the basal $[Ca^{2+}]_i$, in cell oxidative stress, and in cell death. The amount of mitochondria, determined with anti-cox IV antibodies, was significantly decreased by 68.3% in TSP-1$^{-/-}$ compared to WT lacrimal glands (Fig. 6c). Finally, lacrimal gland protein secretion is dependent on protein exocytosis. Rab3D marks secretory vesicles that fuse to the apical membrane and release their cargo (secretory proteins) into the lacrimal gland duct system. The amount of Rab3D protein was significantly decreased 53.2% in TSP-1$^{-/-}$ compared to WT lacrimal glands (Fig. 6d).

Our results show comparable expression of ER and a significant reduction in markers for expression of lysosomes, mitochondria, and secretory vesicles in TSP-1$^{-/-}$ compared to WT lacrimal glands. These data suggest a reduction of select intracellular organelles in TSP-1$^{-/-}$ lacrimal gland acini and might contribute toward an altered morphology and function of TSP-1$^{-/-}$ lacrimal glands.

Intracellular Ca^{2+} signaling is altered in TSP-1$^{-/-}$ compared to WT lacrimal gland acini

The reduction in organelles associated with protein secretion in TSP-1-deficient acini prompted us to assess $Ca^{2+}{}_i$ signaling that is integral part of lacrimal gland protein and fluid secretion. Any aberrations in Ca^{2+}-handling mechanisms might directly affect lacrimal gland protein secretion. Therefore, we first determined the concentration dependency of the rise in $[Ca^{2+}]_i$ evoked by both parasympathetic and sympathetic agonists, major stimuli of secretion, in lacrimal gland acini from 24-week-old WT mice, an age in which the lymphocytic infiltration is extensive. In addition, we also determined Ca^{2+} increase evoked by NANC candidates: ATP, an activator of purinergic (P) P2X receptors, and 2MeSATP, an activator of P2Y receptors.

Carbachol, a M3AchR agonist, increased the $[Ca^{2+}]_i$ in a concentration-dependent manner with a maximum $[Ca^{2+}]_i$ of 1216.05 ± 76.81 nM at 10^{-4} M (Fig. 7a). The α_{1D}-adrenergic agonist phenylephrine also increased $[Ca^{2+}]_i$ in a concentration-dependent manner with a maximum increase of 712.27 ± 202.58 nM at 10^{-4} M (Fig. 7b). ATP and 2MeSATP each increased $[Ca^{2+}]_i$, but a maximum response was not obtained (Fig. 7c, d). The highest response was 437.02 ± 54.90 nM at 10^{-3} M ATP and 314.72 ± 42.33 nM at 10^{-3} M 2MeSATP. We used the concentration of agonist which gave the maximal or highest Ca^{2+} response for subsequent experiments.

We next compared the peak agonist-stimulated rise in $[Ca^{2+}]_i$ in TSP-1$^{-/-}$ versus WT lacrimal gland acini at 4, 12, and 24 weeks of age (Fig. 8). When carbachol was used at 10^{-4} M, the increase in $[Ca^{2+}]_i$ was not different between TSP-1$^{-/-}$ and WT acini at any age measured (Fig. 8a). In contrast, we found that the phenylephrine (Ph)-stimulated increase in $[Ca^{2+}]_i$ was affected in all three age groups (Fig. 8b). In WT mice, a time-dependent increase in Ph-stimulated increase in $[Ca^{2+}]_i$ was observed in WT mice, with

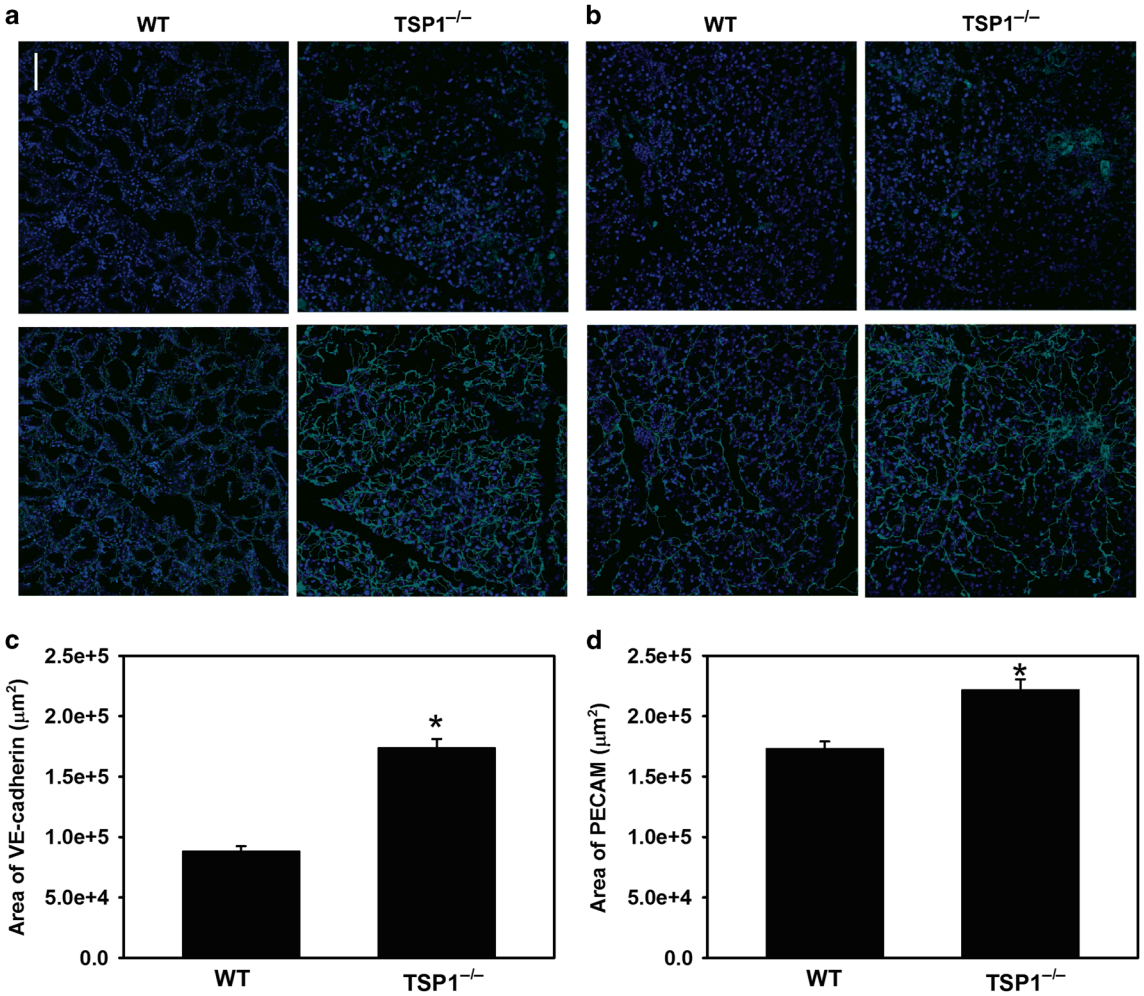

Fig. 3 Area of VE-cadherin and PECAM is decreased in lacrimal glands in TSP-1$^{-/-}$ compared to WT mice. Female WT and TSP-1$^{-/-}$ mouse lacrimal glands were removed at 12 weeks of age, fixed, and stained with VE-cadherin (**a**) and PECAM (**b**) antibodies to study blood vessels. Representative micrographs are shown in **a** and **b**. Quantification of positive staining was performed and the total area was calculated. Data are mean ± SEM from four independent animals and shown in **c** and **d**. Asterisks indicates a significant difference from WT

