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Sjögren's Syndrome-Like Ocular Surface Disease in Thrombospondin-1 Deficient Mice

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Thrombospondin-1 (TSP-1) is a major activator of latent transforming growth factor-β *in vitro* as well as *in vivo*. Mice deficient in TSP-1, despite appearing normal at birth, develop a chronic form of ocular surface disease that is marked by increased apoptosis and deterioration in the lacrimal gland, associated dysfunction, and development of inflammatory infiltrates that result in abnormal tears. The increase in $CD4^+$ T cells in the inflammatory infiltrates of the lacrimal gland, and the presence of anti-Sjögren's syndrome antigen A and anti-Sjögren's syndrome antigen B antibodies in the serum resemble autoimmune Sjögren's syndrome. These mice develop an ocular surface disorder dry eye that includes disruption of the corneal epithelial layer, corneal edema, and a significant decline in conjuctival goblet cells. Externally, several mice develop dry crusty eyes that eventually close. The inflammatory $CD4^+$ T cells detected in the lacrimal gland, as well as those in the periphery of older TSP-1 null mice, secrete interleukin-17A, a cytokine associated with chronic inflammatory diseases. Antigen-presenting cells, derived from TSP-1 null, but not from wild-type mice, activate T cells to promote the Th17 response. Together, these results indicate that TSP-1 deficiency results in a spontaneous form of chronic dry eye and aberrant histopathology associated with Sjögren's syndrome. ***(Am J Pathol 2009, 175:1136–1147; DOI: 10.2353/ajpath.2009.081058)***

Thrombospondin-1 (TSP-1) is a large (450 kDa) matricellular protein capable of activating latent transforming growth factor (TGF)-β *in vitro* and *in vivo*.[1,2] Accordingly, mice with TSP-1 deficiency resemble TGF-$\beta1^{-/-}$ mice, in terms of developing inflammatory infiltrates in multiple organs. However, TSP-1 deficiency leads to a milder inflammation than in TGF-$\beta1^{-/-}$ mice.[3] The resolution of the lung pathology in TSP-1 null mice by a TSP-1-derived peptide, capable of activating latent TGF-β, established the significance of TSP-1 driven activation of TGF-β *in vivo*. This observation confirms our previous report that evaluated the significance of TSP-1 in the TGF-β-rich eye environment. We demonstrated that ocular immune privilege, a phenomenon known to be dependent on active TGF-β, was lost in TSP-1 deficient mice.[4] Furthermore, we reported that TSP-1 is essential for the function of antigen presenting cells (APCs) that contribute to this immune privilege by inducing a form of peripheral tolerance.[5] Thus TSP-1 plays a significant role in preventing inflammatory responses in the TGF-β rich ocular environment, such that inflammation during experimental autoimmune uveitis in TSP-1 deficient mice led to irreversible destruction of the retina.[4]

Absence of TGF-β, although lethal for the animals, causes a severe ocular pathology (in mice <4 weeks old), due to intense inflammatory infiltration of lacrimal glands, followed by their functional loss. Histopathology in these animals resembles that of autoimmune Sjögren's syndrome, characterized by inflammation of exocrine glands and associated dry eye and dry mouth.[6] Similarly mice with disrupted TGF-β receptor mediated signaling in the salivary glands were reported to develop severe inflammation in the gland.[7] These reports indicate that TGF-β plays a key role in the onset and resolution of inflammation in the exocrine glands. The anti-inflammatory effect of TGF-β is also evident from its well-established role in the induction of regulatory T cells that can suppress inflammatory responses.[8] A potential role of such regulatory T cells in the regulation of ocular surface inflammation was recently demonstrated in animals exposed to dessicating environmental stress.[9] We have now identified an important role played by APC-derived TSP-1 in promoting the development of regulatory T cells

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while inhibiting chronic inflammation associated with an interleukin (IL)-17A-producing subset of T cells, Th17 (manuscript in preparation).

Considering the significance of TSP-1 in ocular immune privilege and its ability to activate latent TGF-β and influence the generation of regulatory T cells that mediate peripheral immunological tolerance, we speculated development of an ocular pathology in TSP-1-deficient mice. Although born with no apparent abnormalities of the eye, over a period of time, we noted the development of crusty eyes, relatively reduced in size from typical wild-type eyes, which eventually closed completely. This observation resembled that reported in TGF-$\beta 1^{-/-}$ mice; therefore, we investigated the presence of lacrimal gland inflammation, associated functional loss, and the possibility of ocular surface disease in TSP-1-deficient mice. Ocular surface disease is typically a chronic condition that develops over a period of time. Since TSP-1 null mice do not exhibit a shortened life span like TGF-β-deficient mice, it is possible to examine TSP-1 null mice for the development of chronic ocular surface disease like dry eye, in particular the condition associated with autoimmune Sjögren's syndrome.

The ocular pathology in TGF-$\beta 1^{-/-}$ mice was attributed to a severe inflammation in the lacrimal glands.[6] Considering that TSP-1 is a major activator of latent TGF-β, we expected its absence to likely reduce the biologically active TGF-β in the glandular microenvironment and lead to the lacrimal gland inflammation. Indeed, we detected progressively exacerbated infiltration of the lacrimal glands in TSP-1 null mice, with inflammatory infiltrates containing CD4 and CD8 T cells with a distinct increase in the former subset, as typically reported in autoimmune Sjögren's syndrome. Further extensive analysis in this report demonstrates the presence of an autoimmune disorder in TSP-1-deficient mice that involves a chronic inflammatory immune response targeted to lacrimal gland antigens, resulting in functional loss and subsequent ocular surface disease that resembles Sjögren's syndrome.

Materials and Methods

Mice

C57BL/6 (*H-2*^{b}) mice, 6 to 8 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6-Tg(*TcraTcrb*)*425Cbn/J* OT-II (transgenic for TCR specific for chicken ovalbumin 323-339 in the context of *I-A*^{b}) mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). TSP-1 null mice (C57BL/6 background), originally received from Dr. J. Lawler (BIDMC, Harvard Medical School, Boston, MA) were bred in house in a pathogen-free facility at Schepens Eye Research Institute, Boston, MA. All animal experiments were conducted in accordance with institutional guidelines.

Lacrimal Gland Functional Assessment

The secretory function of lacrimal glands was tested in an *in vitro* assay as described before.[10] Lacrimal glands harvested from wild-type or TSP-1 null mice were cut into small lobules (~2 mm diameter). These lobules were placed in cell strainers in 0.8 ml of Krebs-Ringer bicarbonate buffer (containing in mM/L: 120 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, and 25 $NaHCO_3$) supplemented with 10 mmol/L Hepes and 5.5 mmol/L glucose, pH 7.4 and incubated at 37°C. After 20 minutes, the medium was collected as representative of basal peroxidase levels and replaced with the same volume of Krebs-Ringer bicarbonate buffer containing high KCl (75 mmol/L) or phenylephrine (an α_1-adrenergic agonist, 10^{-4} M/L). Lobules were incubated in these media for an interval of 20 minutes each. High KCl buffer was used to depolarize nerve termini in the tissue to release their neurotransmitters. Following incubation, media were collected and lobules were homogenized in 10 mmol/L Tris-HCl, pH 7.5. The amount of peroxidase in the media and tissue homogenate was determined by spectrophotometric assay (Amplex Red; Invitrogen, Carlsbad, CA). The amount of secreted peroxidase was expressed as a percentage of total: peroxidase in media/(peroxidase in media + peroxidase in tissue) $\times$ 100.

