Recombinant adeno-associated virus carrying thymosin β4 suppresses experimental colitis in mice

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Abstract

AIM
To investigate the protective effect of a recombinant adeno-associated virus carrying thymosin β4 (AAV-Tβ4) on murine colitis via intracolonic administration.

METHODS
AAV-Tβ4 was prepared and intracolonically used to mediate the secretory expression of Tβ4 in mouse colons. Dextran sulfate sodium (DSS) was applied to induce the murine ulcerative colitis, and 2,4,6-trinitrobenzene sulfonic acid (TNBS) was used to establish a mouse colitis model resembling Crohn’s disease. The disease severity and colon injuries were observed and graded to reveal the effects of AAV-Tβ4 on colitis. The activities of myeloperoxidase (MPO) and superoxide dismutase (SOD) and the content of malondialdehyde (MDA) were determined using biochemical assays. Colonic levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-10 were measured using ELISA, and mucosal epithelial cell apoptosis and...
RESULTS
Recombinant AAVs efficiently delivered LacZ and Tβ4 into the colonic tissues of the mice, and AAV-Tβ4 led to a strong expression of Tβ4 in mouse colons. In both the DSS and TNBS colitis models, AAV-Tβ4-treated mice displayed distinctly attenuated colon injuries and reduced apoptosis rate of colonic mucosal epithelia. AAV-Tβ4 significantly reduced inflammatory cell infiltrations and relieved oxidative stress in the inflamed colons of the mice, as evidenced by decreases in MPO activity and MDA content and increases in SOD activity. AAV-Tβ4 also modulated colonic TNF-α, IL-1β and IL-10 levels and suppressed the compensatory proliferation of colonic epithelial cells in DSS- and TNBS-treated mice.

CONCLUSION
Tβ4 exerts a protective effect on murine colitis, indicating that AAV-Tβ4 could potentially be developed into a promising agent for the therapy of inflammatory bowel diseases.

Key words: Thymosin β4; Mice; Colitis; Dextran sulfate sodium; 2,4,6-trinitrobenzene sulfonic acid

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Core tip: We confirmed that intracolonically administered recombinant adeno-associated virus (AAVs) efficiently mediated the ectopic expression of LacZ and thymosin β4 (Tβ4) in mouse colonic mucosa. The current study first indicated that AAV-Tβ4 could improve murine colitis induced either by dextran sulfate sodium or 2,4,6-trinitrobenzene sulfonic acid by suppressing inflammatory cell infiltration, alleviating oxidative stress and epithelial apoptosis, and modulating the production of inflammatory mediators in the inflamed colon. Furthermore, locally overexpressed Tβ4 could attenuate the proliferation of colonic mucosal epithelia. In summary, these results suggest a protective role of Tβ4 in inflammatory bowel diseases (IBD) and indicate that AAV-Tβ4 has therapeutic potential for IBD patients.

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INTRODUCTION
Thymosin β4 (Tβ4) consists of 43 amino acids and is a highly conserved protein belonging to the beta-thymosins family. This small molecule spreads in nearly all cells and exists in various body fluids, such as tears, saliva, blood, plasma and wound fluid[1,2]. Following translation, Tβ4 undergoes complex modifications, such as transglutamination, phosphorylation, N acetylation, and proteolysis[3]. Extracellular Tβ4, released via either active secretion or cell lysis[4], can be rapidly internalized via an unknown mechanism and acts as a paracrine growth factor or cytokine[5,6]. The biological actions of Tβ4 are partially mediated via the activation of integrin-linked kinase[7], upregulation of Bcl-2 production and inhibition of caspase activity[8]. Systemic administration of Tβ4 decreases lethality and inflammatory responses in sepsis, prevents myocardial rupture and improves cardiac function after myocardial infarction, rehabilitates neural function following stroke and encephalomyelitis, and accelerates fracture healing[9-13]. Topical use of Tβ4 improves corneal injury, promotes skin wound healing, and accelerates hair growth[14-16]. These data suggest a great therapeutic potential for Tβ4 in wound healing and inflammatory diseases.