maximum increase occurring at 12 weeks, likely due to the maturation of the mice. In TSP-1$^{-/-}$, the increase in $[Ca^{2+}]_i$ does not change with age. The α_{1D}-adrenergic pathway-mediated increase in $[Ca^{2+}]_i$ at 12 weeks was significantly diminished by 57.9% in TSP-1$^{-/-}$ lacrimal acini in contrast to the other two age groups (4 and 24 weeks), where the stimulated $[Ca^{2+}]_i$ was significantly enhanced in TSP-1$^{-/-}$ by 223.4% and 91.9%, respectively, when compared to the $[Ca^{2+}]_i$ responses in WT acini.

Finally, in mice at 4 and 12 weeks of age, both of the purinergic receptor (P2X and P2Y) mediated stimulation of the $[Ca^{2+}]_i$ was unchanged, but at 24 weeks of age P2X and P2Y responses were both significantly diminished by 63.5% for ATP and 82.2% for 2MeSATP in TSP-1$^{-/-}$ compared to WT acini (Fig. 8c, d). Although our findings show different agonist-specific alterations in the change in peak $[Ca^{2+}]_i$, α_{1D}-adrenergic, P2X, and P2Y agonist-mediated changes in $[Ca^{2+}]_i$ all were altered in TSP-1$^{-/-}$ compared to WT acini at the oldest age measured, indicating alterations in lacrimal glands of TSP-1$^{-/-}$ mice and possible defective cellular Ca^{2+} handling with disease progression.

Protein secretion is affected in TSP-1$^{-/-}$ when compared to WT lacrimal gland acini

To determine whether changes in peak $[Ca^{2+}]_i$ correspond with protein secretion, we compared ex vivo stimulation of lacrimal glands with M_3AchR, α_{1D}-adrenergic, P2X, and P2Y agonists. We measured protein secretion in TSP-1$^{-/-}$ versus WT lacrimal gland pieces at 4, 12, and 24 weeks of age (Fig. 9). We found that in WT mice, carbachol-evoked secretion increased, though not significantly ($p = 0.07$, power 0.36) at 4 weeks and was significantly increased at 12 and 24 weeks ($p = 0.05$ and $p = 0.03$, respectively). There was no significant difference in TSP-1$^{-/-}$ compared to WT mice, at 4 weeks ($p = 0.14$, power 0.33), but was significantly diminished by 93.9% ($p = 0.05$) and 139.0% ($p = 0.013$) in 12-week-old and 24-week-old TSP-1$^{-/-}$ compared to WT pieces (Fig. 9a). In contrast to carbachol-stimulated, phenylephrine-stimulated, and ATP-stimulated secretion were not significantly different between the three ages in TSP-1$^{-/-}$ compared to WT lacrimal gland pieces with powers ranging from 0.07 to 0.4. (Fig. 9b, c). Maximum secretion in response to ATP and 2MeSATP was observed in 12-week-old WT mice (Fig. 9c, d). 2MeSATP stimulation of protein secretion was not significantly different between the two strains at 4-week-old or 24-week-old. However, secretion was significantly diminished by 86.4% ($p = 0.002$), in 12-week-old TSP-1$^{-/-}$ compared to WT pieces (Fig. 9d). Secretion from TSP-1-1$^{-/-}$ mice was reduced compared to secretion from WT mice regardless of the stimuli.

TSP-1$^{-/-}$ mouse lacrimal gland acini exhibit decreased intracellular Ca^{2+} stores compared to WT acini

Protein secretion experiments demonstrated a significant decrease in M3AchR protein secretion in 12-week-old and

