

## Basic Study

## Recombinant adeno-associated virus carrying thymosin $\beta_4$ suppresses experimental colitis in mice

Xiao-Yan Zheng, Yi-Fei Lv, Shuang Li, Qian Li, Qian-Nan Zhang, Xue-Ting Zhang, Zhi-Ming Hao

Xiao-Yan Zheng, Qian Li, Zhi-Ming Hao, Department of Rheumatology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Yi-Fei Lv, Department of Gastroenterology, Shaanxi Provincial People's Hospital, Xi'an 710061, Shaanxi Province, China

Shuang Li, Qian-Nan Zhang, Xue-Ting Zhang, Department of Gastroenterology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

**Author contributions:** Zheng XY and Hao ZM designed and coordinated the research; Li S and Zhang XT prepared the recombinant AAVs; Zheng XY, Lv YF and Zhang QN performed the animal experiments; Zheng XY, Lv YF, Li Q and Zhang QN performed the immunohistochemistry, TUNEL, ELISA and Western blot; Zheng XY and Li Q analyzed the data; Zheng XY and Hao ZM wrote the manuscript.

**Supported by** National Foundation of Natural Sciences, China, No. 81300293.

**Institutional review board statement:** The study was reviewed and approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China (Permit No. XJTULAC2016-404).

**Institutional animal care and use committee statement:** All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China.

**Conflict-of-interest statement:** The authors declare that they have no conflict of interest to disclose concerning this manuscript.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Unsolicited manuscript

**Correspondence to:** Zhi-Ming Hao, MD, PhD, Professor, Department of Rheumatology, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yantaxilu, Xi'an 710061, Shaanxi Province, China. haozhm66@126.com  
Telephone: +86-18991232223

**Received:** June 25, 2016

**Peer-review started:** June 28, 2016

**First decision:** September 6, 2016

**Revised:** October 4, 2016

**Accepted:** November 13, 2016

**Article in press:** November 13, 2016

**Published online:** January 14, 2017

### Abstract

#### AIM

To investigate the protective effect of a recombinant adeno-associated virus carrying thymosin  $\beta_4$  (AAV-T $\beta_4$ ) on murine colitis *via* intracolonic administration.

#### METHODS

AAV-T $\beta_4$  was prepared and intracolonic used to mediate the secretory expression of T $\beta_4$  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-T $\beta_4$  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- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-10 were measured using ELISA, and mucosal epithelial cell apoptosis and

proliferation were detected by TUNEL assay and immunohistochemistry, respectively.

## RESULTS

Recombinant AAVs efficiently delivered LacZ and T $\beta_4$  into the colonic tissues of the mice, and AAV-T $\beta_4$  led to a strong expression of T $\beta_4$  in mouse colons. In both the DSS and TNBS colitis models, AAV-T $\beta_4$ -treated mice displayed distinctly attenuated colon injuries and reduced apoptosis rate of colonic mucosal epithelia. AAV-T $\beta_4$  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-T $\beta_4$  also modulated colonic TNF- $\alpha$ , IL-1 $\beta$  and IL-10 levels and suppressed the compensatory proliferation of colonic epithelial cells in DSS- and TNBS-treated mice.

## CONCLUSION

T $\beta_4$  exerts a protective effect on murine colitis, indicating that AAV-T $\beta_4$  could potentially be developed into a promising agent for the therapy of inflammatory bowel diseases.

**Key words:** Thymosin  $\beta_4$ ; Mice; Colitis; Dextran sulfate sodium; 2,4,6-trinitrobenzene sulfonic acid

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**Core tip:** We confirmed that intracolonic administered recombinant adeno-associated virus (AAVs) efficiently mediated the ectopic expression of LacZ and thymosin  $\beta_4$  (T $\beta_4$ ) in mouse colonic mucosa. The current study first indicated that AAV-T $\beta_4$  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 T $\beta_4$  could attenuate the proliferation of colonic mucosal epithelia. In summary, these results suggest a protective role of T $\beta_4$  in inflammatory bowel diseases (IBD) and indicate that AAV-T $\beta_4$  has therapeutic potential for IBD patients.

