TSP-1 Deficiency Alters Ocular Microbiota: Implications for Sjögren's Syndrome Pathogenesis

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Abstract

Purpose: The potential role of commensals as triggering factors that promote inflammation in dry eye disease has not been explored. The objective of this study was to evaluate whether ocular microbiota changes with the onset of dry eye disease in thrombospondin-1-deficient (*TSP-1*^{-/-}) mice, a strain that develops Sjögren's syndrome-like disease.

Methods: Conjunctival swabs were collected from *TSP-1*^{-/-} and C57BL/6 mice and analyzed for bacterial presence. Opsonophagocytosis of the bacterial conjunctival isolates derived from the aged *TSP-1*^{-/-} mice by neutrophils derived from either *TSP-1*^{-/-} or C57BL/6 bone marrow was evaluated. The bactericidal activities of TSP-1-derived peptide were examined.

Results: We found that in *TSP-1*^{-/-} mice, the conjunctival colonization with *Staphylococcus aureus* and coagulase negative staphylococci sp (*CNS*) species was significantly increased with aging and preceded that of the wild-type C57BL/6 control mice. This correlated with increased neutrophil infiltration into the conjunctiva of the *TSP-1*^{-/-} mice, suggesting that TSP-1 plays a significant role in regulating immunity to commensals. Accordingly, the *TSP-1*^{-/-} PMNs opsonophagocytosed the ocular commensals less efficiently than the TSP-1-sufficient neutrophils. Furthermore, a *TSP-1*-derived peptide, 4N1K, exhibited significant antimicrobial activity when compared to a control peptide against commensal sp.

Conclusion: These studies illustrate that alterations in the commensal frequency occur in the early stages of development of Sjögren's-like pathology and suggest that interventions that limit commensal outgrowth such as the use of TSP-1-derived peptides could be used for treatment during the early stages of the disease to reduce the commensal burden and ensuing inflammation.

Introduction

SJÖGREN'S SYNDROME (SS) is a chronic autoimmune disease that is characterized by lymphocytic infiltration of exocrine glands and results in classic sicca symptoms of dry eyes and dry mouth. The disease prevalence has been calculated at 3.9 per 100,000 (95% confidence interval 2.8–4.9), making it a common health problem and one for which treatment options are limited. The primary form of the syndrome has a characteristic inflammatory cell involvement of the salivary and lacrimal glands, while secondary SS involves various systemic autoimmune disorders in addition to its exocrine gland manifestations.

As it has, for majority of autoimmune diseases, genome-wide association study (GWAS) analysis has identified genetic traits that appear significantly associated with the development of the disease.¹ For the primary form of Sjögren's, these include genes involved in antigen processing (HLA), T-cell responses (STAT4), and B-cell survival

(BLNK), suggestive that dysregulation of the adaptive immune response is a significant contributor to disease pathogenesis.² These data are consistent with the pathological findings, which demonstrate that elevated infiltration of CD4⁺ T cells and B cells into the lacrimal and salivary glands is associated with a compromised gland function.

As for many autoimmune conditions, it is considered that to develop pathology, the specific genetic susceptibility traits require appropriate environmental signals. Given the significant increase of interferon responses in SS patients, viral stimuli have been suggested as such stimuli.³ Cross-reactivity between the SSA-specific autoantibodies, which comprise a class of clinical marker for SS, and Epstein Barr Virus (EBV) EBNA-1 protein was reported, illustrating a molecular mimicry between pathogenic antigenic determinants and host self-proteins.⁴ Furthermore, EBV-encoded small RNA is released by virally infected cells in complex with La/SSB protein and reinforces the connection between autoantibody generation and EBV infection.⁵