Tβ4 is expressed in human intestines where it modulates the intestinal immune system[17,18]. Moreover, Tβ4 has been considered to be an applicable treatment of gastrointestinal disorders[19]. Increased serum Tβ4 levels have been demonstrated in patients with inflammatory bowel diseases (IBDs)[20]. However, the role and function of Tβ4 in IBDs have not been elucidated. Given the anti-inflammatory, anti-apoptotic, and pro-tissue regeneration properties of Tβ4 that have been previously reported, Tβ4 is supposed to be a beneficial factor for IBDs. To verify this speculation, we constructed a recombinant adeno-associated virus (AAV) to achieve persistent secretory expression of Tβ4 in mouse colons and assessed its effects in two murine colitis models.

MATERIALS AND METHODS
Preparation of recombinant AAV
Self-complementary recombinant adeno-associated virus (subtype 2) were constructed by applying an AAV Helper-Free System (Cell Bioslabs, Inc., San Diego, CA, United States). For secretory expression of Tβ4, the N-terminal 80 amino acid signal peptide of human neurotrophin-4 preproprotein was fused to the N-terminus of human Tβ4 (GenBank NM_021109.3). The coding DNA of the fusion protein was synthesized with the addition of EcoRI and BamHI restriction-enzyme sites (AuGCT DNA-SYN Biotechnology Inc., Beijing, China) and inserted into pscAAV-MCS to yield the pscAAV-NT-4-Tβ4 plasmid. Recombinant AAV containing Tβ4 (AAV-Tβ4) was generated via cotransfection of pscAAV-NT-4-Tβ4, pHelper and pAAV-RC5 into AAV-293 cells using polyethyleneimine (PEI). Similarly, a recombinant AAV carrying LacZ (AAV-LacZ) was constructed as a control virus. Seventy-two hours after transfection, the cells were collected for viral proliferation were detected by TUNEL assay and immunochemistry, respectively.
Zheng XY et al. Effects of thymosin β4 on colitis

particle isolation, purification and quantitative analysis.

qPCR
TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, United States) was employed to determine the rAAVs titters and the abundance of the rAAVs in the colon. The primers against the cytomegalovirus promoter region were as follows: 5'-AGACCTGGAAATCCCGTGAGT-3' (forward) and 5'-CGTATAGTCATGCTTACCATGAGT-3' (reverse). The sequence of the probe was 5'-6FAM-AACCGCTATCCACGCCCATTGAGT-TAMRA-3'. The collected data were analyzed by the standard curve method.

Animal experiments
Male BALB/c mice, 6 wk of age, were purchased from the Experimental Animal Centre of Xi’an Jiaotong University and housed under pathogen-free conditions at 22 ± 2 ℃ with a 12:12 h light-dark cycle. All animal experiments were approved by the Institutional Animal Ethics Committee of Xi’an Jiaotong University.

To verify the transduction efficiency of the recombinant AAV (rAAV) in mouse colons, 12 mice were divided into the following 3 equal groups: phosphate-buffered saline (PBS), AAV-LacZ and AAV-Tβ4. After a 12-h fast, the mice were given an enema of PBS or rAAVs accordingly. Under anesthesia with chloral hydrate[21], a soft catheter was inserted into the mouse anus at a depth of 4 centimeters, and 0.2 mL of PBS, AAV-LacZ [4 × 10^10 viral genome (vg) or AAV-Tβ4 (4 × 10^10 vg)] was instilled into the mouse colon via this catheter. The mouse was then held in an upside-down position for 1 min to allow the enema to distribute throughout the colon. After recovery from the anesthesia, water and food were provided. Two weeks later, mice of all of the groups were sacrificed for their colons. The colon was longitudinally opened, washed with cold normal saline, and cut into three parts as follows: the first was for DNA extraction and subsequent qPCR assays, the second was cryosectioned for X-Gal staining as described previously[22], and the third was homogenized in radioimmunoprecipitation assay lysis buffer for Western blotting.

The following two models were studied: dextran sulfate sodium (DSS) colitis to simulate human ulcerative colitis (UC) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis to model Crohn’s disease (CD).