Zheng XY, Lv YF, Li S, Li Q, Zhang QN, Zhang XT, Hao ZM. Recombinant adeno-associated virus carrying thymosin  $\beta_4$  suppresses experimental colitis in mice. *World J Gastroenterol* 2017; 23(2): 242-255 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i2/242.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i2.242>

## INTRODUCTION

Thymosin  $\beta_4$  (T $\beta_4$ ) 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<sup>[1,2]</sup>. Following translation, T $\beta_4$  undergoes complex modifications, such as transglutamination, phosphorylation, N acetylation, and proteolysis<sup>[3]</sup>. Extracellular T $\beta_4$ , released *via* either active secretion or cell lysis<sup>[4]</sup>, can be rapidly internalized *via* an unknown mechanism and acts as a paracrine growth factor or cytokine<sup>[5,6]</sup>. The biological actions of T $\beta_4$  are partially mediated *via* the activation of integrin-linked kinase<sup>[7]</sup>, upregulation of Bcl-2 production and inhibition of caspase activity<sup>[8]</sup>. Systemic administration of T $\beta_4$  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<sup>[1,9-13]</sup>. Topical use of T $\beta_4$  improves corneal injury, promotes skin wound healing, and accelerates hair growth<sup>[1,14-16]</sup>. These data suggest a great therapeutic potential for T $\beta_4$  in wound healing and inflammatory diseases.

T $\beta_4$  is expressed in human intestines where it modulates the intestinal immune system<sup>[17,18]</sup>. Moreover, T $\beta_4$  has been considered to be an applicable treatment of gastrointestinal disorders<sup>[19]</sup>. Increased serum T $\beta_4$  levels have been demonstrated in patients with inflammatory bowel diseases (IBDs)<sup>[20]</sup>. However, the role and function of T $\beta_4$  in IBDs have not been elucidated. Given the anti-inflammatory, anti-apoptotic, and pro-tissue regeneration properties of T $\beta_4$  that have been previously reported, T $\beta_4$  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 T $\beta_4$  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 Biolabs, Inc., San Diego, CA, United States). For secretory expression of T $\beta_4$ , the N-terminal 80 amino acid signal peptide of human neurotrophin-4 preproprotein was fused to the N-terminus of human T $\beta_4$  (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-T $\beta_4$  plasmid. Recombinant AAV containing T $\beta_4$  (AAV-T $\beta_4$ ) was generated *via* co-transfection of pscAAV-NT-4-T $\beta_4$ , pHelper and pAAV-RC5 into AAV-293 cells using polyethylenimine (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

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 titers and the abundance of the rAAVs in the colon. The primers against the cytomegalovirus promoter region were as follows: 5'-AGACTTGGAAATCCCCGTGAGT-3' (forward) and 5'-CGTATTAGTCATCGCTATTACCATGGT-3' (reverse). The sequence of the probe was 5'-6FAM-AACCGCTATCCACGCCATTGATG-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  $22 \pm 2^\circ\text{C}$  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-T $\beta_4$ . After a 12-h fast, the mice were given an enema of PBS or rAAVs accordingly. Under anesthesia with chloral hydrate<sup>[21]</sup>, a soft catheter was inserted into the mouse anus at a depth of 4 centimeters, and 0.2 mL of PBS, AAV-LacZ [ $4 \times 10^{10}$  viral genome (vg) or AAV-T $\beta_4$  ( $4 \times 10^{10}$  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 sequent qPCR assays, the second was cryosectioned for X-Gal staining as described previously<sup>[22]</sup>, 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-T $\beta_4$ /DSS group (10 mice per group). Prior to inducing DSS colitis, 0.2 mL of AAV-LacZ ( $4 \times 10^{10}$  vg) and AAV-T $\beta_4$  ( $4 \times 10^{10}$  vg) were intracolonic (ic) dosed for the mice of the AAV-LacZ/DSS and AAV-T $\beta_4$ /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-T $\beta_4$ /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  $4 \times 10^{10}$  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<sup>[23]</sup>.

