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Thymosin $\beta4$ Alleviates Autoimmune Dacryoadenitis via Suppressing Th17 Cell Response

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Purpose. We investigated the therapeutic effect of recombinant thymosin $\beta4$ (rT$\beta4$) on rabbit autoimmune dacryoadenitis, an animal model of SS dry eye, and explore its mechanisms.

Methods. Rabbits were treated topically with rT$\beta4$ or PBS solution after disease onset for 28 days, and clinical scores were determined by assessing tear secretion, break-up time, fluorescein, hematoxylin and eosin staining, and periodic acid-Schiff. The expression of inflammatory mediators in the lacrimal glands were measured by real-time PCR. The expression of T helper 17 (Th17) cell-related transcription factors and cytokines were detected by real-time PCR and Western blotting. The molecular mechanism underlying the effects of rT$\beta4$ on Th17 cell responses was investigated by Western blotting.

Results. Topical administration of rT$\beta4$ after disease onset efficiently ameliorated the ocular surface inflammation and relieved the clinical symptoms. Further analysis revealed that rT$\beta4$ treatment significantly inhibited the expression of Th17-related genes (RORC, IL-17A, IL-17F, IL-1R1, IL-23R, and granulocyte-macrophage colony-stimulating factor) and IL-17 protein in lacrimal glands, and meanwhile decreased the inflammatory mediators expression. Mechanistically, we demonstrated that rT$\beta4$ repressed the phosphorylation of signal transducer and activator of transcription 3 (STAT3) both in vivo and in vitro. Activation of the STAT3 signal pathway by Colivelin partly reversed the suppressive effects of rT$\beta4$ on IL-17 expression in vitro.

Conclusions. rT$\beta4$ could alleviate ongoing autoimmune dacryoadenitis in rabbits, probably by suppressing Th17 response via partly affecting the STAT3 pathway. These data may provide a new insight into the therapeutic effect and mechanism of rT$\beta4$ in dry eye associated with Sjögren's syndrome.

Keywords: recombinant thymosin $\beta4$, autoimmune dacryoadenitis, Th17 cells, STAT3, inflammatory mediators

Sjögren's syndrome (SS) dry eye is a chronic autoimmune eye disease characterized by lymphocytic infiltration of lacrimal glands (LGs) and ocular surface, causing severe gland dysfunction and visual impairment.[1–3] The pathogenesis of the disease remains unknown, and adequate therapies have been lacking until now. Current available interventions include tear substitutes that provide only temporally symptom improvement and conventional drugs such as immunomodulatory agents and corticosteroids may yield non-negligible side effects.[4,5] Hence, developing more effective and safe drugs is of great need.

Accumulating evidence suggested that pathological T cells, especially T helper 17 (Th17) cells, play an important role in the inducing and development of SS dry eye.[6–9] Elevated levels of Th17 cells and IL-17 expression in exocrine glands and peripheral blood were correlated positively with disease severity in patients with SS,[10,11] and increased expression of Th17-related cytokines such as IL-17 was observed in tears of SS patients with dry eye.[7,12] Clinical trials using inhibitors of the IL-17–Th17 pathway have obtained positive results in several chronic inflammatory diseases, such as ankylosing spondylitis and rheumatoid arthritis.[13] Some studies proposed that inhibition of Th17 response might benefit for SS treatment.[14,15] These findings indicated that inhibiting Th17 cells and their signature cytokines may be a potential therapeutic strategy for SS dry eye.

Thymosin $\beta4$ (T$\beta4$) is a highly conserved and water-soluble actin chelating peptide with multiple physiological properties, including promoting wound healing, inhibiting apoptosis and oxidation, and downregulating inflammatory responses.[16,17] It has been reported previously that topical T$\beta4$ administration relieved signs in non-SS dry eye mice.[18,19] In clinical studies, administration of T$\beta4$ eye

drops significantly ameliorated corneal staining, tear film break-up time (BUT), tear volume production, and ocular discomfort level in patients with graft or host disease-related dry eye disease.[20,21] However, the therapeutic efficacy and mechanism of Tβ4 on SS dry eye have been rarely studied.

Here, we investigated the therapeutic effect of recombinant Tβ4 (rTβ4) on an autoimmune dacryoadenitis rabbit model and explored the potential mechanism, especially the regulation on Th17 cells. We first demonstrated that rTβ4 could alleviate rabbit autoimmune dacryoadenitis and suppress Th17 cell response, which might be associated with inhibiting the signal transducer and activator of transcription 3 (STAT3) pathway.

Materials and Methods

Animal Experiments

Animals. Adult female New Zealand white rabbits (2.5–3.0 kg) were purchased from Vital River Laboratory Animal Technology (Beijing, China). All animal experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by Institutional Animal Care and Use Committee of Tianjin Medical University. All rabbits were raised under pathogen-free conditions with the cycle of 12-hour light–dark (8 AM to 8 PM) and a relative humidity of 50% to 75% in a temperature-controlled room. Careful ocular examinations were performed on all animals to exclude any preexisting eye defects.

Induction of Autoimmune Dacryoadenitis. The left inferior LGs of rabbits were surgically resected under anesthesia for the isolation of purified LG epithelial cells (pLGECs), as described previously.[22] After 2 days of culturing, the pLGECs were irradiated and then cocultured with an equal number of autologous peripheral blood lymphocytes (PBLs) for 5 days. In the following, the activated PBLs (2×10^6 cells) were harvested gently and injected into the ear veins of the donor rabbits to induce autoimmune dacryoadenitis.