DSS-induced colitis: The mice were divided into a normal control (NC) group, a PBS/DSS group, an AAV-LacZ/DSS group and an AAV-Tβ4/DSS group (10 mice per group). Prior to inducing DSS colitis, 0.2 mL of AAV-LacZ (4 × 10^10 vg) and AAV-Tβ4 (4 × 10^10 vg) were intracolonically (ic) dosed for the mice of the AAV-LacZ/DSS and AAV-Tβ4/DSS groups, respectively. Meanwhile, the NC and PBS/DSS mice received an enema of PBS. One week later, mice from the three DSS groups were given 50 g/L DSS (MW: 5000, Sigma-Aldrich, St. Louis, MO, United States) as drinking water for 7 d to establish the colitis model, while the mice in the NC group received DSS-free drinking water.

TNBS-induced colitis: The mice, 10 per group, were randomly assigned to the ethanol control (EC), PBS/TNBS, AAV-LacZ/TNBS and AAV-Tβ4/TNBS groups. Mice of the three TNBS groups were given a 0.2 mL enema of TNBS (Sigma-Aldrich, St. Louis, MO, United States, dissolved in 500 mL/L ethanol) once per week for 6 wk. TNBS was dosed at 50 mg/kg for the first 2 wk, 100 mg/kg for the next 2 wk and 150 mg/kg for the last 2 wk. Meanwhile, mice of the EC group received an equal volume of enema with 500 mL/L ethanol. AAVs were ic administered at a dose of 4 × 10^{10} vg in 0.2 mL, 3 d after the first TNBS instillation, while mice in the other two groups were given PBS instead. At the end of the modeling, mice of all of the groups were sacrificed and colonic tissues were isolated.

Evaluation of disease activity
The health status of all the mice was observed during the modeling. The disease activity index (DAI) was estimated daily (DSS-induced colitis) or weekly (TNBS-induced colitis) in accordance with a well-established scoring system[23].

Assessment of colon damage
Macroscopic damage of the colon was carefully observed under an anatomic microscope (Nikon, Tokyo, Japan) and graded according to a previously used scoring system[23]. Small colon segments were fixed, embedded and sectioned for hematoxylin and eosin staining and immunohistochemistry. Histological alterations were graded by observing the H&E stained sections according to a previously established criteria[24].

Sample preparation
Genomic DNA (gDNA) was isolated from the colonic tissues using a DNeasy Kit (Qiagen, Valencia, CA, United States). For the qPCR analysis, 1 μg of the total gDNA was used in each PCR assay. Small pieces of colonic tissue were weighed and mechanically homogenized in normal saline on an ice-bath to obtain 100 mg/L colonic homogenates for biochemical detections and ELISA.

Western blot analysis
Briefly, proteins were extracted from the colonic tissue samples, applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris-glycine system for β-actin; Tris-Tricine system for Tβ4) and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, United States). Western blots were performed with specific antibodies targeting β-actin (Sigma-Aldrich, St. Louis, MO, United States) and Tβ4 (Abcam, San Francisco, CA, United States).
Biochemical detection
Myeloperoxidase (MPO) activity, malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in the colonic homogenates were assessed by applying the test kits (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to the manufacturer's protocols. The results of the MPO activity, MDA content and SOD activity were, respectively, represented as units per gram of tissue (U/g), nmol per mg protein (nmol/mgprot) and U/mgprot.

ELISA
The supernatants of the colonic homogenates were collected to determine the tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-10 levels. Three ELISA kits (TNF-α, R&D Systems, Minneapolis, MN, United States; IL-1β and IL-10, eBioscience, San Diego, CA, United States) were used in accordance with the manufacturers' instructions. The results were expressed as picogram per mg protein (pg/mgprot).

Immunohistochemistry
Immunohistochemistry was performed with primary antibodies against PCNA and Tβ4, according to a previously reported method[2].

The proliferation of mucosal epithelial cells was estimated by PCNA immunostaining of the colonic sections. PCNA labeling index (PCNA LI) was determined by calculating the ratio of the number of PCNA-positive cells to the total number of counted cells in 10 randomly chosen fields at × 400 magnification.

TUNEL assay
Epithelial cell apoptosis in colonic mucosa was assessed using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, United States) based on the manufacturer’s protocol. One thousand mucosal epithelial cells from 10 randomly selected fields (magnification × 400) were counted in each colonic section. The percentage of TUNEL-labelled epithelia was used to present the apoptotic index (AI).