### 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<sup>[23]</sup>. 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<sup>[24]</sup>.

### 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  $\mu\text{g}$  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  $\beta$ -actin; Tris-Tricine system for T $\beta_4$ ) and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, United States). Western blots were performed with specific antibodies targeting  $\beta$ -actin (Sigma-Aldrich, St. Louis, MO, United States) and T $\beta_4$  (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- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-10 levels. Three ELISA kits (TNF- $\alpha$ , R&D Systems, Minneapolis, MN, United States; IL-1 $\beta$  and IL-10, eBioscience, San Diego, CA, United States) were used in accordance with the manufacturers' instructions. The results were expressed as pictogram per mg protein (pg/mgprot).

**Immunohistochemistry**

Immunohistochemistry was performed with primary antibodies against PCNA and T $\beta_4$ , according to a previously reported method<sup>[22]</sup>.

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  $\times 400$  magnification.

**TUNEL assay**

Epithelial cell apoptosis in colonic mucosa was assessed by applying 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  $\times 400$ ) 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 T $\beta_4$  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 T $\beta_4$  immunostaining to determine the AAV-mediated expression of foreign genes. X-gal staining localized the ectopic  $\beta$ -galactosidase expression in the colonic epithelium and lamina propria of the AAV-LacZ-treated mice (Figure 1B). Immunohistochemistry showed an increased expression of T $\beta_4$  in the AAV-T $\beta_4$ -treated mice compared to the mice administered with PBS or AAV-LacZ (Figure 1C). Moreover, we measured the colonic expression of T $\beta_4$  using Western blot in the two sets of colitic mice. The results showed that the colonic expression of T $\beta_4$  was notably upregulated after DSS treatment while slightly increased after enemas of TNBS. Intracolonic AAV-T $\beta_4$  led to a drastically increased colonic expression of T $\beta_4$  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-T $\beta_4$  could mediate the secretory expression of T $\beta_4$ .