Grouping and Treatment Procedures. To study the effect of Tβ4 administration on autoimmune dacryoadenitis, topical eye drops were administered from week 2 after adoptive transfer and continued for 4 weeks. Rabbits were divided randomly into four groups: (1) normal group (normal) ($n = 6$), (2) untreated autoimmune dacryoadenitis group (model) ($n = 6$), (3) PBS-treated group (PBS) ($n = 6$), and (4) 1000 µg/mL rTβ4-treated group (rTβ4) ($n = 6$). The eye drops were administrated 4 times per day (8 AM, 12 AM, 4 PM, 8 PM), and this rTβ4 therapeutic concentration was based on the results of our preliminary experiments, which has been found to be most effective for autoimmune dacryoadenitis in rabbits (Supplementary Fig. S1). A total of 50 µL of solution were administrated to both eyes of rabbits each time. rTβ4 (Lot. Y-20190701) was prepared by Beijing Northland Biotech Co., Ltd. (Beijing, China). The concentrated solutions (11.99 mg/mL) of rTβ4 were stored at −20°C in pH 7.0 phosphate buffer solution, then diluted and applied. The 1000 µg/mL rTβ4 eye drop was made by diluting aliquots of the 11.99 mg/mL rTβ4 with sterilized PBS. All formulations administered to the rabbits were sterilized using 0.2-µm sterile syringe filters before use.

Clinical Assessment of Autoimmune Dacryoadenitis and Histologic Analysis

Clinical Assessment of Autoimmune Dacryoadenitis. The assessment was performed on the right eyes of all rabbits every 2 weeks after initial rTβ4 eye drops management, as described previously.[22,23] Schirmer's test was carried out to test tear production with a Schirmer strip. The wetted area of the strip was measured 1 minute after insertion into the lower fornix of the eye. Tear BUT was measured to assess tear film stability. After dripping 2% fluorescein into the middle of the lower eyelid, the BUT was recorded as the number of seconds that elapse between the last blink and the appearance of the first dry spot in the tear film. The evaluation of the punctate corneal staining was performed with a slit-lamp biomicroscope under cobalt blue light in a masked manner. The observer graded the disease by assigning between 0 and 4 for each of the four areas and the scores from four regions were summed to a final grade (16 points).

Histologic Analysis of LGs and Conjunctivas. Rabbits were sacrificed after 4 weeks of rTβ4 treatment, and the right inferior LGs were harvested and fixed in 10% formalin and embedded in paraffin wax using standard methods. LG sections were stained with hematoxylin and eosin and scanned with light microscopy (BX51; Olympus Corporation, Tokyo, Japan). The pictures were photographed with Cell Sen software (Olympus). According to the definition, a focus is an aggregate of more than 50 lymphocytes. The total number of focus/4 mm^2 of LG tissue was calculated by a pathologist masked to animal grouping. Samples of conjunctiva were also treated as described elsewhere in this article.[22,23] According to previous reports,[24,25] the levels of acini atrophy were analyzed by semiquantitative analyses that from the LG samples, 20 acini per sample were randomly selected and the mean area (in square micrometers) was measured using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Measurement of Conjunctival Goblet Cells Density. In the fourth week after rTβ4 treatment, all groups of rabbits were sacrificed and a bulbar conjunctival biopsy was performed. To avoid the influence of different conjunctival topographical location on conjunctival goblet cells density, we obtained the conjunctival biopsies superior to the cornea between the rectus lateralis and rectus medialis.[26] The slices were processed by conventional techniques. The sections of conjunctival surface were rehydrated and stained by periodic acid-Schiff. The total number of conjunctival goblet cells/4 mm^2 was counted under an optical microscope by a pathologist masked to the experimental group.

Cell Culture

The PBLs were isolated from the dry eye model rabbits and cocultured with 100 ng/mL rTβ4 in the presence of irradiated pLGECs for 72 hours. rTβ4 concentration used for in vitro experiment was chosen according to our preliminary experiments (Supplementary Fig. S3). Colivelin (sc-361153, Santa Cruz Biotechnology Inc. Dallas, TX, USA) was dissolved in dimethyl sulfoxide, stored in 1.5-mL sterile plastic tubes at −20°C, thawed, and used on the day of the experiment at a final concentration of 0.5 µM based on previous studies.[27,28]

Table. Gene-Specific Primers Used for qRT-PCR (Rabbit)

Gene	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	5′-GGGTGGTGGACCTCATGGT-3′	5′-CGGTGGTTTGAGGGCTCTTA-3′
IL-17A	5′-GGAATGAGGACCACCACATGA-3′	5′-CTGCGTAGGACCAGGATCTCTT-3′
IL-17F	5′-CCCCCTCTGGAGGACAACA-3′	5′-TCCGTGGTTTTGACTGAGGAT-3′
RORC	5′-GGCCTACCACGCCGA-3′	5′-TCCATGCCACCGTATTTGC-3′
IL-6	5′-GCAGAAAAACCAGTGGCTGAA-3′	5′-GGCCGCGCAGGATGA-3′
TNF-α	5′-AGCTTCTCGGGCCCTGAGT-3′	5′-CCACTTGCGGGTTTGCTACT-3′
IL-1β	5′-CTCCTGCCAACCCTACAACAA-3′	5′-TCCAGAGCCACAACGACTGA-3′
MMP-9	5′-CCAGTACCGAGAGAAAGCCTACTT-3′	5′-CCTCGTTCCGGGTACTCACA-3′
MMP-2	5′-GCGCGCCTTCCAAGTCT-3′	5′-CATCGTGGATTCGAGAAAACC-3′
IL-23R	5′-TGTGGCATAGCCGGTAAAGC-3′	5′-AACTGGCGCCCATATGGAA-3′
IL-1R1	5′-TGTACAGGAAAAGGCATTCATGA-3′	5′-GCAGGGCTACGACACAAACA-3′
GM-CSF	5′-CCAGCCCTTGAAGCATGTG-3′	5′-TTACTGCGGCTCAGGATGATC-3′
IL-23	5′-AGGAGTGTCTTGCGAATGTGAT-3′	5′-AGCAGGAGCAGGGTTGATG-3′
CXCL-2	5′-GTGTCATTCTTCCGTGACCAGA-3′	5′-CCCCTTTTATGCCCATTCG-3′
CXCL-8	5′-GACACGGATTGGTACAGAGCTT-3′	5′-TTGGGGTCCAGGCAGAGTT-3′
CCL-20	5′-TAACTTTGACTGCTGCCTTCG-3′	5′-TTTTCACCCATTCCTTCTTCG-3′

GADPH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase.