Amplex Red Assay to Measure Peroxidase Activity

Oxidation of Amplex Red by peroxidase in the presence of hydrogen peroxidase produces a highly fluorescent molecule, resorufin. Amplex Red reagent (Invitrogen, Carlsbad, CA) was used to measure peroxidase levels. For the measurement of peroxidase secreted by lacrimal gland lobules, 0.1 ml medium and 0.01 ml sample or standards were placed in triplicates in 96-well microplates. To each well 0.1 ml assay buffer (50 mmol/L Tris-HCl pH 7.5) was added containing 0.2 M/L Amplex Red reagent and 0.2 M/L hydrogen peroxide. After incubation in the dark for 30 minutes at room temperature, the fluorescence was determined in a fluorescence microplate reader (model FL600; Bio-Tek, Winooski, VT) with 530-nm excitation wavelength and 590-nm emission wavelength.

To determine peroxidase content of the tears, tears collected were diluted with PBS. The assay was performed with 50 μl of samples or standards per well in triplicates to which 50 μl of Amplex Red reagent mixed with hydrogen peroxide as per the manufacturer's instructions was added. The fluorescence was determined as described above.

Aqueous Tear Measurement

Mice were anesthetized with ketamine/xylazine and were injected with pilocarpine hydrochloride (0.5 μg/g in saline). One minute after the injection, phenol red-stained cotton thread (Zone-quick; Oasis Glendora, CA) was gently applied to the ocular surface in the lateral canthus

for 60 seconds. Wetting of the thread was measured in mm, using the scale provided with the cotton thread. Tear volume was normalized against body weight of each mouse. In some experiments, tears from both the eyes were collected using a 5-μl microcapillary pipette and analyzed for their peroxidase content.

Corneal Fluorescein Staining

Corneal fluorescein staining was performed as described by Rashid et al.[11] Sodium fluorescein (1%), 1 μl, was applied to the cornea of mice under anesthesia. Three minutes later, eyes were flushed with PBS to remove excess fluorescein, and corneal staining was evaluated and photographed with a slit lamp biomicroscope (Humphrey-Zeiss, Dublin, CA) using a cobalt blue light. Punctate staining was recorded using a standardized National Eye Institute grading system of 0 to 3 for each of the five areas of the cornea.[12]

Flow Cytometry

Lacrimal gland tissue was digested with collagenase (0.1%, Sigma-Aldrich, St. Louis, MO) at room temperature for 3 $\times$ 20 minutes. The cells were filtered through 70-μm nylon mesh filter (BD Biosciences, San Jose, CA). The single cell suspension was washed and resuspended in cold PBS containing 0.1% bovine serum albumen, and stained with fluorescence labeled anti-CD4 or anti-CD8 antibodies (BD Biosciences, San Jose, CA). Intracellular IL-17A was stained with fluorescence labeled anti-IL-17A antibodies (eBioscience, San Diego, CA) and an intracellular staining kit (eBioscience, San Diego, CA) as per the manufacturer's instructions. To detect apoptosis, cells were labeled with fluorescein isothiocyanate-conjugated Annexin-V and propidium iodide as per the instructions in the kit (BD Bioscience, CA). Fluorescence-labeled cells were analyzed using Coulter Epics XL flow cytometer (Beckman-Coulter, Miami, FL). Data were collected on 25,000 to 50,000 events in each experiment. Further analysis of the data was performed using FlowJo v8.8.4 software. In overlaid histograms "% of Max" label of *y* axis indicates data normalized to match cell counts in each histogram. Typically 10,000 gated events were analyzed.

Enzyme-Linked Immunosorbent Assay

Lacrimal gland extracts were prepared by homogenizing the tissue samples in a lysis buffer (Active Motif, Carlsbad, CA). Glandular extracts, serum samples or culture supernatants were analyzed using enzyme-linked immunosorbent assay (ELISA) kits for IL-17A, interferon (IFN)-γ (eBioscience, San Diego, CA), or anti-SSA or anti-SSB autoantibodies (α Diagnostic, San Antonio, TX). The assay was performed as per the manufacturers' instructions.

Histology

Lacrimal glands, or whole eyes and lids, harvested were fixed immediately in 4% paraformaldehyde and embedded in paraffin. Sections (6 to 8 μm) were cut and stained with H&E or alcian blue/PAS reagent. For the sections of the conjuctiva we used sagittal sections from the middle of the eye as representative of the whole conjuctiva to control for the variations in goblet cell density over the surface of the eye. Both bulbar and palpebral conjunctiva were examined on two slides with six serial sections each. For transmission electron microscopy sections, lacrimal glands were postfixed in half-strength Karnovsky fixative for 24 hours and embedded in Epon-Araldite. Semithin sections (1 to 2 μm) were stained with toluidine blue; ultrathin sections (60 to 90 A) were stained with uranyl acetate and lead citrate before examination on a transmission electron microscope (EM410; Philips, Eindhoven, the Netherlands).

In Vitro *Assay to Detect Cytokines Released by Activated T Cells*

APCs (F4/80+ macrophages) were harvested from the peritoneal lavage of thioglycollate-injected (3% *i.p.*) wild-type or TSP-1 null mice. For co-culture experiments APCs (1×10^6) were cultured with antigen ovalbumin (Sigma, 100 μg/ml) and OT-II T cells (3×10^5). In some experiments, splenocytes from wild-type or TSP-1 null mice (3×10^5) were cultured with anti-CD3 antibody (2CII, BD Biosciences, San Jose, CA). Culture supernatants collected at 48 hours interval were tested for levels of IL-17A or IFN-γ by ELISA.

Real-Time PCR

Total RNA was isolated from the corneas or lacrimal glands harvested from wild-type or TSP-1 null mice (8 or 24 weeks, $n = 4$ to 5) using RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions. cDNA was synthesized by reverse transcribing RNA using oligo dT and M-MLV RT (Promega, Madison, WI). SYBR green real-time PCR assay was used to determine relative quantitative expression of selected genes. Sequences of the primers used for these genes are as follows: *IFN-γ*, F-5′- TCAGCAACAACATAAGCGTCAT-3′, R-5′- GACCTCAAACTTGGCAATACTCAT-3′; *IL-1β*, F-5′- TCTGAAGCAGCTATGGCAACTGTT-3′, R-5′- CATCTTTTGGGGTCCGTCAACT-3′; *IL-6*, F-5′- AGTCAATTCCAGAAACCGCTATGA-3′, R-5′- TAGGGAAGGCCGTGG TTGT-3′; tumor necrosis factor *(TNF)-α,* F-5′-GGCCTCCCTCTCATCAGTTCTATG-3′, R-5′-GTTTGCTACGACGTGGGCTACA-3′; *Monocyte chemoattractant protein-1* (*MCP-1*), F-5′- AACTGCATCTGCCCTAAGGTCTT-3′, R-5′- GCTTCAGATTTACGGGTCAACTTC-3′; *Macrophage inflammatory protein-2* (*MIP-2*), F-5′- TACTGAACAAAGGCAAGGCTAACT-3′, R-5′- CGAGGCACATCAGGTACGA-3′; *glyceraldehyde-3-phosphate dehydrogenase*, F-5′-CGAGAATGGGAAGCTTGTCA-3′, R-5′-AGACACCAGTAGACTCCACGACAT-3′. Amplification reactions were set up using SurePRIME-&GO mastermix (MP Biomedicals, Solon,