Statistical analysis
The Kruskal-Wallis test was applied to analyze the nonparametric data, and the one-way analysis of variance followed by Tukey’s post hoc test was adopted for the quantitative data. P < 0.05 was considered to represent statistical significance.

RESULTS
Ic administered AAV efficiently mediates secretory expression of Tβ4 in the mouse colon
To verify the transduction efficiency of ic AAV, we first used real-time PCR to determine the abundance of vector DNA in the mouse colon. As shown in Figure 1A, real-time PCR revealed the presence of AAV vector DNA in the mouse colon after the administration of rAAVs. Next, we performed X-gal staining and Tβ4 immunostaining to determine the AAV-mediated expression of foreign genes. X-gal staining localized the ectopic β-galactosidase expression in the colonic epithelium and lamina propria of the AAV-LacZ-treated mice (Figure 1B). Immunohistochemistry showed an increased expression of Tβ4 in the AAV-Tβ4-treated mice compared to the mice administered with PBS or AAV-LacZ (Figure 1C). Moreover, we measured the colonic expression of Tβ4 using Western blot in the two sets of colitic mice. The results showed that the colonic expression of Tβ4 was notably upregulated after DSS treatment while slightly increased after enemas of TNBS. Intracolonic AAV-Tβ4 led to a drastically increased colonic expression of Tβ4 in both colitis models (Figure 1D). These findings provide further evidence that rAAV by ic could efficiently deliver foreign genes into the mouse colonic tissue, and AAV-Tβ4 could mediate the secretory expression of Tβ4.

AAV-Tβ4 ameliorates experimental colitis in mice
Mice in the PBS/DSS and AAV-LacZ/DSS groups suffered distinct weight loss, diarrhea and hemorrhage, with higher DAI scores than normal mice. AAV-Tβ4 administration ameliorated DSS-induced colitic manifestations and DAI scores (Figure 2A). Macroscopic examination revealed that DSS treatment led to extensive colonic damages characterized by hyperemia, edema and ulceration (Figure 2B), while pre-treatment with AAV-Tβ4 significantly attenuated the colonic damages, presenting low macroscopic scores (Figure 2B and C). Similarly, DSS-induced microscopic lesions, such as loss of goblet cells and crypts, epithelial necrosis, inflammatory cell infiltrations and crypt abscesses, were also markedly alleviated by AAV-Tβ4 treatment (Figure 2D and E).

Repeated TNBS enemas led to similar but milder symptoms as those caused by the DSS treatment. Mice in the PBS/TNBS and AAV-LacZ/TNBS groups presented notably higher DAI scores compared with the EC mice. The AAV-Tβ4 treatment alleviated mouse suffering, as indicated by the significantly lower DAI scores after the fifth week of modeling (Figure 3A). Similar to the findings in the DSS colitis model, AAV-Tβ4 administration markedly alleviated TNBS-induced mucosal damage both macroscopically (Figure 3B and C) and microscopically (Figure 3D and E).

Colonic MPO activity, a common index for neutrophil and macrophage infiltrations, was increased in both colitis models compared to their respective control groups. The significantly decreased colonic MPO activities in the colitic mice administered with AAV-Tβ4 indicate the efficacy of Tβ4 in attenuating inflammatory cell infiltration (Figure 4).

AAV-Tβ4 attenuates epithelial apoptosis in inflamed colonic mucosa
Apoptosis of the colonic mucosal epithelia disrupts
Zheng XY et al. Effects of thymosin β4 on colitis

Page 245
mucosal integrity and, consequently, affects the barrier function of the colonic mucosa. TUNEL staining was performed to assess the effect of Tβ4 on colonic epithelial apoptosis in the two colitis models. As shown in Figure 5, only a few apoptotic cells were distributed on the surface of the normal colonic mucosa. Both DSS and TNBS treatments remarkably led to the apoptosis of the mucosal epithelial cells, while AAV-Tβ4 administration significantly attenuated these changes. The AI of the AAV-Tβ4 groups was markedly lower than that of the PBS or AAV-LacZ groups.

**Tβ4 reduces oxidative damage in colonic tissues**

Figure 6 shows that MDA content was distinctly increased after DSS treatment, while it was mildly increased in the TNBS model. AAV-Tβ4 markedly decreased the colonic MDA levels in both DSS- and TNBS-treated mice. DSS and TNBS treatments decreased the colonic SOD activities similarly, while AAV-Tβ4 reversed these changes. These results demonstrate the efficacy of Tβ4 in the alleviation of oxidative stress in colitis.