Quantitative RT-PCR (qRT-PCR)

Total RNA from harvested cells or tissues was isolated by EZ-press RNA Purification Kit (EZBioscience, Roseville, CA, USA). The purity and quality of RNA were measured by a NanoDrop ND-2000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA). Then, the first strand of cDNA was synthesized using a reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was then performed with FastStart Universal SYBR Green PCR Master mix (Applied Biosystems, Waltham, MA, USA) using a LightCycler 480 II System (Roche Diagnostics GmbH, Mannheim, Germany). The gene-specific primers are listed in Table. Glyceraldehyde 3-phosphate dehydrogenase as used as the endogenous control for each sample. The relative target mRNA expression was calculated using the following equation: relative expression $= 2^{[\Delta Ct(control) - \Delta Ct(target)]}$.

Western Blot Analysis

Total protein from cells or tissues was obtained using ice-cold lysis buffer and quantified with the Bicinchoninic Acid Protein Assay Kit (Solarbio, Beijing, China). After protein extraction, equal amounts of protein were loaded on SDS-PAGE and transferred to polyvinylidene fluoride membranes (MilliporeSigma, Burlington, MA, USA). The membranes were incubated in 5% fat-free milk for 2 hours. We used primary antibodies against IL-17 (1:666, cat. MAA063Rb21; Cloud-Clone Corp, Wuhan, China), phosphostat3 (Y705) (1:500, cat. MAB4607; R&D Systems, Minneapolis, MN, USA), IL-1β (1:250, cat. PAA563Rb51; Cloud-Clone Corp), IL-6 (1:1000, cat. MAA079Rb21; Cloud-Clone Corp), and β-actin antibody (1:2000, cat. ZB-5301; ZSGB-BIO, Beijing, China). Horseradish peroxidase (HRP)-linked anti-mouse IgG (1:5000, cat. #7076S; Cell Signaling Technology, Danvers, MA, USA), HRP-linked anti-cavia IgG (1:8000, cat. SAA544Gu09; Cloud-Clone Corp), and HRP-linked anti-rat IgG (1:2000, cat. SA00001-15; Proteintech, Wuhan, China) were used as secondary antibodies. After incubating the membranes with primary antibodies and secondary antibodies, we detected positive bands with Multispectral Imaging System (UVP, Tanon, Beijing, China) and analyzed them with Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analyses

All experiments were performed in triplicate. The data were analyzed using SPSS 25.0 software (IBM Corporation, Somers, NY, USA) and Graph Pad Prism 8.0 (GraphPad, San Diego, CA, USA), with presentation as mean ± SD. We performed the Shapiro–Wilk test to test the normality of the data. When normality was not rejected, comparisons between two groups were made using the Student t test and comparisons of three groups were analyzed by ANOVA. As for nonparametric data, we performed the Mann–Whitney *U* test (comparison of two groups) or the Kruskal–Wallis test (comparison of three groups) for the intergroup differences. A *P* value of less than 0.05 was considered statistically significant.

Results

rTβ4 Attenuated Autoimmune Dacryoadenitis Clinically and Histologically

Rabbits with autoimmune dacryoadenitis usually showed evident clinical dry eye symptoms from the second week after adoptive transfer of activated PBLs.[22,29] To investigate the therapeutic effect of rTβ4 on rabbit autoimmune dacryoadenitis, 1000 μg/mL rTβ4 or PBS was administered topical instillation on both eyes four times per day after disease onset (2 weeks after transfer) for continued 4 weeks (Fig. 1A). Disease severity was evaluated every 2 weeks by assessing tear production, tear BUT, and corneal fluorescein staining. As summarized in Figures 1B through E, the untreated autoimmune dacryoadenitis group and the PBS-treated group displayed a severe disease, manifested as decreased tear production, shortened tear BUT, and increased corneal fluorescein staining score after disease induction. By contrast, the rTβ4-treated group showed significantly attenuated clinical symptoms at the second and fourth weeks after rTβ4 treatment.

To further validate the effect of rTβ4, the histological examination was performed after 4 weeks of treatment. As shown in Figures 2A through C, rTβ4 treatment significantly decreased the infiltration of inflammatory cells in LGs and conjunctivas, as well as reversing the loss of conjunc-