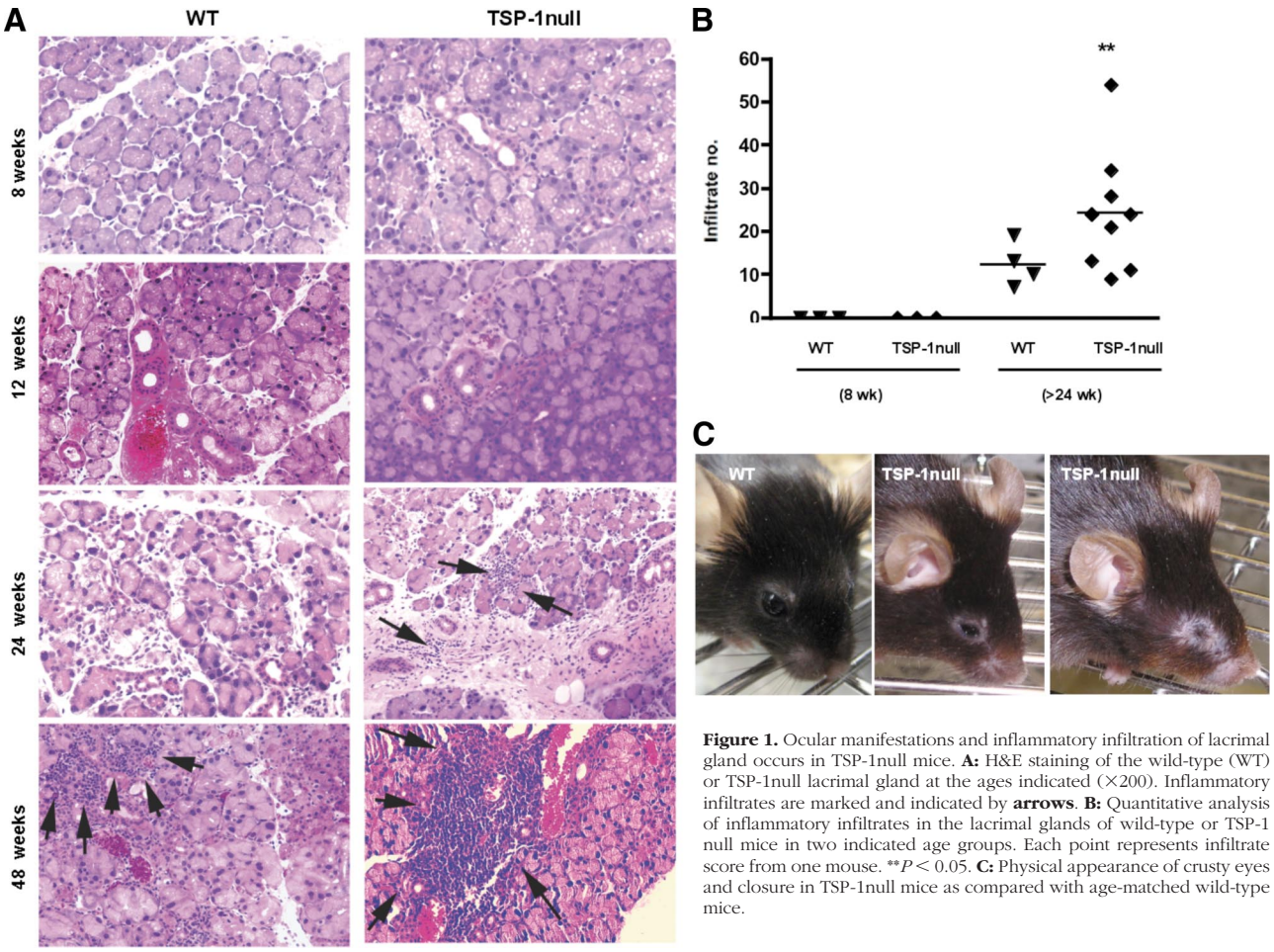

Figure 1. Ocular manifestations and inflammatory infiltration of lacrimal gland occurs in TSP-1null mice. **A:** H&E staining of the wild-type (WT) or TSP-1null lacrimal gland at the ages indicated (×200). Inflammatory infiltrates are marked and indicated by **arrows**. **B:** Quantitative analysis of inflammatory infiltrates in the lacrimal glands of wild-type or TSP-1 null mice in two indicated age groups. Each point represents infiltrate score from one mouse. $^{**}P < 0.05$. **C:** Physical appearance of crusty eyes and closure in TSP-1null mice as compared with age-matched wild-type mice.

OH) in triplicates with the thermal profile: 50°C for 2 minutes; 1 cycle, 95°C for 15 minutes; 1 cycle, 52°C to 55°C for 1 minute, 40 cycles, 72°C for 30 seconds; 1 cycle on ABI Prism analyzer (Applied Biosystems Inc., Foster City, CA). Fluorescence signal generated at each cycle was analyzed using system software. The threshold cycle values were used to determine relative quantitation of gene expression with glyceraldehyde-3-phosphate dehydrogenase as a reference gene.

Results

Lacrimal Gland Inflammation and Ocular Manifestations in TSP-1 Null Mice

To examine the possibility of inflammation, we harvested lacrimal glands from TSP-1null and age matched wild-type control mice and examined histology by H&E staining. As shown in Figure 1A, mononuclear infiltrates were detectable in the lacrimal glands of TSP-1null mice by the age of 24 weeks (at younger ages, such infiltrates were not detectable by histology). These infiltrates were significantly increased in TSP-1 null mice, as compared with those detected in age-matched wild-type controls (Figure 1B). While age related mononuclear infiltrates were detectable in lacrimal gland from 48 weeks old wild-type mice, much larger aggregates of such infiltrates were detectable in TSP-1 null mice of the same age. We also noted ocular manifestations that are typically associated with Sjögren's syndrome in TSP-1 null mice. As shown in Figure 1C, generally size of the eyes in TSP-1 null mice appeared reduced relative to that normally seen in wild-type control mice. In some TSP-1 null mice this change progressively led to a complete closure and loss of the eye. To determine whether the glandular infiltrates contained immune effector $CD4^+$ or CD8+ T cells, we prepared single cell suspensions from the collagenase digests of lacrimal glands obtained from 24-week-old TSP-1 null or wild-type mice and analyzed by flow cytometry to detect and measure the number of T cells in each subset. A representative staining is shown in Figure 2A. We noted a significant increase in the number of $CD4^+$, but not CD8+ T cells, in TSP-1 null lacrimal glands as compared with those detected in age matched control glands (Figure 2, A and B). An increase in $CD4^+$ T cells is commonly described in the histopathology of Sjögren's syndrome.[15] Therefore we examined sera collected from wild-type, as well as TSP-1 null mice (12 to 16 weeks), for the presence of autoantibodies associated with Sjögren's syndrome; anti-SSA and anti-SSB using an ELISA (no autoantibodies were detected at 8 weeks of age in Anti Nuclear Antibodies screening of samples, data not