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However, viral stimuli are not the sole potential environmental triggers or exacerbating factors for autoimmune disease development; alterations in the repertoire and frequency of commensal microbiota have also been considered as contributing factors. Analysis of oral microbiota from primary Sjögren's patients showed an altered microbial diversity when compared to healthy individuals. Increased prevalence in commensal *Streptococci* sp. and a tendency for higher abundance of *Lactobacillus* sp. were reported in the saliva of primary SS patients⁶ and demonstrate the outgrowth of bacterial commensals. A comprehensive study of surface gingival plaque-associated microbiota reported a higher abundance of *Lactobacillus acidophilus* and a significant decrease in gram-negative species. Some of the isolated bacterial species did not belong to the normal healthy flora, such as *Collinsella aerofaciens* and genera *Tissierella* (previously known as a member of *Clostridium*), and demonstrate that SS patients harbor distinct oral tooth-associated microflora.⁷ Very few reports address whether the repertoire of ocular microbiota in primary Sjögren's patients is altered when compared to healthy individuals.⁸

The objective of our work was to define the biodiversity of microbial presence at the ocular surface with the expectation that alterations in ocular commensal abundance might precede disease-associated pathology or contribute to disease severity. To achieve this, we used a mouse model of SS: the thrombospondin-1-deficient (*TSP-1*^{-/-}) mice. Although born without any significant abnormalities, these mice develop spontaneous disease with aging as in humans with ocular clinical manifestations that closely resemble those reported in primary Sjögren's patients. The disease in these mice is characterized by increased apoptosis and deterioration of the lacrimal glands associated with CD4⁺ T-cell infiltration and development of SSA- and SSB-specific autoantibody responses.^{7,9} The significant loss of lacrimal gland secretory function is detectable at 8 weeks and precedes elevated expressions of IL-17, IL-6, and IFN- γ . These findings make the *TSP-1*^{-/-} mice an attractive and new model to study changes in ocular commensal and their contribution to SS disease pathogenesis.

Methods

Mouse strains

Ethics statement. All studies were performed in accordance with the Boston University Animal Care and Use Committee guidelines, Harvard University Animal Care protocols, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

C57BL/6 (H-2b) mice, 6–20 weeks old, were purchased from Charles River Laboratories. *TSP-1*^{-/-} mice (C57BL/6 background) were bred in-house in a pathogen-free facility at Boston University (Boston, MA).

Conjunctival swabs and bacterial growth

Conjunctival swabs were collected from wild-type (WT) (C57BL/6) mice and *TSP-1*^{-/-} mice at 7, 13, and 20 weeks of age. For the microbiological analysis, each swab was placed in 0.5 mL Luria-Bertani broth for 1 h at 37°C to enrich for bacteria; next, the bacterial culture was serially diluted down to 10⁻² and plated on 4 selective solid media plates: sheep's blood, chocolate, MacConkey, and mannitol

salt agar. Plates were incubated at 37°C and the colonies were counted the following day to enumerate the bacterial load in CFU/mL for each conjunctival swab.

Neutrophil isolation

Neutrophils were isolated as previously described.^{10–15} Briefly, bone marrow was isolated from hindquarters by flushing the femurs and tibias with 10 mL sterile phosphate-buffered saline (PBS) per mouse. This PBS suspension was run through a 70- μ m cell strainer and the cell pellet recollected with a subsequent 10-min centrifugation at 600g. To isolate polymorphonuclear cells (PMNs), a gradient of 3 mL 81%/2 mL 62%/2 mL 55%/2 mL 50% Percoll (GE Healthcare Life Sciences) was assembled and layered with the cells in a suspension of 3 mL 45% Percoll on top. This gradient was centrifuged without brake for 30 min at 1600g. The layer that lies between the 81% and 62% Percoll bands and contains mature neutrophils was collected and washed thrice with PBS. The collected cells were then layered over an equal amount of Histopaque 1119 (Sigma) and spun down for the removal of red blood cells.¹⁰ PMNs were suspended to a final working solution in Hanks' balanced salt solution without calcium and magnesium (HBSS^{-/-}; GIBCO) unless they were to be used immediately for the purpose of opsonophagocytic assay. The cell number and viability were evaluated by counting trypan blue dye impermeable and permeable cells. This purification procedure yielded 99% viable bone marrow-derived neutrophils with 90% purity as described in Siemsen et al.¹⁰ and Shan et al.¹⁶