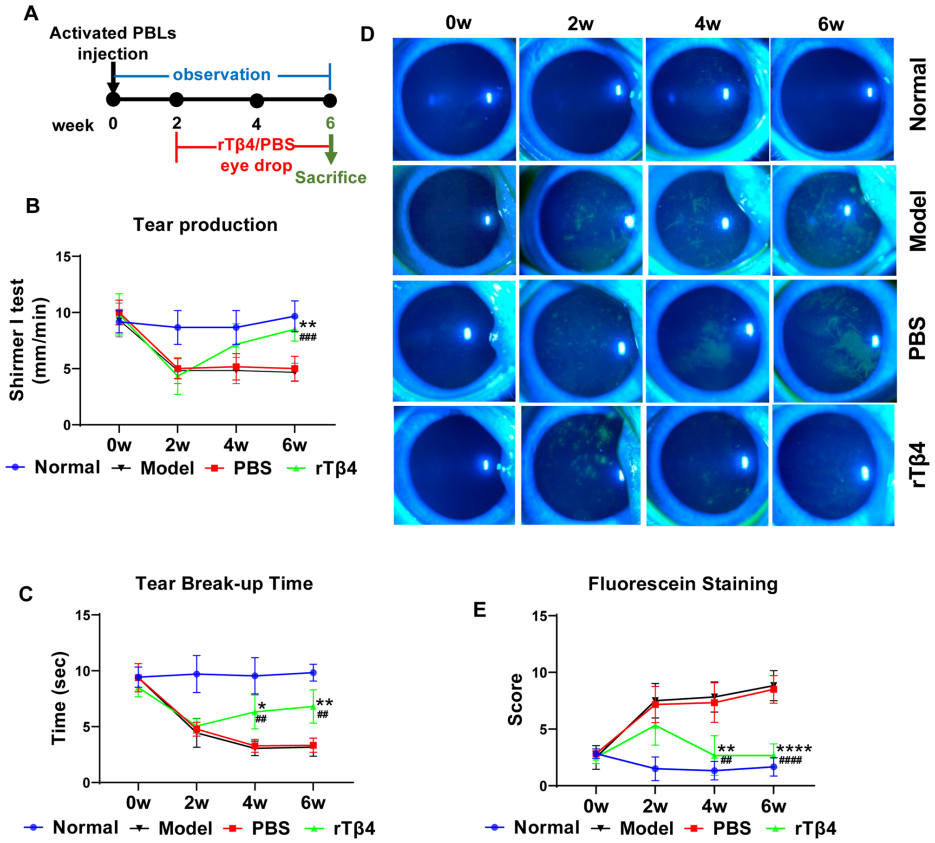

Figure 1. rTβ4 reduced clinical signs of rabbit autoimmune dacryoadenitis ($n = 6$). (**A**) Schema of 1000 µg/mL rTβ4 treatment in rabbit autoimmune dacryoadenitis. (**B**) Tear production was measured using Schirmer's test before and after 1000 µg/mL rTβ4 administration. (**C**) Tear BUT was measured using slit-lamp examination to evaluate the tear stability. (**D** and **E**) Corneal fluorescein staining was imaged by a slit lamp biomicroscope and scores were measured. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ vs. the corresponding value in the PBS group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs. the corresponding value in the model group. The statistical significance of corneal fluorescein staining scores, Schirmer's test, and tear BUT were determined by two-way ANOVA.

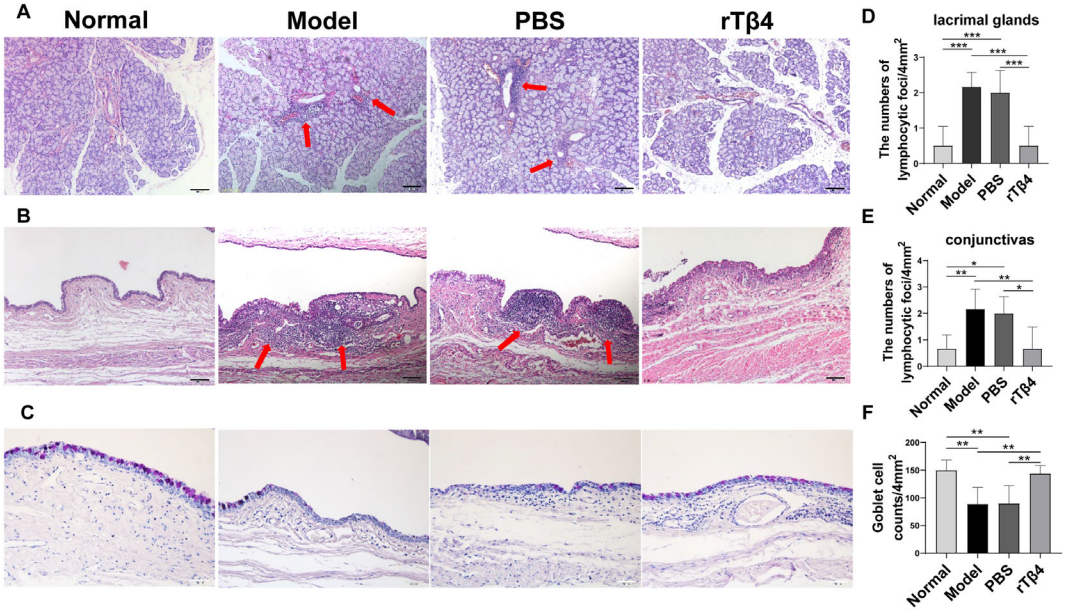

Figure 2. rTβ4 treatment suppressed inflammatory cell infiltration in LG and conjunctiva and increased conjunctival goblet cell density ($n = 6$). (**A**, **B**) Hematoxylin and eosin–stained photographs of LGs and conjunctivas were represented. *Arrow* indicates local lymphocytic foci (>50 infiltrating lymphocytes) around vascular or ductal. (**C**) Conjunctival goblet cell density (mean ± SD) as detected by enumerating filled goblet cells in periodic acid-Schiff (PAS)-stained histological sections of conjunctiva. (**D**, **E**) Numbers of lymphocytic foci per 4 mm2 in LGs and conjunctivas were evaluated. (**F**) Numbers of filled goblet cells per 4 mm2 in conjunctivas were evaluated ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The statistical significance was determined by one-way ANOVA.