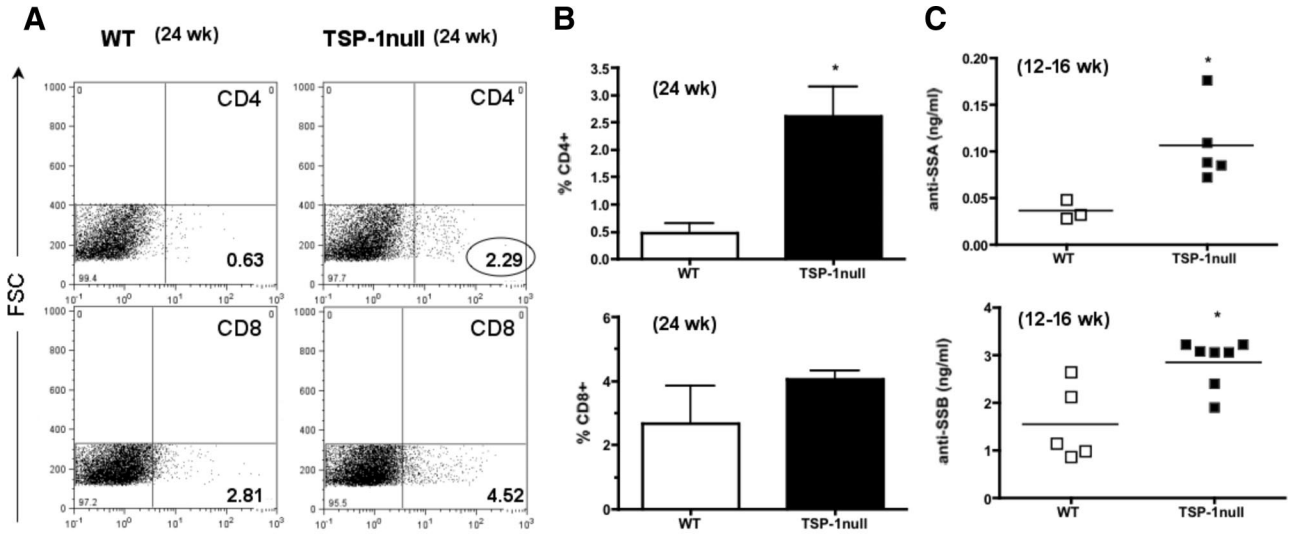

Figure 2. Cellular phenotype of infiltrating cells in the lacrimal glands and the presence of autoantibodies in TSP-1 null mice. **A:** Flow cytometric analysis of single cell suspensions prepared from either 24-week-old wild-type or TSP-1 null lacrimal glands were stained for CD4 or CD8 T cells as described in *Materials and Methods*. Analysis is normalized to 10,000 cells in each dotplot. Circled number indicates > 3 fold increase in detectable CD4 + cells. **B:** Percentage of $CD4^+$ and CD8+ T cells in the TSP-1 null lacrimal glands containing inflammatory infiltrates, as compared with those detected in wild-type gland ± SEM, $n = 3$ to 4. **C:** Sera collected from wild-type or TSP-1 null mice (12 to 16 weeks) were tested for the presence of Sjögren's associated anti-SSA and anti-SSB antibodies in an ELISA. $^*P < 0.05$.

shown). As shown in Figure 2C, significantly increased levels of both autoantibodies were detectable in serum samples collected from TSP-1 null mice as compared with those detected in wild-type controls. Thus detection of mononuclear infiltrates in the lacrimal glands with an increase in $CD4^+$ subset of T cells along with the presence of autoantibodies associated with Sjögren's syndrome suggest the possibility of autoimmune mechanisms underlying the lacrimal gland pathology in TSP-1 null mice.

Loss of Lacrimal Gland Function in TSP-1 Null Mice

To determine whether the histopathological findings in the lacrimal glands of TSP-1 null mice were associated with secretory impairment of the gland, we began with functional assessment of lacrimal glands using an *ex vivo* peroxidase secretion assay.[10] In this assay, we stimulated lacrimal gland fragments derived from 8-week-old wild-type and TSP-1 null mice, with the α_1-adrenergic agonist phenylephrine (10^{-4}M/L) or depolorizing high KCl buffer (that induces release of neurotransmitters from nerve termini within the tissue) and measured peroxidase secreted in the medium. As shown in Figure 3A, consistent with previous reports, wild-type lacrimal gland fragments secreted significantly increased levels of peroxidase in response to both methods of stimulation, as compared with the basal level. However, TSP-1 null lacrimal gland fragments failed to do so. There was no significant difference in pilocarpine-stimulated tear secretion in young or old wild-type and TSP-1 null mice (Figure 3B). In primary Sjögren's syndrome patients,[14] as well as in a mouse model,[15] it has been reported that the functional loss of lacrimal glands is associated with altered protein composition of tear fluid and that peroxidase levels are reduced in Sjögren's syndrome. Therefore we assessed the peroxidase content of the stimulated tears collected from both wild-type and TSP-1 null mice. While there was no significant difference noted in peroxidase levels in tears collected from younger mice, we noted a significant decline in tear peroxidase content of older TSP-1 null mice as compared with the wild-type controls (Figure 3C). Goblet cells located in the conjunctiva are also known to contribute to peroxidase in tears[16] and a significant decline in their numbers is reported in Sjögren's syndrome.[17] Therefore we assessed goblet cell density in the conjunctiva of young (<24 weeks) and old (>24 weeks) wild-type and TSP-1 null mice in serial sections stained with alcian blue/PAS to detect mucin filled goblet cells. At a younger age, we detected significantly increased density of such cells in TSP-1 null mice, while the goblet cell density significantly dropped in older mice (Figure 3D) with a larger (56.2%) decline detectable in TSP-1 null mice, as compared with that seen in wild-type (39.5%). Thus in TSP-1 null mice, the age-related decline in goblet cell density appears to be exacerbated. Here it is worth noting that the significant increase in goblet cell density in young TSP-1 null mice is likely to mask the tear peroxidase deficit at this age. Furthermore, in older mice, the goblet cell numbers appear to become comparable in wild-type and TSP-1 null mice, which also coincides with the significant decline detected in tear peroxidase in this age group. This suggests that the lacrimal gland functional deficit is clearly reflected in tear abnormality detectable in older TSP-1 null mice in the absence of a presumably compensatory increase in goblet cell density. Therefore these results indicate that although the functional deficit exists at an earlier age in TSP-1 null lacrimal glands, it is likely compensated for initially while