Opsonophagocytic assay

The opsonophagocytosis of *Staphylococcus aureus* and *Staphylococcus coagulase-negative* sp by *TSP-1*-deficient and *TSP-1*-sufficient PMNs was compared. The assay was carried out as described by Gadjeva et al.¹⁵ and Dwyer and Gadjeva.¹⁷ Briefly, 1 $\times$ 10⁶ PMNs for each genotypic condition were resuspended in HBSS supplemented with Ca²⁺ and Mg²⁺ (HBSS^{+/+}; GIBCO) to support complement activation¹⁸ and incubated with target bacteria at multiplicity of infection and 25% autologous serum (extracted pericardially and purified with a microtainer) as a source of opsonins. A total reaction volume of 300- μ L reaction was assembled. Each condition was brought to volume with HBSS^{+/+} and incubated with rotation at 37°C for 90 min. Mixtures of bacteria and serum in the absence of neutrophils provided appropriate experimental controls. Additional controls included heat-inactivated serum to abrogate complement activation. Appropriate selective plates, including those with blood agar and *Staphylococcus*-selective mannitol agar solid media, were used to quantify bacteria at 0 and 90 min. The percent opsonophagocytic killing was calculated as in Dwyer and Gadjeva.¹⁷

Whole-cell enzyme-linked immunosorbent assay for detection of IgG

Bacterial suspensions made from *CNS* sp grown on blood agar plate were washed twice with PBS. Flat-bottom enzyme-linked immunosorbent assay (ELISA) plates (HB, Fisher) were coated with 100 μ L of OD₆₅₀=1 bacterial culture suspension and incubated overnight at 4°C. The plates were washed thrice with PBS-T (PBS/0.05% TWEEN

20) and blocked for 2 h with ELISA Blocker™ at room temperature. Following blocking, the plates were washed thrice with PBS-T and incubated with the serum samples (at 2-fold serial dilutions starting from 1/200 to 1/204800) for 2 h at room temperature. Following washing, the plates were incubated with 100 μ L of 1:1500 alkaline phosphatase-conjugated anti-mouse IgG (Sigma) for 2 h and developed using p-nitrophenyl phosphate (Sigma) (1 mg/mL) for 30 min at room temperature. The absorbance was measured using a TECAN multiplate reader. Titers were determined as the highest serum dilution factor that displayed a positive reading with a value 2-fold higher than the background as in Gadjeva et al.¹⁹

Assays to evaluate antimicrobial activity of TSP-1-derived 4N1K and 4NGG peptides

The 4N1K peptide (KRFYVVMWKK) was derived from the C-terminal receptor-binding domain of TSP-1 and has been identified as a ligand for the integrin-associated protein (IAP, CD47).²⁰ The 4NGG (KRFYGGMWKK) peptide was designed to lack the receptor-binding properties.²⁰ Both peptides were purchased from Bio Basic (BBI). A web-based antimicrobial peptide prediction (APD) tool (http://aps.unmc.edu/AP/prediction/prediction_main.php) was used to evaluate whether the 4N1K and 4NGG peptides have antimicrobial capacity. TSP-1-derived peptides—4N1K and 4NGG—were incubated in a 100 μ L total reaction volume at prescribed concentrations ranging from 0.1 μ M through 10 μ M with 0.5×10^4 bacteria of tested *Staphylococcus sp* for 1, 3, and 6 h at 37°C, with shaking. For the *Pseudomonas aeruginosa* peptide-mediated killing experiments, 0.5×10^5 CFU were exposed to 10- μ M and 50- μ M peptide for 1 and 3 h *in vitro*. For peptide killing assays, the Luria-Bertani (Beckton Dickinson) broth was used as the incubation growth medium for the *Staphylococcus* species and the Tryptic Soy Broth (Beckton Dickinson) was used for *P. aeruginosa*. To avoid bacterial clumping, bacterial serial dilutions were prepared in DMEM/F12 (Life Technologies)/5% HI-FBS (GIBCO)/0.01% Triton X-100, and 10 μ L of each reaction was plated onto either a blood agar plate (for *P. aeruginosa*) or a mannitol salt agar plate (for the *Staphylococcus sp*) to quantify bacterial growth.