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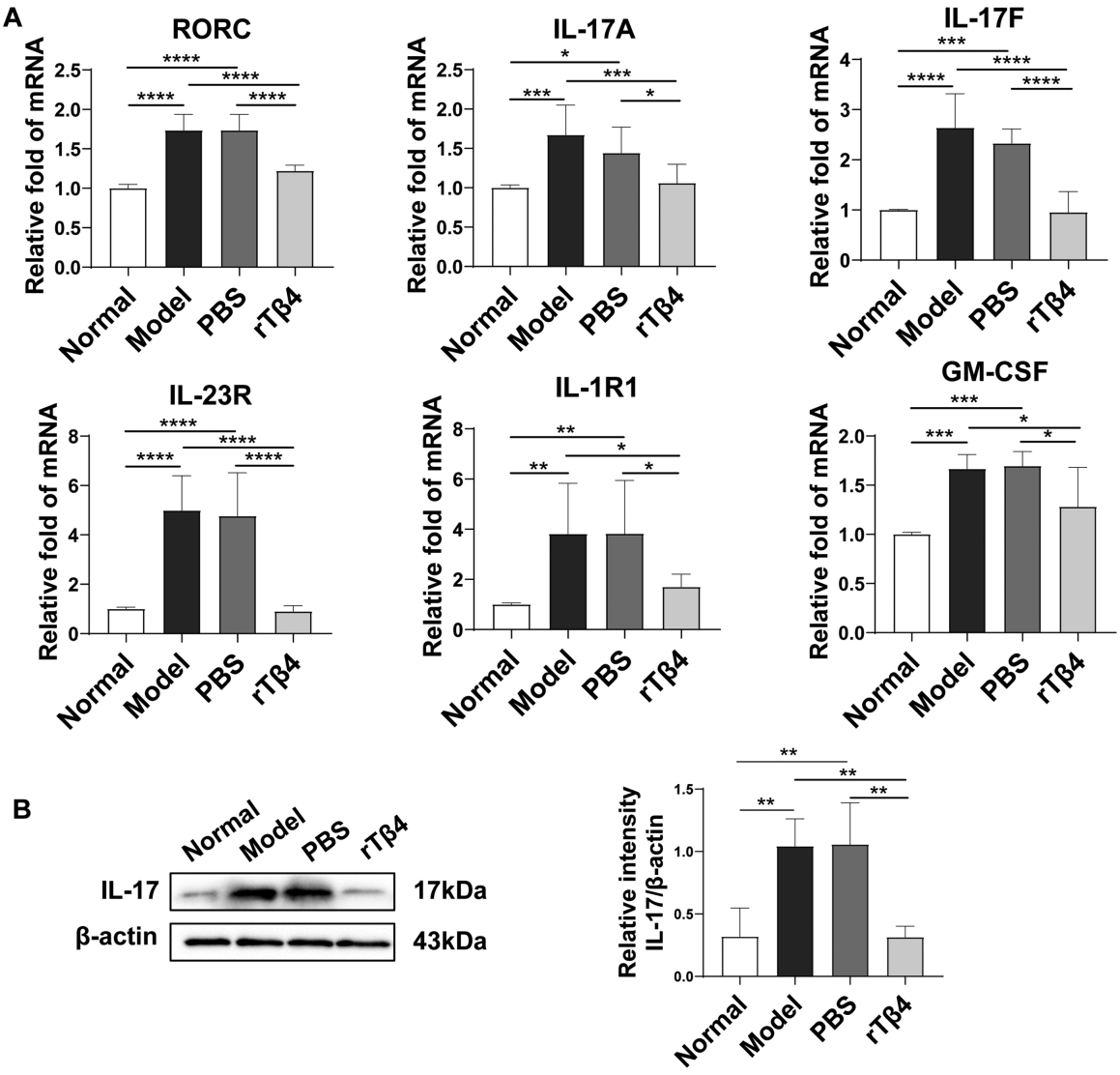

Figure 3. Administration of 1000 µg/mL of rTβ4 regulated Th17 in vivo. Rabbits were sacrificed at the end of treatment and LGs were collected. Tissues were subjected to qRT-PCR or Western blot analysis. (**A**) mRNA expression of Th17-related genes (RORC, IL-17A, IL-17F, IL-23R, IL-1R1, GM-CSF) were analyzed by qRT-PCR. (**B**) The protein level of IL-17 expression was analyzed by Western blot assay. And relative protein ratio of IL-17 to β-actin was quantified. Data were representative of three independent experiments ($n = 3$ rabbits per group in each experiment) and bar graphs indicated mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

tival goblet cell density. The levels of acini atrophy were not significantly different between the PBS-treated group and rTβ4-treated group (Supplementary Fig. S2). Together, administration of rTβ4 eye drops attenuated the severity of rabbit autoimmune dacryoadenitis effectively.

rTβ4 Decreased Th17 Responses in LGs

Th17 cells have been reported to play an essential role in the pathogenesis of SS dry eye.[8] To clarify the underlying mechanism by which rTβ4 relieved the rabbit autoimmune dacryoadenitis, we investigated the effects of rTβ4 on Th17 cell response in inflamed LGs. As shown in Figure 3A, the expression of Th17-related genes, including RORC, IL-17A, IL-17F, IL-23R, IL-1R1, and granulocyte-macrophage colony-stimulating factor (GM-CSF), was all significantly decreased in the inflamed LGs of the rTβ4-treated group compared with those of the PBS-treated group. In addition, Western blot analysis showed that rTβ4 administration led a significantly decreased IL-17 protein expression in LGs (Fig. 3B). Collectively, these findings suggest that rTβ4 may suppress the Th17 immune response, thereby alleviating rabbit autoimmune dacryoadenitis.

rTβ4 Suppressed the Expression of Inflammatory Mediators in LGs

To investigate the effect of rTβ4 on lacrimal inflammatory responses in SS dry eye, the mRNA expression of inflammatory mediators in LGs were determined by qRT-PCR. As indicated in Figure 4A, compared with normal controls, a significant increased expression of inflammatory mediators, including IL-6, IL-1β, IL-23, TNF-α, matrix metalloproteinases-2, matrix metalloproteinase-9,