**Tβ4 modulates TNF-α, IL-1β, and IL-10 levels in the inflamed colon**

Colonic productions of inflammatory cytokines were determined to make a preliminary exploration of the underlying mechanisms of the anti-inflammatory
Zheng XY et al. Effects of thymosin β4 on colitis

Figure 2  Adeno-associated virus carrying thymosin β4 ameliorated dextran sulfate sodium-induced colitis in mice. A: Dynamic changes in DAI [the asterisk indicates *P < 0.05 vs PBS/dextran sulfate sodium (DSS) or adeno-associated virus (AAV)-LacZ/DSS group]; B and C: AAV-thymosin β4 (Tβ4) improved DSS-induced colonic lesions including mucosal edema, hyperemia, and ulceration, resulting in a significantly decreased macroscopic score; D and E: Histological examination (HE staining, magnification × 200) revealed the mice in AAV-Tβ4/DSS group exhibited alleviated colonic lesions such as destruction of mucosal crypts, loss of epithelial cells, deep ulcerations, and inflammatory cell infiltration, leading to a significantly decreased histological score compared with the mice in the other two DSS groups. Error bars indicate the SD. Scale bars = 50 μm. DAI: Disease activity index.

A

B

C

D

E
Zheng XY et al. Effects of thymosin β4 on colitis

As shown in Figure 7, DSS treatment resulted in distinct increases in colonic TNF-α, IL-1β and IL-10 levels, while ectopically expressed Tβ4 significantly inhibited these increases. Repeated TNBS enemas increased colonic TNF-α levels and decreased IL-10 levels, while AAV-Tβ4 administration attenuated...
these alterations (Figure 7A and C). Unlike the findings in the DSS model, neither TNBS treatment nor ic AAV-Tβ4 significantly altered colonic IL-1β levels (Figure 7B, P > 0.05). Collectively, these data indicate that Tβ4 could modulate the production of inflammatory cytokines in inflamed colons.

**Tβ4 attenuates the acceleration of cell proliferation in the inflamed colonic mucosa**

PCNA immunostaining demonstrated that both DSS and TNBS caused notable increases in the proportions of PCNA-positive cells in the colonic mucosa, while ectopic Tβ4 mediated by AAV significantly suppressed these increases (Figure 8), indicating that Tβ4 alleviated the acceleration of cell proliferation in the inflamed colons.

**DISCUSSION**

Although increased levels of serum Tβ4 were reported in IBD patients [20], the role of Tβ4 in the development of IBDs is still elusive. In this study, the colonic expression of Tβ4 was increased after colitis induction, especially in the DSS model, and the increase was positively related to the colitis severity. These findings suggest that differential expressions of Tβ4 might be helpful to predict and assess the severity of IBDs. The present study also showed that AAV-mediated overexpression of Tβ4 in mouse colons alleviated both DSS- and TNBS-induced colitis. Collectively, these data suggest a dose-dependent protective effect of Tβ4 in inflamed colons. The increased production of local Tβ4 in mice with severe colitis reflected an adaptive response of these mice to colonic damages, while the increased expression of endogenous Tβ4 might not be sufficient to withstand the colonic injury and present the protective effect. Only a dramatic increase in the local Tβ4 content as in the present study achieved by AAV delivery, can distinctly attenuate the colitic manifestations in mice. Furthermore, our subsequent results indicated that the protective effect of Tβ4 may involve suppressing oxidant damage and inflammatory cell infiltrations, regulating colonic inflammatory cytokine levels, preventing mucosal inflammatory cytokine levels, preventing mucosal epithelia from apoptosis and regulating cell regeneration in the inflamed colonic tissues.

Inflammation is the core component of IBDs, in which neutrophils and macrophages play crucial roles. MPO is mainly produced by macrophages and neutrophils, and its activity is usually considered as an indicator of mucosal inflammation in IBDs. Previous studies by others demonstrated that Tβ4 treatment reduced the infiltration of MPO-positive neutrophils into the infarct zone after myocardial infarction [25] and reduced the MPO activity in bleomycin-intoxicated lungs in mice [26]. In this study, MPO activity was distinctly decreased after AAV-Tβ4 administration in both DSS- and TNBS-treated mice, enhancing our understanding of the inflammation-inhibitory efficacy of Tβ4.