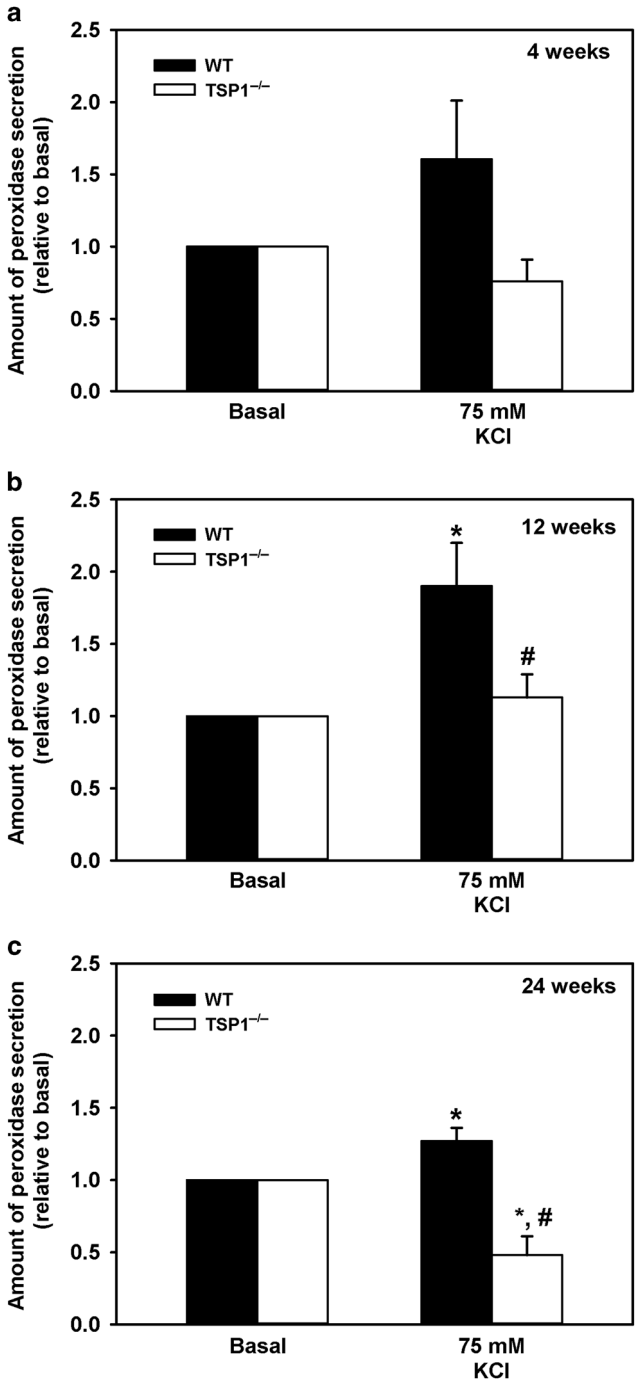

Fig. 4 High KCl Induced Secretion is Decreased with Age in in Lacrimal Glands in TSP-$1^{-/-}$ Compared to WT Mice. Lacrimal gland pieces were stimulated with normal 4 mM KCl (basal) and high 75 mM KCl and protein secretion was measured in 4-week-old (**a**), 12-week-old (**b**), and 24-week-old (**c**) mice. Data are mean ± SEM from at least three independent animals for each condition. Asterisks indicates statistically significant different from basal; hash indicates a significant difference from WT

24-week-old TSP-$1^{-/-}$ compared to WT mouse lacrimal glands. As carbachol, the M3AchR agonist used in the present study primarily evokes protein exocytosis via ER-associated Ca^{2+} release stimulated by IP_3R, we determined whether thapsigargin-releasable intracellular Ca^{2+} stores, most likely located in the ER, were altered in TSP-$1^{-/-}$ compared to WT acini. The Ca^{2+} response to thapsigargin indicates the size of the intracellular Ca^{2+} store.

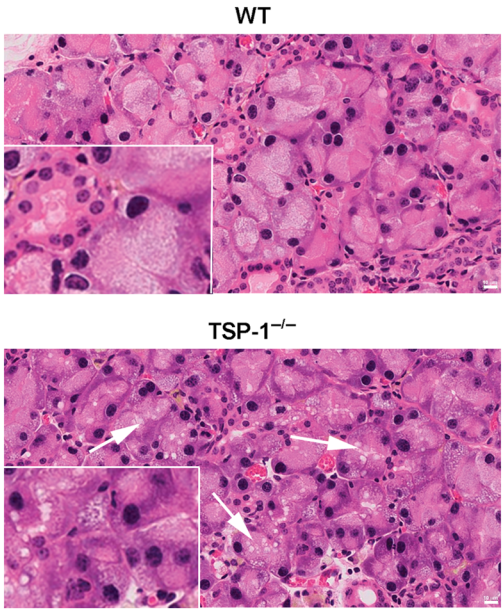

Fig. 5 Structural changes of lacrimal glands in TSP-$1^{-/-}$ compared to WT mice. Lacrimal glands from 12-week-old WT and TSP-$1^{-/-}$ mice were isolated, fixed, and stained with hematoxylin and eosin. High-power magnification shows altered acinar cell morphology in TSP-$1^{-/-}$ mice. Arrows indicate vacuoles inside of cytoplasm of TSP-$1^{-/-}$ acinar cells. The scale bar is 10 microns. Micrographs are representative of three independent animals

When Ca^{2+} is introduced in the bathing solution, this results in refilling of ER stores. The rate of the Ca^{2+} change indicated the rate of influx of extracellular Ca^{2+}. We detected a significant decrease in peak Ca^{2+} amplitude and area under the curve during thapsigargin exposure to lacrimal gland acini in 24-week-old TSP-$1^{-/-}$ compared to WT acini (Fig. 10). There was a 72.1% decrease in peak response (Fig. 10a) and a 74.8% decrease in area under the curve (Fig. 10b) in TSP-$1^{-/-}$ compared to WT acini. Thus, this finding can be interpreted as a reduction in ER store Ca^{2+} content in lacrimal acini of 24-week-old TSP-$1^{-/-}$ when compared to WT mouse acini. In contrast, the rate of refilling of ER stores by extracellular Ca^{2+} influx was unchanged between the two groups (Fig. 10c). Therefore, even though the store operated Ca^{2+} entry mechanism and ER as organelle remains unaltered at 24 weeks in TSP-1-deficient lacrimal gland acini compared to WT acini, the total Ca^{2+} content in the ER stores is drastically depleted and could account for the decrease in cholinergic agonist-stimulated protein secretion.

DISCUSSION

Lacrimal gland dysfunction leading to dry eye has been widely associated with inflammation. Under specific pathophysiological settings, a number of studies have shown upregulation of proinflammatory cytokines, chemokines, and metalloproteinases.[6] This in turn leads to a rapid expansion of autoreactive T helper cells which then migrate to the lacrimal gland tissue and cause inflammation and tissue atrophy.[31] In TSP-$1^{-/-}$ mouse lacrimal glands there is an age-dependent influx of lymphocytes and proinflammatory cytokines that can alter lacrimal gland structure and function leading to decrease secretion and ocular surface disease.

The major mechanism by which lacrimal gland and tear production is increased is by the activation of afferent sensory nerves in the ocular surface that by a reflex activate efferent nerves that release neurotransmitters. The released neurotransmitters interact with their receptors on lacrimal gland cells to