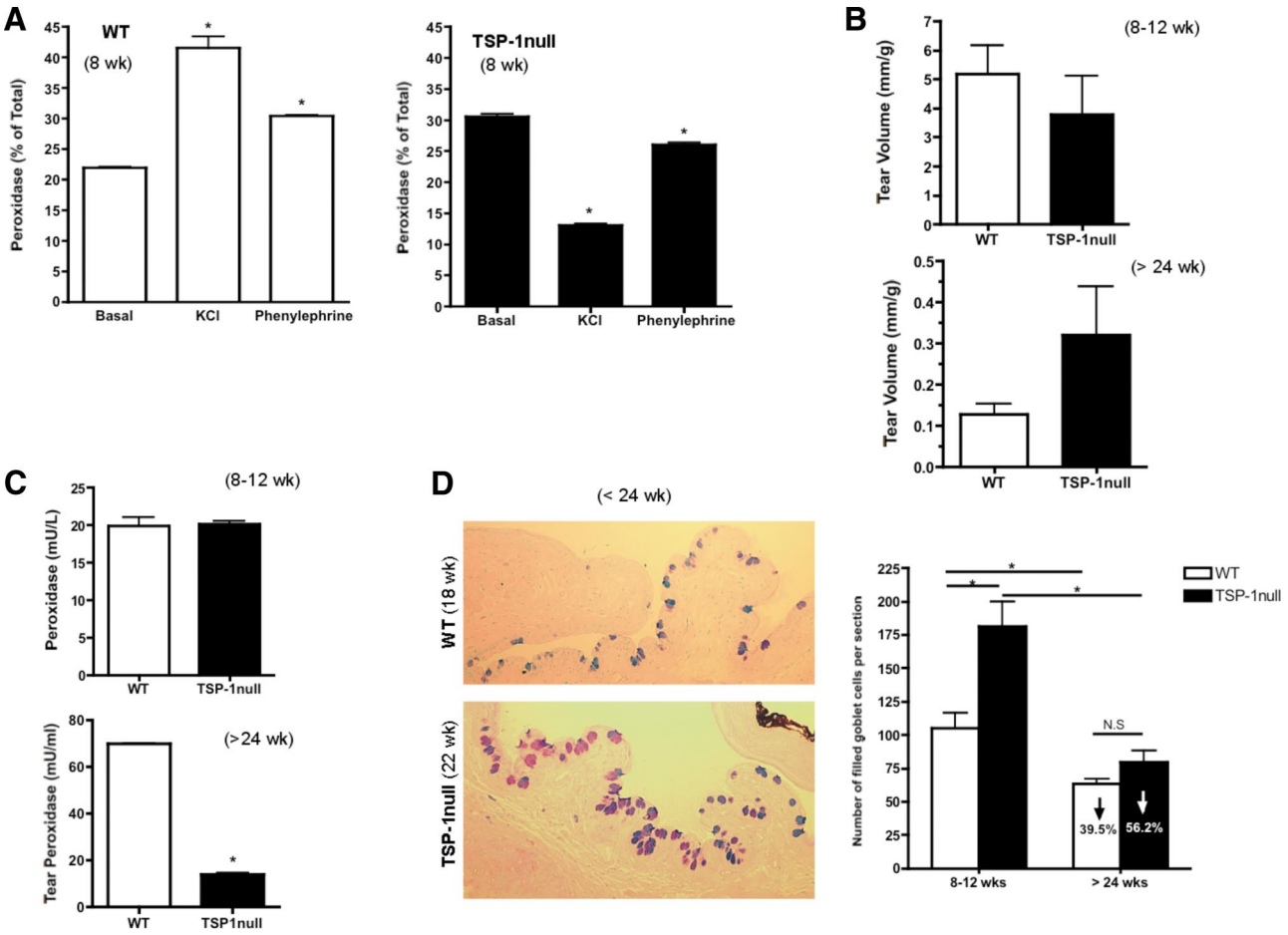

Figure 3. Functional loss of lacrimal gland and decline in conjunctival goblet cell density in TSP-1 null mice. **A:** Lacrimal gland tissues harvested from 8 weeks old wild-type or TSP-1 null mice was tested for their ability to secrete peroxidase on stimulation in an *ex vivo* assay as described in *Materials and Methods*, with high KCl containing buffer or the α_1-adrenergic agonist phenylephrine (10^{-4}M/L). Peroxidase released in the medium was measured using Amplex Red reagent. (*$P < 0.05$ compared with basal levels). Pilocarpine stimulated tears were collected from wild-type or TSP-1null ($n = 5$ to 8) mice in two indicated age groups. **B:** Tear volume was measured using phenol red cotton thread as described in *Materials and Methods* and normalized against the weight of each animal. **C:** To determine tear peroxidase, tears were collected in 5 μl capillaries and their peroxidase content was measured using Amplex Red reagent as described in *Materials and Methods*. **D:** Eye lid sections from wild-type or TSP-1 null mice from the indicated age groups were stained with alcian blue/PAS stain and mucin containing goblet cells stained blue, purple and magenta were counted. (*$P < 0.05$, N.S = not significant).

progressively with age the continued glandular deficit eventually compromises tear quality as the compensatory response wanes. These results clearly indicate that the functional loss of lacrimal glands in TSP-1 null mice precedes the histopathology detected.

Ocular Surface Defect and Inflammation in the Absence of TSP-1

By virtue of the contained growth factors, tears secreted by lacrimal glands are known to play a significant role in maintaining the normal health of the ocular surface, and in particular that of corneal epithelial cells. To determine whether aberrant tear composition detected in TSP-1 null mice affects their ocular surface, we examined its integrity using corneal fluorescein staining. As seen in Figure 4A, wild-type cornea did not show any uptake of the fluorescent dye, indicating an intact epithelial barrier. However TSP-1 null cornea showed a patchy staining pattern, exhibiting a damaged corneal epithelial barrier. Further quantitative analysis (based on the scoring system described in *Materials and Methods*) clearly demonstrated significantly increased corneal fluorescein staining in TSP-1 null, as compared with wild-type mice, as early as 12 weeks of age and persisted until 24 weeks, the oldest age evaluated (Figure 4B). Histological examination of TSP-1 null corneas provided further evidence of an abnormal and damaged epithelial layer, which was also accompanied by corneal edema in older mice, as compared with young (8-week-old) mice (Figure 4C). Together these results correlate with the loss of lacrimal gland function that appears to begin in TSP-1 null mice as early as 8 weeks of age.

To evaluate if the ocular surface changes in TSP-1 null mice were associated with inflammation, we performed real-time PCR analysis on the RNA harvested from the cornea tissue derived from wild-type or TSP-1 null mice to detect levels of message for dry eye-associated inflammatory molecules TNF-α, MCP-1, and MIP-2 (Figure 4D). While no significantly increased expression of these molecules was detectable in corneas harvested from young mice, the expression of these molecules was significantly

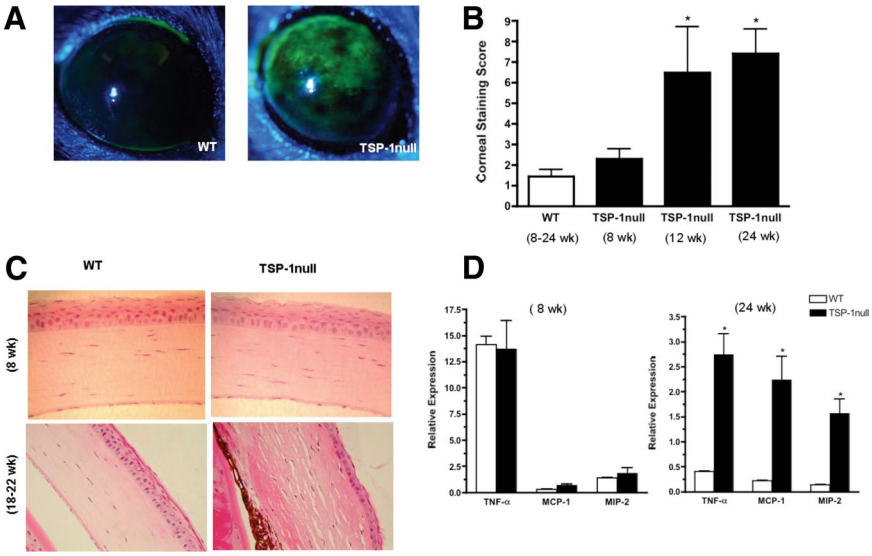

Figure 4. Ocular surface abnormalities in TSP-1 null mice. Corneal fluorescein staining was performed as described in *Materials and Methods*. **A:** A representative photograph of a slit lamp examination of stained cornea in wild-type or TSP-1 null mouse (24 weeks). **B:** Quantitative assessment of corneal staining was performed in wild-type ($n = 5$) and TSP-1 null mice ($n = 6$) at indicated ages. **C:** H&E staining of corneas from wild-type or TSP-1 null mice (×200). **D:** RNA harvested from 8- ($n = 5$) or 24- ($n = 4$) week-old wild-type or TSP-1 null corneas was subjected to SYBRgreen real-time PCR analysis to determine expression of TNF-α, MCP-1, and MIP-2. Results are presented as relative expression of indicated genes to glyceraldehyde-3-phosphate dehydrogenase. (*$P < 0.05$ as compared with wild-type control).

increased in TSP-1 null corneas derived from older mice (>24 weeks) as compared with their age-matched wild-type controls.

Our findings suggest that TSP-1 deficiency in mice results in a loss of lacrimal gland function altering the composition of tears, which in turn leads to ocular surface damage as detected by gradual loss of corneal epithelial barrier and development of associated inflammation. This damage resembles that detected in dry eye associated with Sjögren's syndrome.

Detection of IL-17 in the Lacrimal Glands from TSP-1-Deficient Mice

It is known that a generally immunosuppressive cytokine like TGF-β plays a key role in regulating development of a regulatory Foxp3+, as well as an inflammatory Th17 population. Development of the latter is promoted in the presence of IL-6.[18] The inflammatory cytokine IL-17 has been reported to be part of chronic inflammatory processes of many autoimmune diseases.[18,19,20] More recently it has been reported to be associated with inflammatory responses detected in autoimmune Sjögren's syndrome, as well as in dry eye.[21–24] We therefore investigated if TSP-1 deficiency that significantly decreases the availability of biologically active TGF-β leads to enhanced IL-17 expression in cells in the periphery or inflamed lacrimal glands. To that end, we first assessed splenic $CD4^+$ population in young (8-week-old) wild-type and TSP-1 null mice for their expression of IL-17A and IFN-γ by intracellular staining followed by flow cytometric analysis. As shown in Figure 5A, we detected an over twofold increase in IL-17A-positive cells in TSP-1-deficient splenocytes (0.82%), as compared with the wild-