TNF-α is the most potent pro-inflammatory cytokine involved in IBDs, which was verified by the successful application of TNF-α antibodies for the treatment of IBDs [27-29]. Consistent with the previous study in which systemic administration of Tβ4 decreased the TNF-α level in mice with sepsis [9], our results show that AAV-Tβ4 markedly alleviated the upregulated expression of TNF-α in the injured colonic tissues, suggesting that suppressing TNF-α expression is an essential mechanism for the Tβ4-mediated alleviation of colitis.

IL-1β is an important mediator of inflammation, and its level is often increased during the development of UC. However, reports regarding alterations of IL-1β levels in CD are controversial [28,30-32]. Distinct upregulation of IL-1β was found in the colons of DSS-treated mice, while the IL-1β content was hardly changed in the TNBS model. Tβ4 reversed the DSS-induced increase in colonic IL-1β expression, which suggests that the suppression of IL-1β expression contributes to the protective effect of Tβ4 on colitis.

IL-10, a potent anti-inflammatory cytokine, is considered a negative regulator in IBDs [23,34]. However, the colonic IL-10 expression profile in patients with IBD is controversial. Most reports indicate that mucosal levels of IL-10 are increased in UC [27,30], but normal [38], decreased [36,37] and elevated [38,39] mucosal IL-10 levels are reported for CD. Recent studies have demonstrated that polymorphisms of the IL-10 gene are correlated with the development of UC and CD [40]. In our study, AAV-Tβ4 attenuated the DSS-induced increase and TNBS-induced decrease in colonic IL-10 levels. It is known that TNBS colitis exhibits enhanced Th1-mediated inflammatory response, while DSS

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**Figure 4** Adeno-associated virus carrying thymosin β4 suppressed dextran sulfate sodium- and 2,4,6-trinitrobenzene sulfonic acid-induced inflammation in colonic tissue. Error bars indicate the SD. AAV: Adeno-associated virus; Tβ4: Thymosin β4; DSS: Dextran sulfate sodium; TNBS: 2,4,6-trinitrobenzene sulfonic acid.
colitis is characterized by a predominant Th2 response (increase in IL-10). A previous study reported that Tβ4 had inhibitory effects on human colonic lamina propria lymphocytes\[18\]. Thus, we speculate that the disparate effects of Tβ4 on IL-10 production might be mediated via its suppression on the differently enhanced responses of immune cells in the two models. Overall, our results suggest that restoring the balance of cytokines might be one of the underlying mechanisms of Tβ4 in attenuating colitis.

Oxidant/antioxidant imbalance plays an essential role in the pathogenesis of IBDs. The present study demonstrates that AAV-Tβ4 administration decreased MDA production and increased SOD activity in the inflamed colons, which illustrates the efficacy of Tβ4 in scavenging ROS and boosting anti-oxidative defense during colitis. ROS mainly originate from inflammatory cells. Therefore, the mechanism of Tβ4 in the prevention of oxidant damage in colonic tissue should be mainly attributable to the alleviation of the infiltration of inflammatory cells. In addition, previous studies have revealed that Tβ4 reduced ROS levels in corneal epithelial cells, cardiac fibroblasts and cardiomyocytes via the upregulation of the expression of antioxidant enzyme SOD and catalase\[41-43\]. A direct enhancement of SOD expression by Tβ4 might also contribute to the protection of oxidative damage.

Intestinal epithelial apoptosis plays an essential role in the pathogenesis of IBDs. The present study demonstrates that AAV-Tβ4 administration decreased MDA production and increased SOD activity in the inflamed colons, which illustrates the efficacy of Tβ4 in scavenging ROS and boosting anti-oxidative defense during colitis. ROS mainly originate from inflammatory cells. Therefore, the mechanism of Tβ4 in the prevention of oxidant damage in colonic tissue should be mainly attributable to the alleviation of the infiltration of inflammatory cells. In addition, previous studies have revealed that Tβ4 reduced ROS levels in corneal epithelial cells, cardiac fibroblasts and cardiomyocytes via the upregulation of the expression of antioxidant enzyme SOD and catalase\[41-43\]. A direct enhancement of SOD expression by Tβ4 might also contribute to the protection of oxidative damage.