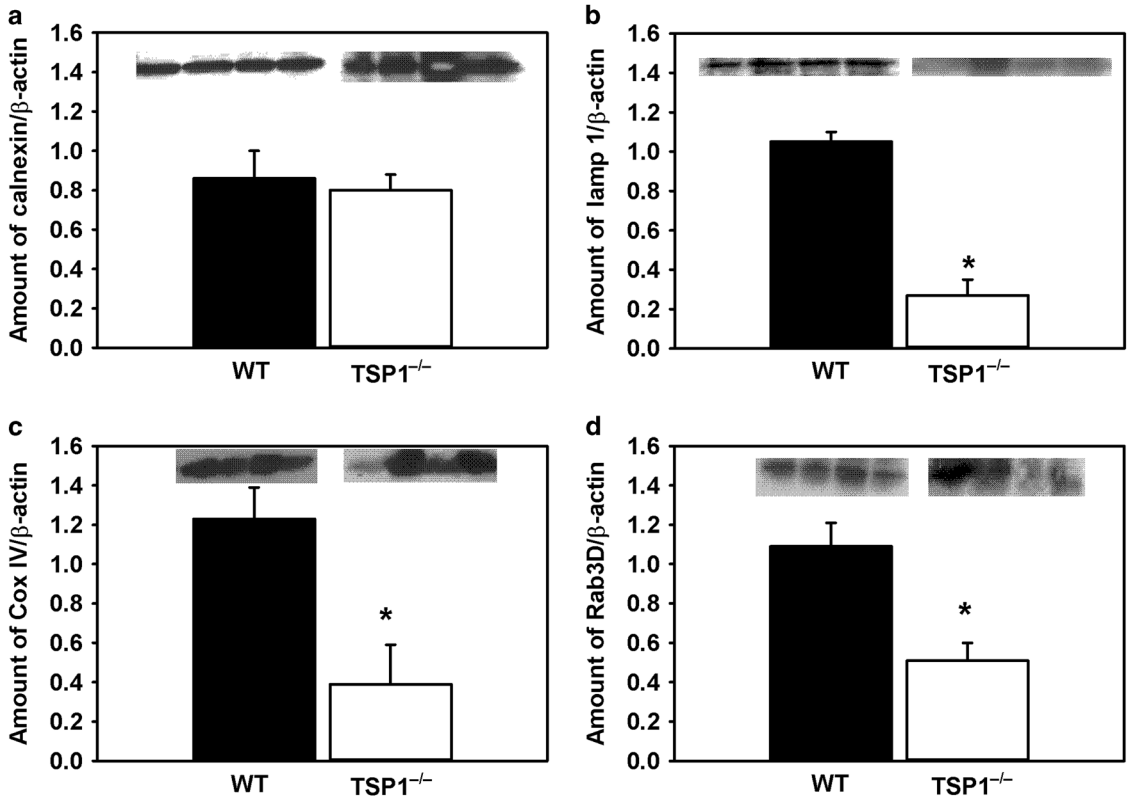

Fig. 6 Amount of organelle-related protein is decreased in lacrimal glands in TSP-1$^{-/-}$ compared to WT mice. The amount of calnexin (**a**), Lamp1 (**b**), Cox IV (**c**), Rab3D (**d**), and β-actin was measured by western blot in lacrimal gland samples from 12-week-old WT and TSP-1$^{-/-}$ mice. Western blots are shown as insets where each lane is a separate animal. The amount of each organelle-related protein was standardized to β-actin (not shown). Data are mean ± SEM from four independent animals. Asterisks indicates significance from WT expression

stimulate secretion, both protein and electrolyte/water. A decrease in total nerves (measured by anti-TUJ-1 antibody) and in parasympathetic nerves (measured by anti-VIP antibody) occurred by the 4 week time point in TSP-1$^{-/-}$ compared to WT lacrimal glands and continued at 12 and 24 weeks of age, when there is also a decrease in protein secretion stimulated by activating the nerves. Thus neural regulation of protein secretion is drastically altered in TSP-1$^{-/-}$ compared to WT lacrimal glands especially at older ages.

Zoukhri and Kublin[32] showed that high KCl stimulation of nerves in pieces of lacrimal gland releases the neurotransmitter acetylcholine from parasympathetic nerves of WT mice. The parasympathomimetic/cholinergic agonist-stimulated signaling pathway is one of the major pathways for lacrimal gland and tear secretion. The decrease in parasympathomimetic agonist (carbachol)-stimulated protein secretion that occurred in mouse lacrimal glands at 12 and 24 weeks of age in the present study is consistent with a major effect of lack of TSP-1 on the parasympathetic agonist-stimulated protein secretion at both the neural and the acinar cell level. These data suggest that TSP-1$^{-/-}$ compared to WT mouse lacrimal glands have a defect in both the nerve/neurotransmitter release mechanism as well as in the signaling pathways activated by parasympathomimetic agonists in acinar cells. Of interest is our finding that neurally mediated protein secretion was decreased at 4 weeks of age, but exogenous agonist-induced secretion was not affected until later in disease progression. This finding suggests that nerves are most sensitive to the loss of TSP-1 and that the neurotransmitter receptors and the signaling pathways they activate in the acinar cells are less sensitive and unchanged until the disease progresses. These results are different from those found in the lacrimal glands of MRL/Mp-Fas-lpr/lpr (MRL/lpr) mice, a murine model for Sjögren's syndrome, where the efferent nerve density was not altered, but the high KCl-stimulated acetylcholine release was impaired.[33] In contrast to the TSP-1$^{-/-}$ model, denervation supersensitivity occurred in the MRL/lpr model, as the acinar cell responses to neurotransmitters were increased compared to control animals.[34] Thus, a different process affects nerves, but not neurotransmitter activation of acinar cells in the TSP-1$^{-/-}$ compared to the MRL/lpr model. As IL-1β is elevated in both of these models, we propose that in the TSP-1$^{-/-}$ lacrimal gland one of the structural domains of TSP-1 rather than the increase in cytokines could be affecting M_3AchR agonist stimulation of secretion.

Cholinergic agonist-stimulated protein secretion is dependent upon an increase in the $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ also directly participates in exocytosis of secretory granules that release secretory proteins. Most of the canonical Ca^{2+} signaling pathways in lacrimal glands are IP_3 mediated and utilize internal ER Ca^{2+} stores for increasing Ca^{2+} signals leading to lacrimal gland protein secretion. Although differences in ER expression could directly affect cellular Ca^{2+} handling, the amount of ER is not altered in TSP-1$^{-/-}$ compared to WT lacrimal glands. Mitochondria also play a vital role in calcium homeostasis by acting as a buffer for any spontaneous rises in $[Ca^{2+}]_i$ that overwhelm the capacity of the ER stores.[35] Mitochondria are also the cellular "powerhouse" and impart energy to carry out cellular functions. In addition, Kawashima et al.[36] demonstrated mitochondrial damage in the lacrimal glands of patients diagnosed with Sjögren's syndrome. The downregulation of a mitochondrial marker (Cox IV) suggests that mitochondrial expression is affected in the TSP-1$^{-/-}$ mouse lacrimal glands thereby hampering Ca^{2+} buffering in these cells. Moreover, an effect of mitochondrial oxidative damage on secretory output can also not be ruled out. A change in the amount of secretory granules could affect protein secretion.