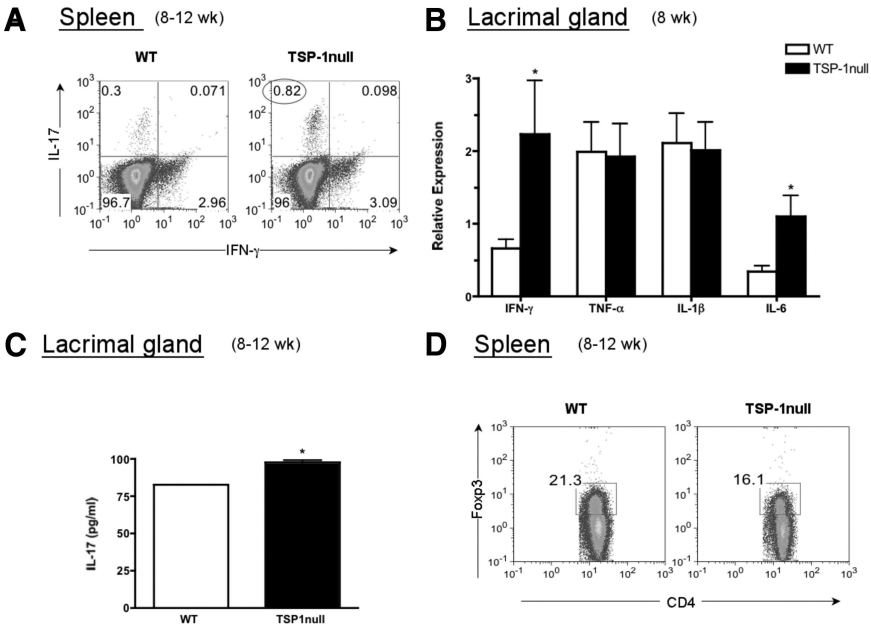

Figure 5. An imbalance in Th17 effectors and Foxp3+ regulatory cells young TSP-1 null mice. **A:** Splenocytes from wild-type or TSP-1 null mice (8 to 12 weeks) were stained for CD4 and intracellular IL-17A and IFN-γ ($n = 5$ each). A representative intracellular staining of IL-17A and IFN- γ in $CD4^+$ cells in the spleens of wild-type and TSP-1 null mice is shown. **B:** Real-time PCR was performed on RNA isolated from lacrimal glands harvested from wild-type or TSP-1 null mice (8 weeks, $n = 5$ each) to detect message levels for the indicated genes. **C:** Lacrimal gland extracts derived from wild-type or TSP-1 null mice (8 to 12 weeks) were tested for the levels of IL-17A in an ELISA. Results presented as mean cytokine level ± SEM. **D:** A representative flow cytometric analysis of CD4+CD25+Foxp3+ regulatory T cells in the spleens of wild-type or TSP-1 null mice (8 to 12 weeks) ($n = 5$ each). *$P < 0.05$.

type controls (0.3%). Expression of inflammatory cytokines in the lacrimal gland was assessed by real-time PCR. This analysis revealed significantly increased message for IFN-γ and IL-6 in TSP-1null mice, as compared with that detected in wild-type controls (Figure 5B). The detectable increase in IFN- γ noted in the lacrimal glands at an age that did not show any detectable inflammatory infiltrates by histology, indicated early signs of the development of inflammation in TSP-1 deficient lacrimal glands, presumably detectable due to a greater sensitivity of the PCR assay relative to histological exams. Furthermore, significantly increased expression of pro-inflammatory IL-6 in these lacrimal glands also suggested potentially Th17-supportive environment in the TSP-1 deficient glands, as compared with wild-type controls. Consistent with this possibility we detected significantly increased amount of IL-17A protein in the tissue homogenates prepared from TSP-1 null lacrimal glands, as compared with the wild-type glands (Figure 5C). As shown in Figure 5D, we also noted a consistent decline in the number of splenic Foxp3+ regulatory T cell population in TSP-1 deficient mice, as compared with those in wild-type mice (15.6% ± 1.6 vs. 20% ± 1.0, $n = 5$, $P < 0.05$). A decline in such regulatory T cells was recently reported in Sjögren's syndrome patients.[25] Together these results indicated a change in the peripheral balance between Th17 and Foxp3+ regulatory population in young TSP-1 null mice, as well as a presence of microenvironment in the lacrimal gland likely to be supportive of Th17 effectors associated with chronic inflammation.

Next, in older TSP-1 null mice, we tested if IL-17A-producing effectors persisted in the periphery or appeared in the inflamed lacrimal gland. Splenic T cells derived from wild-type or TSP-1 null mice were stimulated in culture with anti-CD3 antibodies and culture supernatants collected at 48 hours were tested by ELISA for the levels of IL-17A. Peripheral T cells in older TSP-1 null mice produced significantly reduced levels of IFN-γ and increased levels of IL-17A, a characteristic of Th17 population (Figure 6A). To determine whether inflammatory infiltrates detectable in the lacrimal glands of older TSP-1 null mice included IL-17A-expressing cells, we analyzed single cell suspensions, prepared after collagenase digestion of the tissue, by flow cytometry. Cells positively stained for CD4 marker were gated and analyzed further for their intracellular IL-17A expression. As seen in Figure 6B (top panel), an increased proportion of $CD4^+$ T cells in TSP-1 null lacrimal glands expressed IL-17A, as compared with those from wild-type lacrimal glands. These results were consistent with the significantly increased detection of IL-17A protein in the TSP-1 null glandular homogenates by ELISA, as compared with wild-type controls (Figure 6B, lower panel). Furthermore, expression of inflammatory cytokines assessed in the lacrimal glands of older mice using real-time PCR clearly demonstrated significantly increased levels of all of the tested inflammatory cytokines in TSP-1 null mice than in wild-type controls (Figure 6C). Of note was the decline in IFN-γ with an enhanced IL-6, relative to that seen in lacrimal glands derived from younger TSP-1 null mice. These results in older mice further confirmed the correlation between