Intestinal epithelial apoptosis is a representative indicator of mucosal damage in IBDs. In the present study, the attenuation of colonic cell apoptosis by Tβ4 should be mainly due to its inhibitory effects on inflammation and oxidative reaction, which protect mucosal epithelia against death-inducing factors. Moreover, there is substantial evidence that Tβ4 suppresses

Figure 5  Adeno-associated virus carrying thymosin β4 alleviated dextran sulfate sodium- and 2,4,6-trinitrobenzene sulfonic acid-induced epithelial cell apoptosis in the colonic mucosa. TUNEL staining showed that intracolonic adeno-associated virus (AAV)-thymosin β4 (Tβ4) prevented the colonic mucosal epithelia from undergoing apoptosis and thus led to decreased apoptotic indexes (AIs) in dextran sulfate sodium (DSS)- (A and B) and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced (C and D) colitis in mice. The insets indicate the TUNEL-positive nuclear staining. Error bars indicate the SD. Scale bar: 50 μm and 25 μm (insets).
Figure 6  Adeno-associated virus carrying thymosin β4 relieved colonic oxidant damage in the mice with colitis. MDA content (A) and SOD activity (B) were determined using biochemical assay. Error bars indicate the SD. AAV: Adeno-associated virus; Tβ4: Thymosin β4.

Figure 7  Adeno-associated virus carrying thymosin β4 modulated colonic TNF-α, IL-1β and IL-10 levels in dextran sulfate sodium and 2,4,6-trinitrobenzene sulfonic acid models. Colonic TNF-α (A), IL-1β (B) and IL-10 (C) levels were determined by ELISA. Error bars indicate the SD. AAV: Adeno-associated virus; Tβ4: Thymosin β4; DSS: Dextran sulfate sodium; TNBS: 2,4,6-trinitrobenzene sulfonic acid.
apoptosis of corneal epithelia, cardiomyocytes, neurons, normal intestinal epithelia, and colorectal cancer cells by regulating several intracellular signaling events such as Akt activation, c-Jun phosphorylation, Bcl-2 phosphorylation, and decreased caspase-3 activity [8,44-48].

Thus, a direct anti-apoptotic activity of Tβ4 might also participate in this protective effect. As apoptosis is a dynamic pathophysiologic process with considerable complexity, further investigation is needed to clarify the underlying mechanism(s) of Tβ4 for inhibiting colonic epithelial apoptosis.

Tβ4 can promote the regeneration of the cornea, skin, heart, and intestine epithelial cells [1,46,47]; nevertheless, PCNA immunostaining in the current study revealed that Tβ4 attenuated the accelerated proliferation of colonic mucosal cells following DSS or TNBS treatment. One possible explanation may be that the attenuation of mucosal damage by Tβ4 reduces the compensatory regeneration secondary to tissue damage, which surpasses the proliferation-promoting effect mediated by Tβ4.

Because IBD is a chronic and relapsing disorder mainly involving the intestines, local and persistent expression of therapeutic molecules should be a suitable approach to IBD treatment. AAV is characterized by a wide range of hosts, long-term expression, low immunogenicity and lack of toxicity. Repeated application of AAV is also feasible. Therefore, AAV emerges as a promising vector in gene therapy for chronic diseases, and it has been widely utilized in clinical trials to treat a variety of diseases [49-51]. In the present study, we constructed AAV-Tβ4 and confirmed its efficacy in transducing mouse colonic cells and mediating high expression of secretory Tβ4.

In conclusion, our study verifies that Tβ4 is a protective molecule in murine colitis and suggests that ic
AAV-Tβ4 could potentially be developed into a promising therapeutic approach for IBDs. To further clarify the importance of endogenous Tβ4 in the maintenance of intestinal homeostasis and in the development of IBDs, tissue-specific ablation of Tβ4 in mouse colons or neutralization of extracellular Tβ4 by immunizing animals with a Tβ4-carrier protein conjugates should be carried out. Besides, the "treatment" effect of AAV-Tβ4 on chronic colitis such as IL-10 knock-out mouse model is also under our consideration.

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