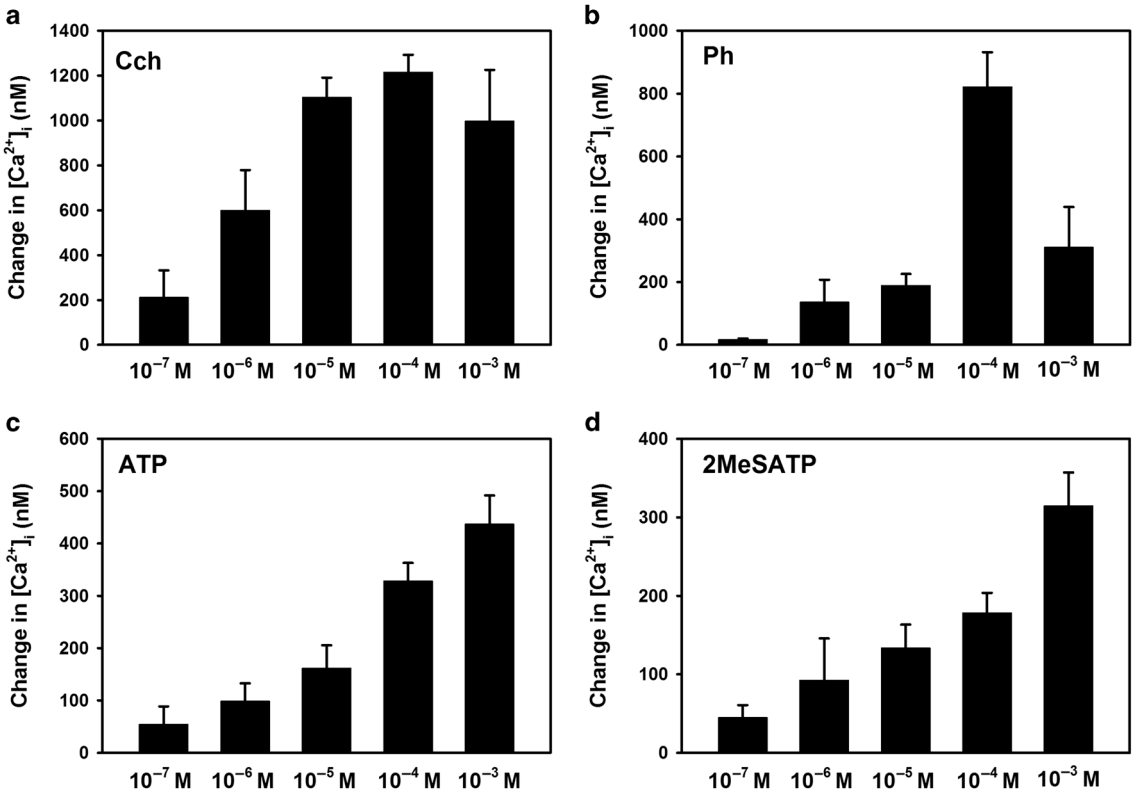

Fig. 7 Concentration dependency of the $[Ca^{2+}]_i$ response in WT lacrimal gland acini. Lacrimal gland acinar cells from 24-week-old WT mice were treated with increasing doses of carbachol (Cch) (**a**), phenylephrine (Ph) (**b**), ATP (**c**), and 2MeSATP (**d**), and the change in peak $[Ca^{2+}]_i$ was measured. Data are mean ± SEM from three independent animals

Decreased expression of Rab3D suggests that this pool of secretory vesicles might not be available for stimulation of secretion. As protein secretion from lacrimal acini is dependent on the intracellular Ca^{2+} stimulus and availability of secretory vesicles, a decrease in secretory granules could directly affect exocytosis and protein secretion.

There are conflicting changes in the use of intracellular Ca^{2+} by cholinergic agonists to stimulate protein secretion in TSP-$1^{-/-}$ compared to WT lacrimal gland acini. Consistent with a decrease in the Ca^{2+}-dependent signaling pathway used by cholinergic agonists in TSP-$1^{-/-}$ mice is a decrease in the thapsigargin-releasable intracellular Ca^{2+} stores, amount of mitochondria, amount secretory vesicles, and a decrease in protein secretion. Inconsistent with this signaling pathway in TSP-$1^{-/-}$ mouse lacrimal glands is no change in the carbachol-induced $[Ca^{2+}]_i$ and in the amount of ER thought to be the site of the thapsigargin-releasable intracellular Ca^{2+} stores. To explain the lack of change in the carbachol-induced $[Ca^{2+}]_i$ that we expected to lower with decreasing protein secretion is the possibility that there could be a decrease in Ca^{2+}-independent signaling pathways used by cholinergic agonists such as the activation of the Ca^{2+}-independent PKC isoform PKCε that is known to stimulate cholinergic agonist protein secretion.[37] Another possibility is that there could be an increase in the inhibitory pathways activated by cholinergic agonists in the lacrimal gland including activation of phospholipase D and ERK1/2[20]. A final possibility is that the rapid increase in $[Ca^{2+}]_i$ that occurs for only minutes does not correlate with the longer term depletion of thapsigargin-releasable intracellular Ca^{2+} stores that occurs over 15 min or with secretion that is measured over 40 min. Thus, use of intracellular Ca^{2+} stores over the 40 min stimulation of secretion could deplete the decreased stores in the TSP-$1^{-/-}$, but not in the normal stores of the WT mouse lacrimal gland. M3AchR agonist-stimulated secretion is dependent on intracellular Ca^{2+} and influx of extracellular Ca^{2+} when these are depleted for the 40 min stimulation of secretion.[18,38,39] To explain the lack of change in the amount of ER with decreasing intracellular Ca^{2+} stores, we suggest that that only a specialized portion of the ER could be involved in the intracellular Ca^{2+} stores or that the proteins that regulate the $[Ca^{2+}]_i$ such as Ca^{2+} ATPase, STIM1, and Orai could be changed in TSP-$1^{-/-}$ compared to WT lacrimal gland acini. Supporting this latter possibility is that the type II and III repeats of TSP-1 bind about 30 Ca^{2+} and that TSP-1 is located inside the ER.[40] Lack of TSP-1 inside the ER and a decrease in Ca^{2+} binding could account for the depletion of intracellular Ca^{2+} stores that we found in the TSP-$1^{-/-}$ mouse lacrimal glands and for the decrease in M_3AchR-stimulated protein secretion. Further studies on protein binding, direct/indirect associations and cellular location of TSP-1 need to be done to specifically determine the different roles of TSP-1 in modulation lacrimal gland function.

The lacrimal gland is also innervated by sympathetic nerves and activation of these nerves releases norepinephrine that interacts with α_{1D}-adrenergic receptors to stimulate protein secretion. Although the sympathetic innervation to the WT mouse lacrimal gland is less dense than the parasympathetic one, sympathomimetic agonists such as the α_{1D}-adrenergic agonist phenylephrine cause an effective if not a more effective increase in secretion than parasympathomimetic ones. In contrast, the increase in agonist-stimulated $[Ca^{2+}]_i$ is less for phenylephrine than for carbachol. Furthermore, the changes in α_{1D}-adrenergic agonist-induced increase in $[Ca^{2+}]_i$ and protein secretion in TSP-$1^{-/-}$ compared to WT lacrimal glands were different when compared to those stimulated by M_3AChR agonists. In WT mice, phenylephrine-induced $[Ca^{2+}]_i$ was increased at 12 weeks of age compared to 4 weeks before declining at 24 weeks. This could be due to the normal maturation process. In TSP-$1^{-/-}$ compared to WT mouse