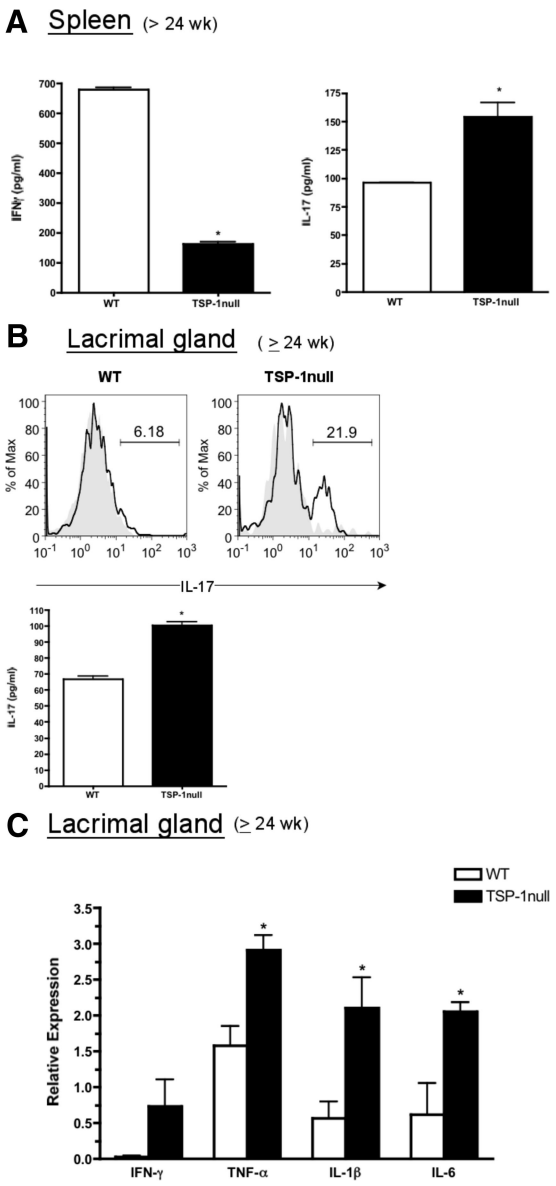

Figure 6. Th17 cells detectable in the lacrimal gland and persists in spleens of older TSP-1 null mice. **A:** Splenocytes harvested from wild-type or TSP-1 null mice (> 24 weeks) were stimulated with anti-CD3 (1 μg/ml) in a 96-well culture plate. Culture supernatants collected at 48 hours were tested for the presence of IFN-γ and IL-17A in an ELISA. **B:** Single cell suspensions prepared form the lacrimal glands of 24-week-old wild-type or TSP-1 null mice were stained with fluorescence conjugated antibodies for the cell surface CD4 and intracellular IL-17A as described in *Materials and Methods*, and analyzed by flow cytometry. Cells stained positively for CD4 were gated and analyzed for their intracellular expression of IL-17A. A representative data of matched pair is shown (top panel). Filled histograms indicate staining with isotype control antibodies and empty histograms show the intracellular IL-17A staining detected in $CD4^+$ cells. The proportion of cells stained positively is indicated as percent positive cells in each histogram. Lacrimal gland extracts derived from mice in the same age group were tested for the levels of IL-17A in an ELISA. Results presented as mean cytokine level ± SEM (bottom panel). **C:** Real-time PCR was performed on RNA isolated from lacrimal glands derived from wild-type or TSP-1 null mice (> 24 weeks) to detect levels of the indicated genes. $^*P < 0.05$.

Th17 and chronic inflammation that develops in TSP-1 null lacrimal glands. Together our results suggest that TSP-1 deficiency renders animals prone to developing chronic inflammation and particularly the lacrimal gland environment becomes supportive of local inflammation,

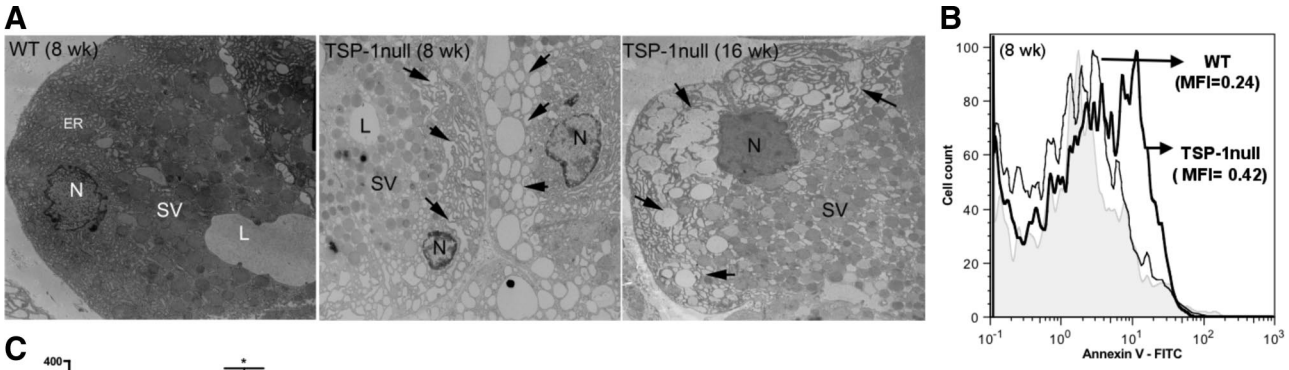

Figure 7. Lacrimal gland deterioration and the ability of antigen presenting cells to promote Th17 development in the absence of TSP-1. **A:** Transmission electron micrograph of lacrimal gland acinar cells in wild-type or TSP-1null glands derived from mice at indicated ages (×3000). **Arrows** indicate vacuoles and signs of deterioration with an acinar cell. ER, endoplasmic reticulum; N, nucleus; SV, secretary vesicle; L, lumen. **B:** Single cell suspensions prepared from collagenase-digested lacrimal glands from wild-type or TSP-1 null mice (8 weeks, $n = 5$ each) were stained with fluorescein isothiocyanate-conjugated annexin-V and propidium iodide. Flow cytometric analysis of the stained cells revealed staining primarily with annexin-V. The histogram shows fluorescence levels detected in unstained (gray filled), wild-type (thin solid line), or TSP-1null cells (thick solid line). Mean fluorescence intensity of each histogram is indicated. **C:** Thioglycollate-elicited macrophages (1×10^6) derived from 8-week-old wild-type or TSP-1 null mice were pulsed with soluble antigen ovalbumin (100 μg/ml) and co-cultured with OT-II T cells (3×10^5) in 24-well culture plate. Culture supernatants were collected at 48 hours and tested for the presence of IL-17A in an ELISA. Results presented as mean cytokine level ± SEM. $^*P < 0.05$.

which eventually causes functional loss and subsequent ocular surface disease.

APCs Deficient in TSP-1 Promote IL-17 Secretion from Activated T Cells

Our results in this study demonstrate a functional loss of lacrimal glands in TSP-1null mice before the appearance of mononuclear infiltrates (Figures 1A and 3A). Examination of transmission electron microscopy sections of lacrimal glands from young wild-type and TSP-1 null mice revealed significant deterioration of acinar cells indicative of apoptotic processes (Figure 7A). To determine whether lacrimal glands derived from young TSP-1 null mice that histologically do not show any signs of inflammation contained any apoptotic cells, lacrimal glands harvested from 8-week-old wild-type or TSP-1 null mice were processed by collagenase digestion, as described in *Materials and Methods*, and cells were stained with propidium iodide and fluorescein isothiocyanate-conjugated annexin-V. Flow cytometric analysis of the stained cells (pooled from five mice in each group) indicated increased staining for annexin-V (with virtually no cells stained with propidium iodide) in TSP-1 null lacrimal gland cells, as compared with the wild-type cells (Figure 7B). These results clearly supported presence of increased apoptosis in the absence of TSP-1 in lacrimal glands of young mice.

Typically APCs involved in the clearance of deteriorating or apoptotic cells are known to increase their TGF-β expression and avoid induction of an inflammatory response to any tissue-derived autoantigens.[26,27] We have previously demonstrated that APCs deficient in TSP-1 are incapable of inducing peripheral tolerance when exposed to TGF-β, thereby supporting inflammatory responses.[5] Therefore, we speculated that APCs in TSP-1 null mice may present lacrimal gland-derived autoantigens in a manner that supports chronic inflammation by developing lacrimal gland specific Th17 subsets. To test such a possibility, we compared APCs derived from wild-type and TSP-1 null mice for their ability to present the soluble antigen ovalbumin to T cells derived from transgenic OT-II mice that express ovalbumin-specific TCR. We used thioglycollate-elicited macrophages as APCs, pulsed them with ovalbumin and co-cultured them with OT-II T cells. Culture supernatants collected at 48 hours were analyzed by ELISA to determine IL-17A levels. As shown in Figure 7C, TSP-1 null APCs activated antigen-specific T cells to secrete significantly increased IL-17A, as compared with the wild-type APCs. Thus APCs in TSP-1 null mice appear to promote development of the inflammatory Th17 subset of effectors. Together our results support a potential aberrant presentation of lacrimal gland antigens by infiltrating macrophages to activate effectors associated with chronic inflammation.

Discussion

In this study, we introduce TSP-1 null mice as a novel animal model with spontaneous development of severe ocular surface disease as seen in Sjögren's syndrome and other non-Sjögren's dry eye diseases. The damaged corneal epithelial barrier in these mice correlates with the lacrimal gland dysfunction and reduced goblet cell density. The development of chronic inflammatory infiltrates and presence of autoantibodies associated with Sjögren's syndrome indicate potential autoimmune mechanisms underlying the ocular surface defect. Detection of increased Th17 cells in the lacrimal gland and periphery of TSP-1 null mice is consistent with the observations reported in other autoimmune diseases.[10,11] Such a profile of inflammatory effectors correlates with the ability of TSP-1 null APCs to promote the development

of a Th17 subset. Our findings thus clearly suggest that TSP-1 plays a significant role in the lacrimal gland function and homeostasis. Use of TSP-1 null mice described in this study therefore opens up multiple opportunities ranging from examining mechanisms underlying secretory functions of cells in the lacrimal gland to developing therapeutic strategies to mitigate severe dry eye.