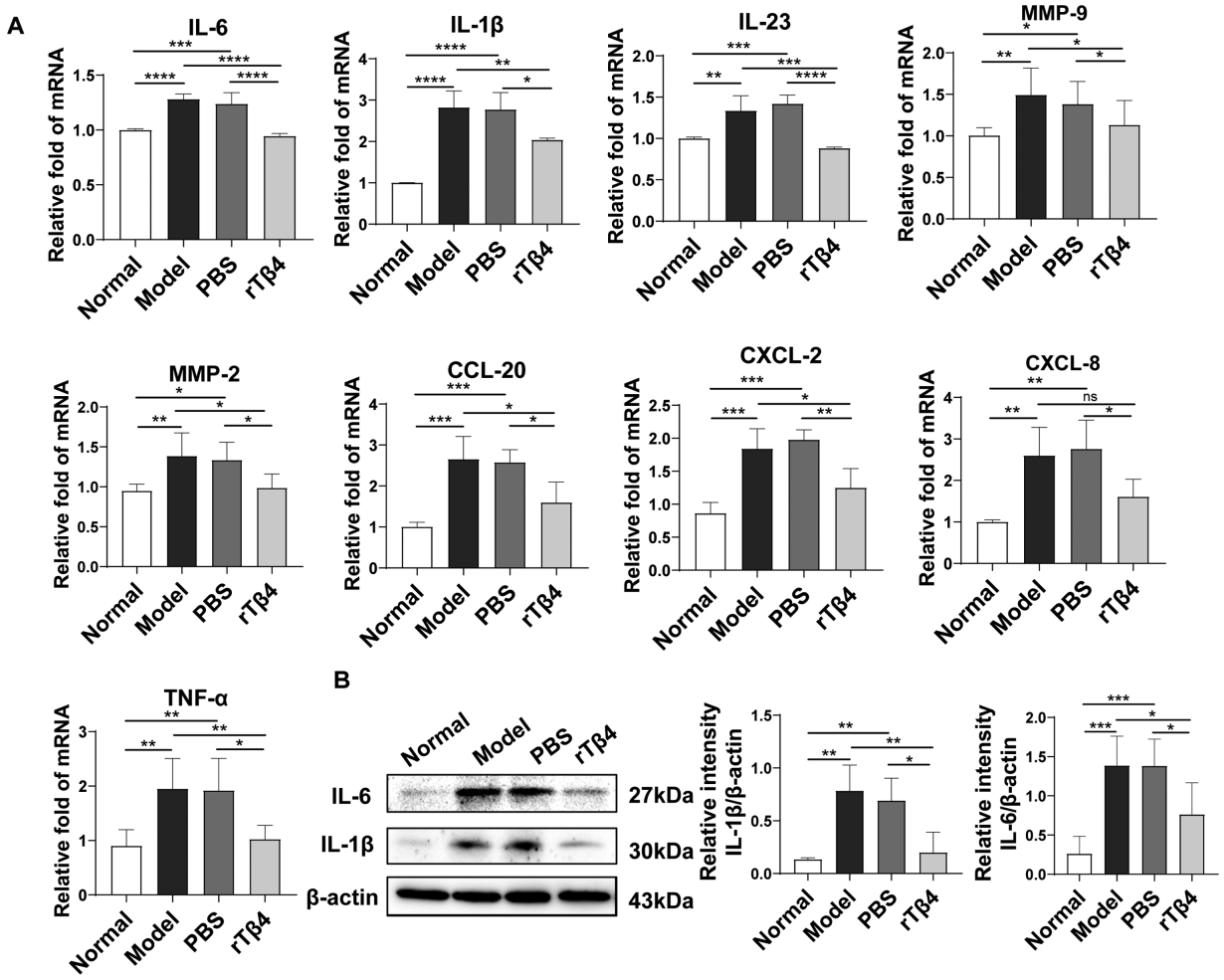

Figure 4. In vivo administration of 1000 μg/mL rTβ4 decreased proinflammatory cytokines. Rabbits were sacrificed at the end of treatment and LGs were collected. Tissues were subjected to qRT-PCR and Western blot. (**A**) Gene expression profiles of inflammatory mediators (IL-1β, IL-6, IL-23, TNF-α, matrix metalloproteinase (MMP)-9, MMP-2, CXCL-2, CXCL-8, and CCL-20). (**B**) The protein levels of IL-6 and IL-1β were measured and quantitatively analyzed by Western blot. The values were expressed as mean ± SD. ns, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Data were representative of at least three independent experiments.

chemokine (C-X-C motif) ligand (CXCL)-2, CXCL-8, and cysteine–cysteine motif chemokine ligand (CCL-20), was observed in the LGs of model group and PBS group, whereas rTβ4 treatment decreased the expression of these inflammatory mediators effectively. In addition, the protein levels of IL-6 and IL-1β in LGs were decreased significantly after rTβ4 treatment (Fig. 4B). These findings indicate that rTβ4 administration regulated the inflammatory milieu in LGs.

rTβ4 Downregulated Th17 Immune Response In Vitro

We further clarified the effect of rTβ4 on Th17 cells in vitro. PBLs isolated from model rabbits stimulated with irradiated pLGECs were treated with or without rTβ4 (100 ng/mL). Seventy-two hours later, cells were collected and prepared for qRT-PCR and Western blot. As shown in Figure 5A, the mRNA levels of Th17-related genes, including RORC, IL-17A, IL-17F, GM-CSF, IL-23R, and IL-1R1 were significantly decreased in the rTβ4-treated group compared with the control group. Consistent with the change in the IL-17 mRNA level, the protein expression of IL-17 was significantly reduced in the rTβ4-treated group (Fig. 5B). Taken together, these data demonstrated that rTβ4 could downregulate Th17 response in stimulated PBLs.

rTβ4 Suppressed Th17 Cells by Inhibiting the STAT3 Pathway

We next defined the underlying molecular mechanism through which rTβ4 modulated the Th17 cell response. Considering that STAT3 is a crucial regulator of Th17 development and cytokine production,[30–32] we investigated whether rTβ4 alleviated SS dry eye via suppressing STAT3 signaling. To test this, LGs were collected after 4 weeks of rTβ4 treatment, and Western blot was performed to determine the protein expression of phosphorylation STAT3 (pSTAT3). We found that the level of pSTAT3 in LGs was significantly decreased in the rTβ4-treated group compared with the PBS-treated group in vivo (Fig. 6A). Further in vitro experiments showed that rTβ4 inhibited pSTAT3 and IL-17 protein levels in stimulated PBLs (Fig. 6B). After activation of pSTAT3 using the pharmacological STAT3 activator Colivelin, the downregulated protein level of IL-17