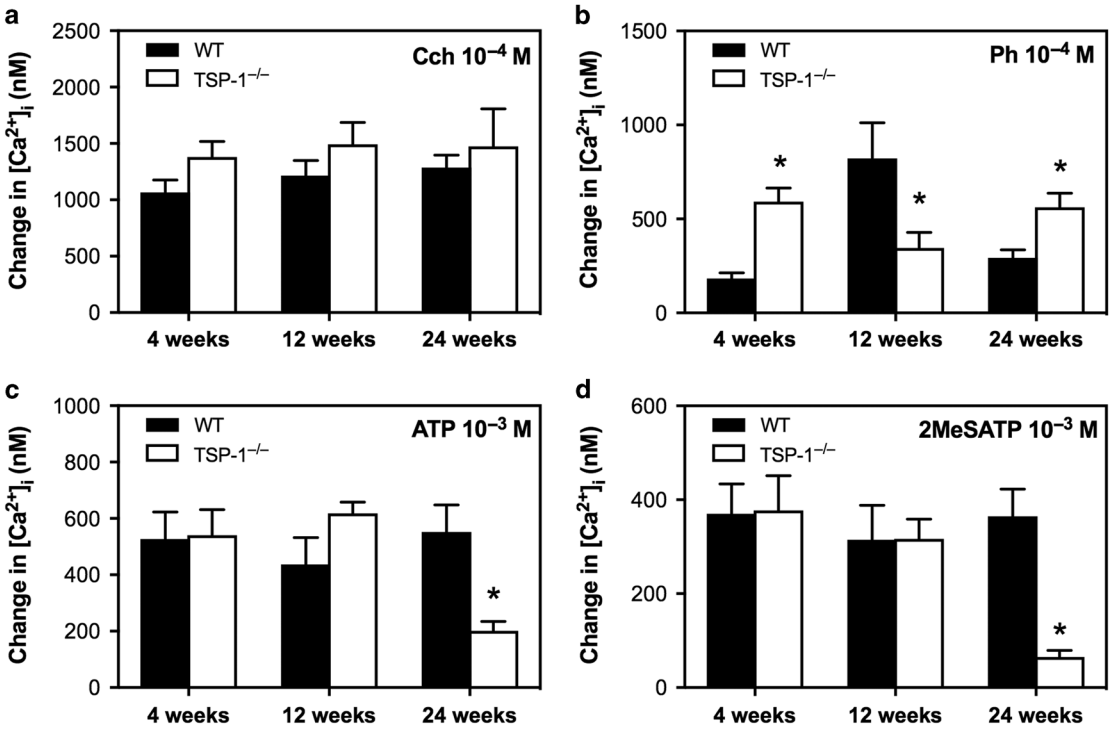

Fig. 8 Increase in $[Ca^{2+}]_i$ mediated by carbachol, phenylephrine, ATP, and 2MeSATP in TSP-$1^{-/-}$ compared to WT mice. Lacrimal gland acini were isolated and the change in $[Ca^{2+}]_i$ mediated by carbachol (Cch) 10^{-4} M (**a**), phenylephrine (Ph) 10^{-4} M (**b**), ATP 10^{-3} M (**c**), and 2MeSATP 10^{-3} M (**d**) was studied in 4-week-old, 12-week-old, and 24-week-old WT and TSP-$1^{-/-}$ mice. Asterisks indicates significance from WT response at the same age. Data are mean ± SEM from three independent animals

glands phenylephrine-induced $[Ca^{2+}]_i$ was increased at 4 weeks, decreased at 12 weeks, and increased at 24 weeks of age; whereas secretion was not altered at any age. This is in contrast to carbachol where the rise in $[Ca^{2+}]_i$ was unchanged but protein secretion was decreased in TSP-$1^{-/-}$ compared to WT mice. It is unclear why the stimulation of the $[Ca^{2+}]_i$ by phenylephrine is inconsistent, as no other marker or function that we measured at the three different ages showed this pattern. Similarly to M_3AChR agonist, α_1-adrenergic agonist-induced protein secretion did not follow the pattern of phenylephrine-stimulated $[Ca^{2+}]_i$ suggesting that phenylephrine-induced protein secretion was not dependent upon the rapid increases in $[Ca^{2+}]_i$ stimulated by agonists. Unlike M_3AchR agonists, α_{1D}-adrenergic agonist-induced secretion is less dependent on intracellular Ca^{2+} and influx of extracellular Ca^{2+} when these are depleted for the 40 min stimulation of secretion.[38,39,41,42] Furthermore, whereas M_3AChR agonist-induced secretion was decreased in TSP-$1^{-/-}$ compared to control lacrimal glands, α_{1D}-adrenergic agonist-induced secretion was unchanged. As M_3AChR agonists use different signaling pathways than α_1-adrenergic agonists, it is not surprising that the secretory responses are opposite. α_{1D}-Adrenergic agonists use eNOS and cGMP to stimulate secretion and transactivate the EGFR to activate ERK1/2 that attenuate secretion; whereas M_3AchR agonists do not. Another possible reason for the difference in α_{1D}-adrenergic agonist secretory response is that only M_3AChR, not α_{1D}-adrenergic agonists use the thapsigargin-dependent intracellular Ca^{2+} stores. The rapid increase in $[Ca^{2+}]_i$ represents the release of Ca^{2+} from intracellular stores, that are decreased in TSP-$1^{-/-}$ compare to WT. M_3AChR agonist-stimulated secretion is dependent upon these stores. The decrease in these stores in TSP-$1^{-/-}$ compare to WT may not be extensive enough to alter the M_3AChR agonist-induced intracellular Ca^{2+} response that occurs over seconds, but is enough to decrease secretion that is measured over 40 min. In contrast the α_{1D}-adrenergic agonist secretory response is less dependent upon intracellular Ca^{2+} and would not be affected by the decrease in the Ca^{2+} store in TSP-$1^{-/-}$ compared to WT mouse lacrimal gland acini.

Purinergic agonists such as ATP can be released from efferent sympathetic and parasympathetic nerves, and in the lacrimal gland the signaling pathways activated by P2X agonists and their interaction with parasympathomimetic and sympathomimetic agonists have been delineated. We determined whether the P2X agonist ATP and the P2Y agonist 2MeSATP stimulation of $[Ca^{2+}]_i$ and protein secretion was altered in TSP-$1^{-/-}$ compared to WT lacrimal glands. There were no consistent changes in these parameters with age in TSP-$1^{-/-}$ compared to WT glands. Thus, release of ATP from nerves and purinergic receptor activation of acinar function probably does not play a role in dry eye that occurs in TSP-$1^{-/-}$ mice.