Compromised corneal epithelial barrier function has been described as a primary characteristic of ocular surface disease like dry eye.[28,29] Depending on the tear production dry eye diseases are classified as either evaporative (aqueous-adequate) or aqueous-deficient. The latter is further divided into non-Sjögren's and Sjögren's syndrome groups. Dry eye in Sjögren's syndrome is associated with systemic immune dysfunction.[30] Our results indicate that although TSP-1null mice are born with a normal corneal surface, significant corneal epithelial abnormalities develop as early as 12 weeks of age. The development of dry eye was also confirmed by detection in the cornea of message for inflammatory molecules, such as TNF-α, MCP-1, and MIP-2, which have been linked to ocular surface disorders.[31,32] These abnormalities are consistent with the functional loss of the lacrimal gland in these animals. Although tear volume measurements in this study indicate no apparent deficiency in TSP-1 null mice, it is quite likely that the damaged ocular surface in these mice induces reflex tear (especially fluid) secretion by remaining undamaged portions of lacrimal gland or other sources such as conjunctiva confounding our results.[33,34] This is especially likely as the tears collected were stimulated with injected cholinergic agonist pilocarpine. In human Sjögren's syndrome, reflex tear deficiency has been reported.[35,36] Such deficiency correlated with the extent of inflammatory infiltration detected in the lacrimal glands. However, reflex tear secretion was still detectable in patients with relatively fewer infiltrates. It has been reported that nerve termini cluster in certain areas in the lacrimal gland.[37] Therefore it is conceivable that while severe inflammatory infiltrates in the gland may damage most areas with the nerve termini, lesser inflammation may spare some areas allowing residual responses. In our experiments tear volume measurements were performed before the maximal infiltration was detected. Therefore, it is quite likely that reflex tears in TSP-1 null mice masked the underlying non-reflex tear deficiency. In addition, the significantly reduced peroxidase content of these tears in TSP-1 null mice matched the abnormal composition of tears and, in particular, the significant decline in peroxidase reported in Sjögren's syndrome.[16,17] Thus our results are consistent with tear protein abnormalities reported in Sjögren's syndrome.

The presence of autoantibodies associated with Sjögren's syndrome in the sera of TSP-1 null mice further suggests an autoimmune pathogenesis of their ocular surface disease. Most other mouse models of Sjögren's syndrome are characterized mainly by lacrimal gland infiltrates and few describe accompanied functional loss of exocrine glands.[38] To our knowledge, no mouse model to date has been described to develop ocular surface disease as seen in dry eye associated with Sjögren's syndrome. Also, in most mouse models the degree of inflammation does not always correlate with the glandular dysfunction, which in some follows inflammatory infiltrates.[17,39,40] Unlike such models, in TSP-1 null mice ocular surface defect and functional loss of lacrimal glands precede detectable inflammatory infiltrates. Therefore TSP-1 null mice depict a chronic and gradual development of an inflammatory disease that leads to dry eye associated with Sjögren's syndrome.

In most autoimmune diseases, the mechanisms that lead to induction of peripheral inflammatory immune response are not clearly understood. Aberrant presentation of autoantigens in the lacrimal glands has been suggested as one possible mechanism in Sjögren's syndrome.[41] It has been hypothesized that the lacrimal gland microenvironment influences local APCs to confer tolerogenic properties under normal conditions.[42,43] Local or systemic perturbations were proposed to initiate autoimmune pathophysiology by impairing local immunohomeostasis. The detection of apoptosis and the functional loss of lacrimal glands in TSP-1 null mice, before the development of inflammatory infiltrates, strongly suggest a potential induction of immune effectors by the antigens derived from the deteriorating lacrimal gland or damaged ocular surface. Our observations in this study are also supported by those reported in the nonobese diabetic-severe compromised immunodeficient mouse model in which morphological and functional abnormalities detectable in target organs were suggested to act as an inciting event for leukocyte infiltration.[44] In fact, examining the microarray data generated in this study (now available in GEO public repository record GDS2177) revealed a loss of TSP-1 expression in lacrimal glands of nonobese diabetic-severe compromised immunodeficient mice, as compared with age-matched C57BL/6-scid controls. The tissues in this study were harvested at an age corresponding to an early inflammation in nonobese diabetic mice. Moreover recently enhanced degradation of extracellular matrix proteins, reported in the lacrimal glands of nonobese diabetic mice, was implicated in the pathogenesis of dry eye disease in Sjögren's syndrome.[45] Our results, together with these reports, suggest that an extracellular matrix protein such as TSP-1 is likely to be important in maintaining the functional integrity of lacrimal glands and its absence results in increased cell death in the tissue that incites inflammatory infiltrates and autoimmune response causing further damage with an eventual functional loss, which in turn leads to ocular surface disease of dry eye. Thus our study strongly implicates TSP-1 deficiency in the pathogenesis of an autoimmune disease like Sjögren's syndrome.

We have previously demonstrated that ocular APCs exposed to TGF-β depend on their ability to express TSP-1 to induce peripheral immune tolerance.[5] Therefore TSP-1-deficient APCs in the lacrimal glands can be expected to be incapable of inducing tolerance. Also, it has been demonstrated that typically tissue macrophages that clear apoptotic cellular debris secrete TGF-β, which inhibits inflammatory responses.[26,27] Therefore, we expected TSP-1-deficient macrophages that fail to activate latent TGF-β, to be supportive of an inflammatory activa-

tion of immune effectors. Our results are consistent with such a possibility, as TSP-1-deficient macrophages were found to promote secretion of inflammatory IL-17A. More recently this cytokine has been linked with many autoimmune diseases including Sjögren's syndrome.[10,11,20,24] Such a property of TSP-1-deficient APCs is also consistent with the detection of an IL-17A-expressing $CD4^+$ T cell subset in the lacrimal glands of TSP-1null mice but not wild-type mice. Besides the presence of Th17 cells, deficiency in a regulatory T cell population has been linked with many autoimmune diseases.[20] Development of CD4+CD25+Foxp3+ regulatory T cells is known to be dependent on TGF-β[8] and more recently this population was indicated to play a role in suppressing immune-mediated ocular surface inflammation.[9] Also, a peripheral decline in the frequency of Foxp3+ T regulatory cells is reported in the Sjögren's syndrome patients.[25] A similar decline in regulatory T cells detected in TSP-1 null mice is supportive of predominance of autoimmune mechanisms. Thus the detection of Th17 cells in combination with reduced regulatory T cells is consistent with the presence of chronic inflammatory autoimmune disease.

Results presented in this study are consistent with the possibility that glandular deterioration in TSP-1 null mice results in peripheral sensitization of immune effectors against lacrimal gland antigens, and that such effectors are capable of secreting IL-17A, a cytokine associated with chronic inflammation. With an altered balance in the proportion of regulatory T cells in the periphery, such a Th17 population may infiltrate the target organ lacrimal gland, which further provides supportive environment for such effectors eventually resulting in deterioration of the glandular function leading to ocular surface disease. Thus together our findings in this study introduce TSP-1 null mice as a model of ocular surface disease associated with Sjögren's syndrome. Additionally, this study reveals a novel significance of TSP-1-dependent mechanisms in the secretory function of lacrimal glands, as well as highlights significance of TSP-1 in regulating chronic inflammation that develops in this gland in Sjögren's syndrome. Further analysis of mechanisms underlying the development of chronic inflammation is likely to reveal therapeutic strategies to treat ocular surface disease and autoimmune Sjögren's syndrome.

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