Statistical analysis

Data were analyzed with GraphPad Prism 5.0 software (GraphPad Software, Inc.). The sample distribution was analyzed for normality. When appropriate, data were compared using Student's *t*-test. The comparisons of bacterial presence in the conjunctival swabs from the TSP-1^{-/-} and WT C57BL/6 control mice were carried out using Fisher's exact test.

Results

TSP-1-deficient mice show alterations in the ocular microbiota

The issue of whether ocular microbiota permanently exists in humans is a contentious one. Various classes of bacteria have been reported to be present using 16S sequencing approaches²¹ and prompt the question whether ocular microbiota is a reflection of skin microbiota and

TABLE 1. FREQUENCY OF *ALPHA-HEMOLYTIC STREPTOCOCCUS SP* DETECTED IN THE CONJUNCTIVAL SWABS FROM THE TSP-1^{-/-} AND C57BL/6 MICE

Genotype	Week 7	Week 13	Week 20
TSP-1	0/12	0/11	0/7
WT (C57BL/6)	1/16	0/12	2/13
Fisher's exact test	Not significant	Not significant	Not significant

Data were pooled from 3 individual experiments. Data are tabulated in a contingency format and are presented as the number of animals showing bacterial presence in the conjunctival swabs out of the total number of evaluated animals (Fisher's exact test).

TSP-1, thrombospondin-1; WT, wild type.

whether it changes during pathological conditions. To monitor ocular microbiota, conjunctival swabs were collected from TSP-1^{-/-} and WT C57BL/6 mice over a span of time of within 20 weeks of age. We observed that the frequency of ocular bacterial presence increased with aging in the WT and TSP-1^{-/-} mice. At 7 weeks of age, individual C57BL/6 mice showed detectable α -hemolytic *Streptococcus sp* growth, although this was cleared at subsequent weeks (Table 1). All of the conjunctival swabs harvested from the 7-week-old TSP-1^{-/-} mice showed no detectable bacterial growth on nonselective rich agar media. In contrast, the conjunctival swabs from the 13-week-old TSP-1^{-/-} mice were enriched for *CNS sp* (Table 2) and individual *S. aureus* colonies were present, whereas fewer WT C57BL/6-derived swabs showed no significant bacterial presence (Table 2). The tendency for enriched presence of *Staphylococcus sp* was preserved at week 20, at which point, all the TSP-1^{-/-} mice maintained colonization with *CNS sp* and few WT mice had *CNS sp*. These results indicate that TSP-1 deficiency results in earlier colonization with commensals (Table 2).

TSP-1-deficient mice have defective opsonophagocytosis of ocular commensals

An increased PMN infiltration was reported by 12 weeks of age in TSP-1-deficient conjunctiva and suggests an ongoing anti-infectious response.⁹ To determine whether TSP-1-dependent regulation of ocular microbiota frequency is a reflection of functionally defective PMNs, we evaluated the degree of phagocytosis of *CNS sp* and *S. aureus* in TSP-1-sufficient and TSP-1-deficient neutrophils. Bone marrow-derived neutrophils were purified, mixed with fresh mouse-derived serum as a source of mouse complement, and exposed to commensal *Staphylococcus sp*. The TSP-1-deficient neutrophils showed a significant reduction in

TABLE 2. FREQUENCY OF *STAPHYLOCOCCUS SP* DETECTED IN THE CONJUNCTIVAL SWABS FROM THE TSP-1^{-/-} AND C57BL/6 MICE

Genotype	Week 7	Week 13	Week 20
TSP-1	1/12	9/11	7/7
WT (C57BL/6)	2/16	1/12	3/13
Fisher's exact test	Not significant	0.0005	0.0031