TSP-$1^{-/-}$ female mice have ADDE progression in a time-dependent fashion allowing us to dissect out the anomalies, which contribute to glandular dysfunction. This study presents a comprehensive and detailed overview of the processes involved in such a scenario. Additional functional and expression studies are needed to delve into the mechanistic intricacies of such a phenotype. Moreover, gain of function or phenotypic rescue experiments will throw light into additional mechanisms that are not apparent. Recent literature on contribution of TSP-1 in ocular inflammation along with the phenotypic characteristics unraveled in this study makes the TSP-1 mouse model very useful for the study of ADDE at its early developmental stages. In addition, this model also might serve be a useful and novel one for rapid screening of drugs to treat ADDE.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Schepens Eye Research

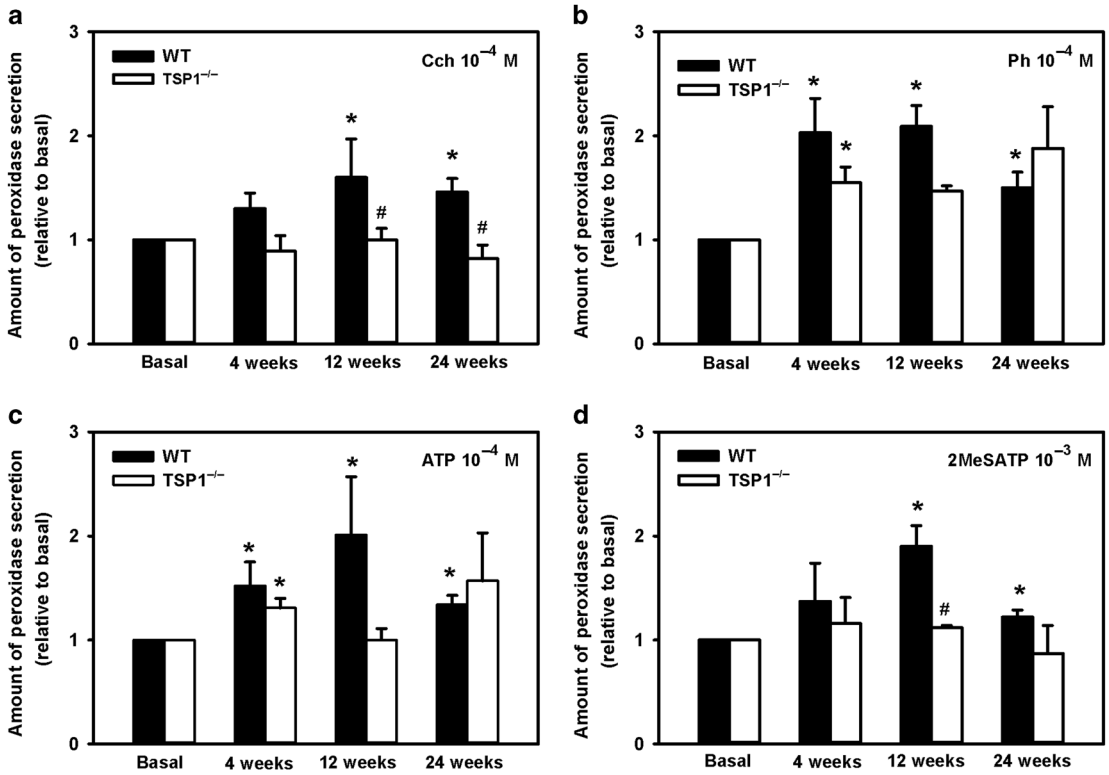

Fig. 9 Lacrimal gland protein secretion in TSP-1$^{-/-}$ compared to WT mice in response to stimuli. Lacrimal gland pieces from 4-week-old, 12-week-old, and 24-week-old WT and TSP-1$^{-/-}$ mice were stimulated for 40 min with carbachol (Cch) (**a**), phenylephrine (Ph) (**b**), ATP (**c**), and 2MeSATP (**d**), and protein secretion was measured. Data are mean ± SEM from three independent animals. Asterisks indicates statistically significant different from basal; Hash indicates a significant difference from WT

Institute Animal Care and Use Committee. TSP-1$^{-/-}$ mice were originally obtained from Dr. J. Lawler (BIDMC, Harvard Medical School, Boston, MA) and bred at the Boston University Medical School Animal Facility. These mice were made on the C57BL/6 J background. For that reason 4-week-old, 12-week-old, and 24-week-old C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor ME) and were used as control. Only female mice were used in this study. For the act of killing, they were anesthetized for 5 min in CO_2. Thereafter, both exorbital lacrimal glands were removed immediately.

Preparation of lacrimal gland acini

Lacrimal glands were fragmented and digested with collagenase (100 U/mL, CLSIII; Worthington Biochemicals) in Krebs-Ringer bicarbonate buffer with HEPES (KRB-HEPES; 119 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, and 5.5 mM glucose, at pH 7.45) plus 0.5% bovine serum albumim (BSA) for 30 min at 37°C. After incubation, digested tissue was filtered through a nylon mesh (150-μM pore size), and centrifuged at 50×*g* for 2 min. The pellet was washed twice through KRB-HEPES containing 4% BSA. The dispersed acini recovered for 60 min at 37 °C before use.

Immunohistochemistry

Lacrimal glands were extracted and fixed in 4% formaldehyde diluted in phosphate-buffered saline (PBS) for 2 days at 4 °C. Then the glands were incubated in 30% sucrose for a day, embedded in OCT and sectioned (10 μm thick) using a cryostat. The sections were stained with H&E or with antibodies against TUJ-1 (1:500, BioLegend, San Diego, CA), VIP (1:100, Abcam, Cambridge, MA), VE-cadherin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), or PECAM (1:100, BD Biosciences, San Jose, CA) overnight at 4 °C in a humidified chamber. The secondary antibody conjugated to either Cy2 or Cy3 (1:300 dilution) was applied for 1 h at room temperature (RT). Coverslips were mounted on slides, viewed by fluorescence microscopy (Eclipse E80i; Nikon, Tokyo, Japan), and micrographs were taken with a digital camera (Spot; Diagnostic Instruments, Inc., Sterling Heights, MI) or viewed by confocal microscopy (TCS-SP2, Leica Microsystems, Bannockburn, IL). Sections incubated in the presence of isotype controls served as the negative control. The length of blood vessels was measured using the Imaris program.

Measurement of blood vessel area

Mouse lacrimal glands were labeled with VE-cadherin (labels endothelial cell junctions) or PECAM (labels vascular endothelial cells) and three to four *z*-stacks per sample that were ~30 microns thick were collected from mouse lacrimal glands sections. Each *z*-stack was collected from different section. Then we used Imaris Image Analysis Software (Bitplane Zurich, Switzerland) to create three-dimensional reconstructions from confocal Z series. To measure total blood vessels length in each *z*-stack, the Filament-Tracer module of Imaris software was used and filament tracing analyses were performed. This information was then used to quantify potential changes in vascular length in the TSP-1$^{-/-}$ lacrimal glands. Two-tailed *t*-test was used to determine whether the difference between TSP-1$^{-/-}$ and WT blood vessels is statistically significant.