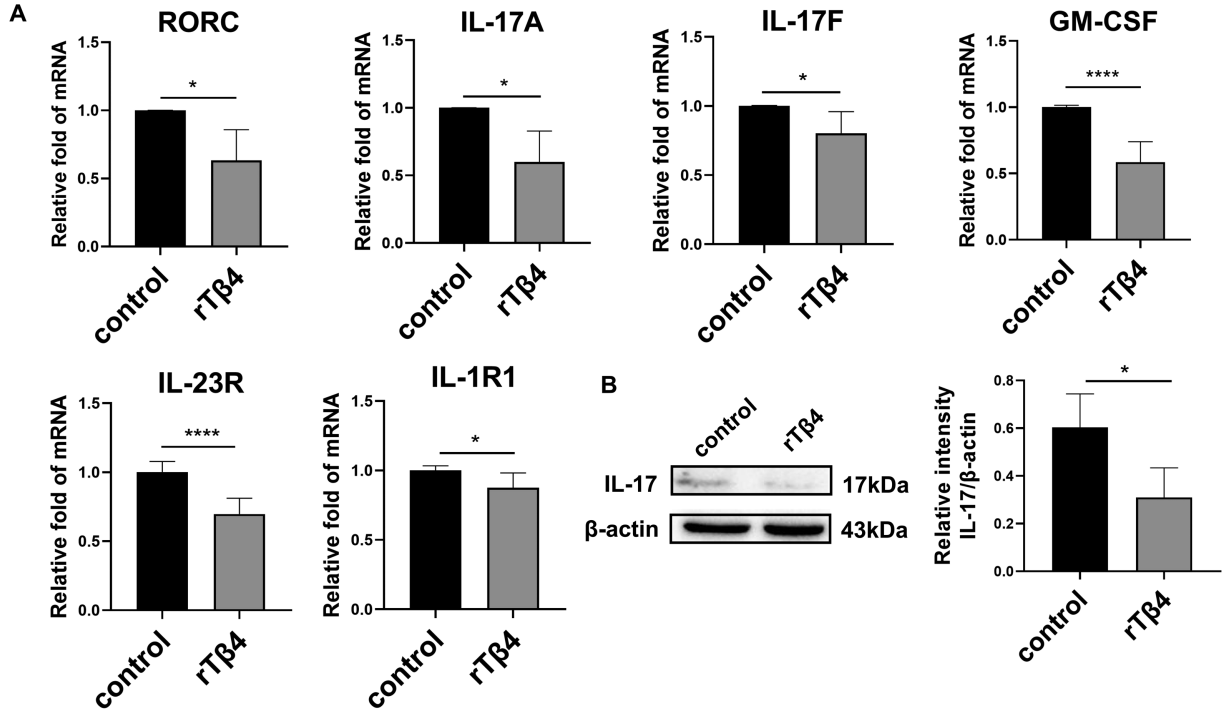

Figure 5. rTβ4 inhibited Th17 immune response in vitro. (**A**) Treatment with 100 ng/mL rTβ4 suppressed Th17 immune response in vitro. PBLs isolated from diseased rabbits were cocultured with irradiated pLGECs in the presence or absence of rTβ4 for 72 hours. Gene expression of RORC, IL-17A, IL-17F, GM-CSF, IL-23R, and IL-1R1 were analyzed by qRT-PCR. (**B**) The protein level of IL-17 expression was analyzed by Western blot assay. The relative protein ratio of IL-17 to β-actin was quantified. Data were representative of three independent experiments ($n = 3$), and bar graphs showed mean ± SD. *$P < 0.05$, ****$P < 0.0001$.

induced by rTβ4 was abolished partly (Fig. 6B). Taken together, these results indicate that STAT3 signaling may mediate partially the suppressive effect of rTβ4 on Th17 cells.

Discussion

Tβ4 is a highly conserved and water-soluble actin chelating peptide demonstrating anti-inflammatory activities and therapeutic effects in several inflammatory and autoimmune diseases, including inflammatory eye diseases.[18,33,34] A recent study demonstrated that topical Tβ4 treatment suppressed the expression of inflammatory mediators (IL-1β, TNF-α, nitric oxide, inducible nitric oxide synthase) and diminished inflammatory cell infiltrates in an experimental model of *Pseudomonas aeruginosa*–induced keratitis.[16,35] As for the dry eye syndrome, topical Tβ4 administration was shown to improve clinical parameters in mouse dry eye model induced by desiccation stress[36] or benzalkonium chloride.[19] However, little attention has been paid to the effect of Tβ4 on SS dry eye. The current study demonstrated that topical administration of rTβ4 after disease onset efficiently relieved the clinical symptoms, increased conjunctival goblet cells, and decreased lacrimal inflammatory cell infiltration in rabbit autoimmune dacryoadenitis during an observation period of 4 weeks. Further mechanistic study revealed that the beneficial effects of rTβ4 might be mediated by inhibiting Th17 cells, probably in part via the downregulation of STAT3 signaling.