Data were pooled from 3 individual experiments. Data are tabulated in a contingency format and are presented as the number of animals showing bacterial presence in the conjunctival swabs out of the total number of evaluated animals (Fisher's exact test).

the ability to opsonophagocytose either the commensal *S. aureus* or the *CNS* sp (Fig. 1). To evaluate whether the defects in the phagocytosis were due to alterations in the natural antibody levels recognizing the commensal strains, *CNS* sp-specific IgG titers were quantified. Surprisingly, the *TSP-1*^{-/-} mice showed a significant increase in the *CNS*-specific titers when compared to the C57BL/6 mice, which corroborated the colonization data, but did not result in improved opsonophagocytosis (Fig. 1). These results suggest that a functional deficit resulting from the TSP-1 deficiency in PMNs permits abundance of ocular commensals.

TSP-1-derived peptide exhibits antimicrobial activity

Homology alignment analysis of the amino acid sequence of 4N1K peptide revealed that it is unique, sharing no existing

homologies with any other currently known antimicrobial peptide. The Wimley–White whole-residue hydrophobicity of the 4N1K peptide was -0.23 kcal/mol, with aliphatic index of 58. The peptide carries 5 hydrophobic residues and 4 charged residues, a composition that fits the requirements for the antimicrobial activity. An APD-based prediction of the peptide structure suggests a possible helical structure that may interact with bacterial membranes and have the antimicrobial activity. Indeed, the 4N1K peptide was active against both commensal *CNS* sp and *S. aureus* strains isolated from the colonized *TSP-1*^{-/-} conjunctivas (Fig. 2). Replacement of 2 amino acids to generate the 4NGG peptide²⁰ lead to loss of the antimicrobial activity of the peptide. At 10 μ M, the 4N1K peptide showed a prominent antimicrobial activity against the *CNS* sp (Fig. 2) and this activity was reduced to 50% at 5 μ M and less than 10% at 1 μ M (data not shown), illustrating a dose–response dependence. Interestingly, the antimicrobial activity of the 4N1K peptide was not limited to gram-positive bacteria, since such could be detected against *P. aeruginosa* strains *PAO1* and *PA14* as well (Fig. 3). However, the activity was much weaker and detectable at 50 μ M. In addition, the antimicrobial activity against the *PA14* strain was weaker than that against the *PAO1* strain, as no killing was observed within the first hour of the reaction.

Discussion

With aging, the *TSP-1*^{-/-} mice develop ocular signs of SS.²² The soluble mucin secretion by the conjunctival goblet cells is reduced at 6 weeks of age, and secretory abnormalities are detected in the lacrimal glands by the eighth week; together, these events result in significantly disrupted protective ocular surface barrier by 12 weeks of age.²² By that time, all of the *TSP-1*^{-/-} mice show significant presence of *CNS* sp, demonstrating that the compromised barrier function in the *TSP-1*^{-/-} mice sensitizes them to increased conjunctival colonization.

Given that TSP-1 binds to the *Staphylococcus* sp peptidoglycan and major autolysin adhesin (Atl),^{23,24} it is tempting to ask what the functional outcome of this interaction is. It was proposed that this promotes bacterial colonization.²⁴ However, we suggest that TSP-1 facilitates commensal clearance, while regulating the inflammatory milieu. Consistently, we detected a significant opsonophagocytic killing of the commensal strains by the C57BL/6-derived neutrophils, while *TSP-1*-deficient neutrophils were defective in phagocytic killing. In agreement with this observation is the finding that CD36, another TSP-1 receptor, promotes internalizations of *Staphylococcus* sp.^{25,26} Since cumulatively these data show impaired *Staphylococcus* sp clearance in the absence of either CD36 or TSP-1, it is likely that CD36 mediated bacterial clearance in the TSP-1-dependent way.