Western blotting analysis

Lacrimal glands were removed from mice and homogenized in RIPA buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) to lyse the cells. Proteins were separated by SDS–PAGE and processed for western blotting. The antibodies against Rab3D,

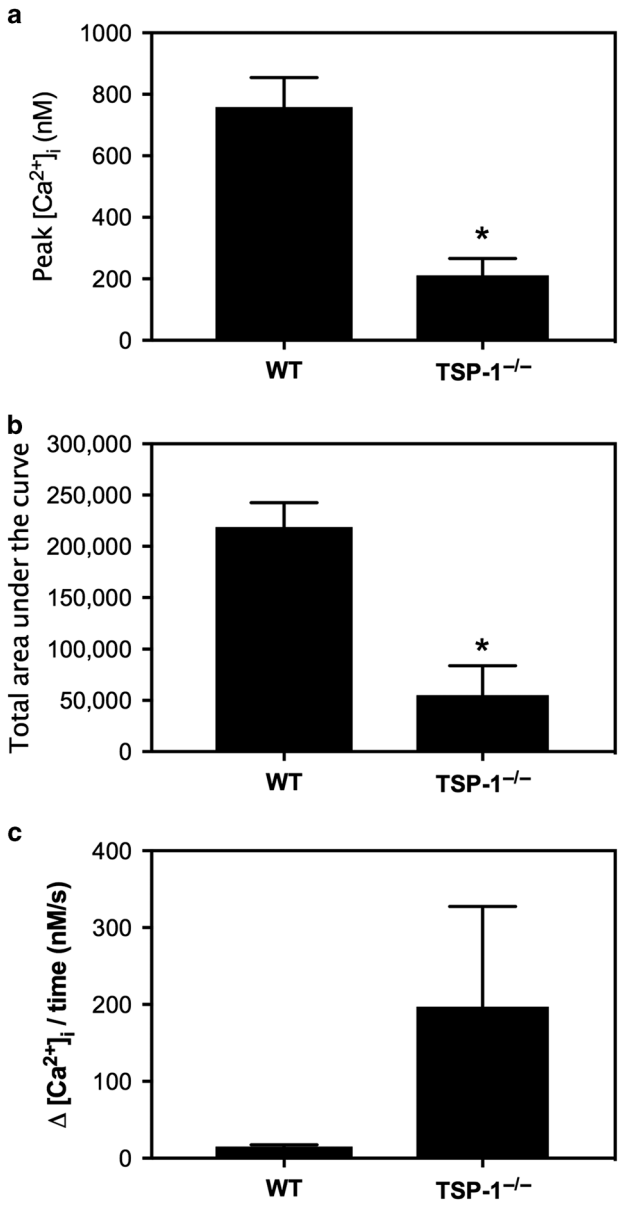

Fig. 10 Intracellular Ca^{2+} stores are decreased in TSP-$1^{-/-}$ compared to WT lacrimal gland acini. The size of intracellular Ca^{2+} stores was measured with a 15 min thapsigargin stimulation (10^{-5} M) in the absence of extracellular Ca^{2+}. The peak $[Ca^{2+}]_i$ (**a**), the total area under the curve (**b**) were measured over the 15 min period, and the rate of refilling was measured with the addition of extracellular Ca^{2+} (**c**) and compared between 24-week-old WT and TSP-$1^{-/-}$ mice. Data are mean ± SEM from three independent animals. Asterisks indicates a significant difference from WT. At least three independent experiments were performed

calnexin, Lamp1, and Cox IV were used at 1:200 dilution (all from Santa Cruz Biotechnology). Immunoreactive bands were visualized by the enhanced chemiluminescence method. The films were analyzed with Image J software (http://rsbweb.nih.gov/ij/).

Measurement of $[Ca^{2+}]_i$

Lacrimal acini were incubated for 30 min at RT in the dark with KRB-HEPES containing 0.5% BSA, 0.5 µM fura-2/AM, 8 µM pluronic acid F127, and 250 µM sulfinpyrazone followed by washing in KRB-HEPES containing sulfinpyrazone. Lacrimal acini were stimulated with carbachol, phenylephrine, ATP, and 2MeSATP (all from Sigma-Aldrich). Calcium measurements were made with a ratio imaging system (InCyt Im2; Intracellular Imaging, Cincinnati, OH) using excimer wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Experiments were repeated in at least three animals. After the addition of agonists, data were collected in real time. Data are presented as the actual $[Ca^{2+}]_i$ with time or as the change in peak $[Ca^{2+}]_i$. Change in peak $[Ca^{2+}]_i$ was calculated by subtracting the average of the basal value (no added agonist) from the peak $[Ca^{2+}]_i$. Although data are not shown, the plateau $[Ca^{2+}]_i$ was affected similarly to the peak $[Ca^{2+}]_i$, except where indicated. To calculate intracellular Ca^{2+} stores, extracellular Ca^{2+} was removed. Then, cells were treated for 15 min with 10^{-5} M thapsigargin. Peak $[Ca^{2+}]_i$ and total area under the curve were measured. After that, Ca^{2+} was reintroduced in the bath and the rate of refilling was analyzed.

Measurement of peroxidase secretion

Lacrimal pieces were incubated for 40 min in KRB-HEPES containing 4% BSA at 37 °C in the presence of agonists carbachol, phenylephrine, ATP, and 2MeSATP. To terminate the incubation, pieces were pelleted by centrifugation, and the supernatant was collected. The pellet was homogenized in 10 mM Tris-HCl (pH 7.5). Peroxidase activity, an index of protein secretion, was measured in duplicate in both the supernatant and the pellet. Peroxidase was measured using Amplex Red reagent (Invitrogen), which, when oxidized by peroxidase in the presence of hydrogen peroxide, produces a highly fluorescent molecule. The amount of fluorescence in the supernatant and pellet was quantified using a fluorescence microplate reader (model FL600; BioTek) with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Peroxidase was expressed as a percentage of peroxidase secreted into the media (supernatant) compared with total peroxidase present in the cells before stimulation (pellet plus supernatant). Data were expressed as fold increase over basal, which was set to 1.

Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTIONS

S.B., R.R.H., and H.P.M. carried out the experiments and analyzed data. D.A.D. and S.B. designed the experiments. S.B., D.A.D., and L.G.-P. drafted the manuscript, S.M. consulted and advised on mouse model.

ADDITIONAL INFORMATION

Conflict of interest: The authors declare no competing financial interests.

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