Tβ4 was reported to relieve several immune-related diseases through modulating inflammatory cells, including polymorphonuclear leukocytes (neutrophils) and macrophages.[16,37] However, whether Tβ4 exerted its therapeutic effects by modulating Th17 cells in SS dry eye remains unknown. RORC is required for the differentiation of Th17 cells and the expression of IL-17, which can recruit inflammatory cells into inflamed LG and, therefore, exacerbate disease progression of SS dry eye.[9,38] Here, we found that rTβ4 significantly decreased RORC mRNA expression and IL-17 production, suggesting that rTβ4 concurrently affected Th17 cell lineage commitment and function. Of note, IL-23R and IL-1R signalings induce STAT3 phosphorylation to facilitate pathogenic cytokine GM-CSF expression, thereby endowing Th17 cells with pathogenic effector functions.[39–41] Our data that rTβ4 suppressed the expression of GM-CSF, IL-23R, and IL-1R1 significantly in the LGs of rabbits with autoimmune dacryoadenitis highlighted the crucial role of rTβ4 in modulating pathogenic Th17 cell responses. Considering that the contribution of Th17 cells to the pathogenesis of autoimmune dacryoadenitis, rTβ4 may become a promising therapeutic agent for treating autoimmune dry eye diseases.

Cytokines produced by innate immune cells such as dendritic cells or macrophages play an important role in activating pathogenic Th17 program. IL-6 is essential for priming pathogenic Th17 cell responses,[42] and IL-23 and IL-1β signaling can promote stabilization and expansion of pathogenic Th17 cells.[39,43] Here, we observed a significant decrease in the expression of IL-6, IL-1β, and IL-23 in the LGs of an rTβ4-treated rabbit disease model. By decreasing IL-6, IL-1β, and IL-23, rTβ4 might form a cytokine milieu that suppressed the generation and function of Th17 cells, which need further investigation. CCL-20 is a key chemokine that can induce the migration of Th17 cells to ocular surface

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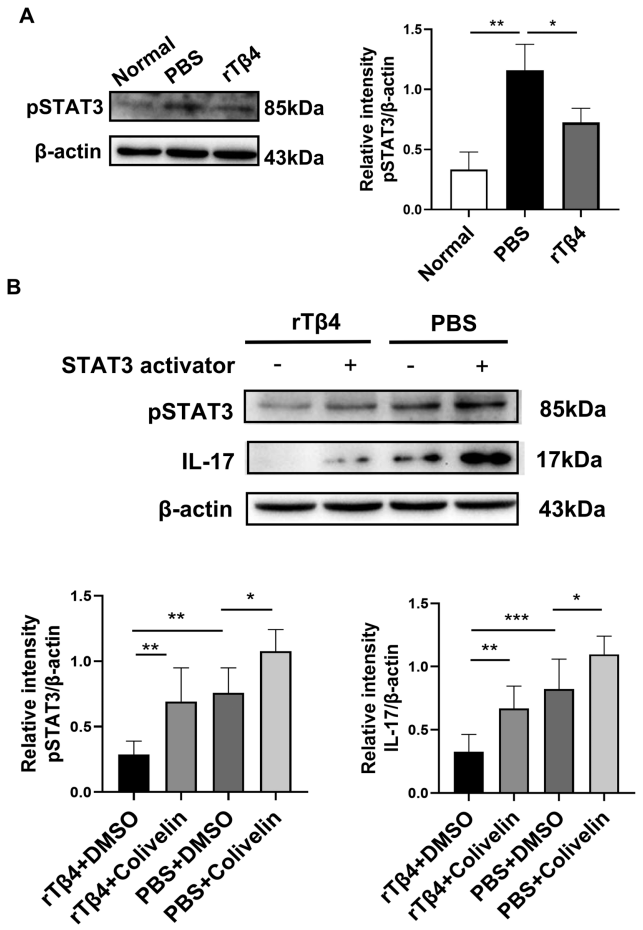

Figure 6. rTβ4 inhibited Th17 immune response via suppressing STAT3 phosphorylation. (**A**) Administration of 1000 µg/mL rTβ4 suppressed the protein level of phosphorylated STAT3 in vivo. (**B**) PBLs induced by pLGECs were pretreated with the STAT3 activator (Colivelin) for 1 hour and coincubated with rTβ4 for 72 hours. The protein expression of IL-17 and pSTAT3 was measured and quantitatively analyzed by Western blot. Data were representative of three independent experiments ($n = 3$), and bar graphs showed mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

inflammatory sites in dry eye disease.[44,45] In this study, we observed a decreased expression of CCL-20 in the LGs of the rTβ4-treated group compared with the untreated group, suggesting that rTβ4 treatment may have resulted in decreased recruitment of Th17 cells to the inflammatory sites, leading to alleviated rabbit dacryoadenitis in the Tβ4-treated group.

STAT3 is a transcription factor essential for Th17 cell development. After activation, pSTAT3 translocates to the nucleus and induces the expression of the transcription factor RORC. STAT3 and RORC synergize to regulate transcription of the Th17 hallmark molecules such as IL-17A, IL-17F, and IL-23R.[46,47] A previous study demonstrated that Tβ4 suppressed murine embryonic stem cell proliferation by repressing the activity of STAT3 signaling.[48] However, the effect of Tβ4 on STAT3 signaling in SS dry eye is unclear. Here, we showed that pSTAT3 and IL-17 protein levels were upregulated dramatically in the inflamed LGs of rabbit autoimmune dacryoadenitis, and rTβ4 could reverse the increases, indicating the important role of the STAT3 pathway in the modulatory effects of rTβ4 on Th17 cells. Indeed, activation of STAT3 with the pharmacological STAT3 activator Colivelin can rescue the inhibitory effect of rTβ4 on IL-17 expression partly, supporting the idea that rTβ4 might suppress the Th17 response partially by inhibiting the activation of STAT3 signaling. However, the precise mechanism by which Tβ4 regulates STAT3 phosphorylation still needs further investigation.

In conclusion, our work demonstrated that rTβ4 alleviated the clinical symptoms of rabbit autoimmune dacryoadenitis efficiently and diminished the lacrimal inflammation, and this effect may be attributable partly to suppressing the Th17 response through repressing STAT3 signaling. These findings highlight that rTβ4 can serve as a candidate immune-regulatory drug for the treatment of SS dry eye and other immune-mediated ocular surface diseases.

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