Furthermore, we provide evidence that the TSP-1-derived peptide, 4N1K, has an antimicrobial activity against a commensal *CNS* sp and *S. aureus* sp in the presence of physiologically relevant salt concentrations (300 mOsmol/L). Many other tear proteins, including α - and β -defensins, and cathelicidin have antimicrobial activities against commensals and pathogens; however, only few (eg, lipocalin-derived peptide) are insensitive to the ionic strength of normal tear fluid.^{27–29} Similarly, other TSP-derived peptides were previously reported to carry an antimicrobial activity against *Escherichia*

FIG. 1. Thrombospondin-1 (*TSP-1*^{-/-}) neutrophils fail to effectively phagocytose commensal *Staphylococcus aureus* and *CNS* sp. (A) Bone marrow-derived PMNs were exposed to commensals in the presence of 25% serum for 90 min, and aliquots were plated on selective mannitol agar plates. Data are representative of 2 experiments carried out in duplicate. Student's *t*-test. (B) *CNS* sp-specific IgG titers in serum of *TSP-1*^{-/-} ($n=7$) and C57BL/6 ($n=7$) mice. To determine the antibody titers, serial dilutions of serum derived from the *TSP-1*^{-/-} and wild-type (WT) mice were exposed to bind to the *CNS* sp. Data are representative of 2 experiments carried out in duplicate. Student's *t*-test. *****p*-values <0.05 . *CNS* sp, coagulase negative staphylococci sp; PMN, polymorphonuclear.

FIG. 2. TSP-1-derived peptide 4N1K has an antimicrobial activity against the commensal strains derived from the conjunctival swabs. (A) 4N1K has an antimicrobial activity against commensal *S. aureus*. Reactions with 10- μ M peptide and 0.5×10^4 bacteria were set up for 1, 2, and 6 h, and aliquots were plated. (B) 4N1K has an antimicrobial activity against commensal *CNS sp*. The percent bacterial killing by 4N1K was compared with that of the control 4NGG peptide. Aliquots of the reactions were plated at 1, 2, and 6 h. Data are representative of experiments carried out in duplicate. Each condition was evaluated in triplicate. Student's *t*-test.

FIG. 3. TSP-1-derived peptide 4N1K has an antimicrobial activity against *Pseudomonas aeruginosa* PAO1 and PA14. The percent bacterial killing by 4N1K was compared with that of the control 4NGG peptide. Aliquots of the reactions were plated at 1 and 3 h. Data are representative of experiments carried out in duplicate, where each condition is evaluated in triplicate. Student's *t*-test. Bacterial growth was observed in the samples containing *PAO1* treated with 4NGG peptide for 3 h and in the samples containing *PA14* at 1 h. Bacterial killing was observed in all samples treated with the 50- μ M 4N1K peptide.

coli and *P. aeruginosa*, but these peptides displayed no activity against *Staphylococcus sp* and were functional under low-salt conditions.³⁰ Indeed, the 4N1K peptide is active against both the commensal *CNS sp* and *S. aureus* isolates. Also, the antimicrobial activity of the peptide is not limited to those strains, as 4N1K has an antimicrobial activity against *P. aeruginosa* PAO1 and PA14. More than 90% killing of *P. aeruginosa* was achieved with 138 μ g/mL 4N1K peptide. Cumulatively, these data show that TSP-1 has a detectable antimicrobial activity against commensal bacterial strains typically found at the conjunctival surfaces.

The elevated presence of *CNS sp* in the *TSP-1*^{-/-} mice resembled what was reported by Albiets and Lenton, who showed elevated numbers of *CNS sp* and *S. aureus* species isolated from the lid and conjunctiva in primary Sjögren's patients.³¹ These findings provoke an interesting question, namely, whether microbial abundance and repertoire are altered during SS and whether any alterations in ocular

microbiota exacerbate the disease severity. Although there are only few studies addressing potential alterations in bacterial microbiota at the ocular surface in SS patients,^{8,31} the data support the supposition that an elevated commensal presence exists, which may exacerbate inflammatory responses at the ocular surface, suggesting that the therapeutic strategies should adopt a modality to control commensal outgrowth.

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Author Disclosure Statement

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