***AAV-T $\beta_4$  ameliorates experimental colitis in mice***

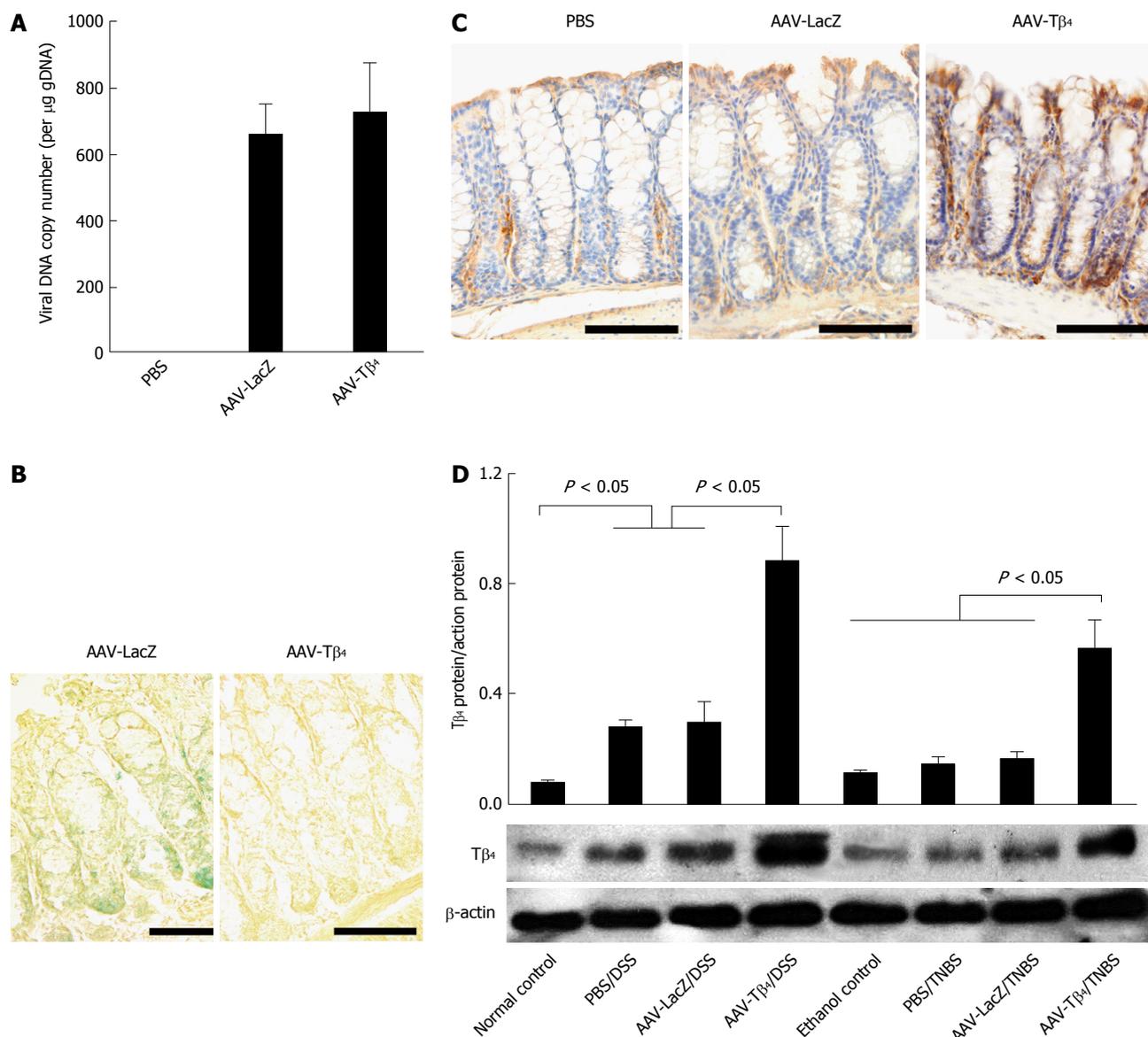
Mice in the PBS/DSS and AAV-LacZ/DSS groups suffered distinct weight loss, diarrhea and hematochezia, with higher DAI scores than normal mice. AAV-T $\beta_4$  administration ameliorated DSS-induced colitic manifestations and DAI scores (Figure 2A). Macroscopic examination revealed that DSS treatment led to extensive colonic damages characterized by hyperemia, edema and ulceration (Figure 2B), while pre-treatment with AAV-T $\beta_4$  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, epithelia necrosis, inflammatory cell infiltrations and crypt abscesses, were also markedly alleviated by AAV-T $\beta_4$  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-T $\beta_4$  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-T $\beta_4$  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-T $\beta_4$  indicate the efficacy of T $\beta_4$  in attenuating inflammatory cell infiltration (Figure 4).

***AAV-T $\beta_4$  attenuates epithelial apoptosis in inflamed colonic mucosa***

Apoptosis of the colonic mucosal epithelia disrupts



**Figure 1** Intracolonic administration of adeno-associated virus carrying thymosin  $\beta_4$  mediated secretory expression of thymosin  $\beta_4$  in mouse colons. Two weeks after intracolonic (ic) adeno-associated virus (AAV)-LacZ, AAV-thymosin  $\beta_4$  (T $\beta_4$ ) or PBS, the mice were euthanized to harvest their colons. A: Real-time PCR showed the presence of viral DNA in the colonic tissues of the AAV-treated mice; B: X-gal staining verified AAV-mediated expression of LacZ in the colonic tissues. C: Immunohistochemistry revealed that ic AAV-T $\beta_4$  resulted in markedly increased expression of T $\beta_4$  in the mouse colon; D: In the mice with experimental colitis, Western blot showed that dextran sulfate sodium (DSS) treatment remarkably upregulated the colonic T $\beta_4$  expression while TNBS enemas only slightly increased colonic T $\beta_4$ . Intracolonic AAV-T $\beta_4$  led to substantially increased expression of T $\beta_4$  in the colonic tissues in both DSS- and TNBS-treated mice. Error bars indicate SD. Scale bars = 50  $\mu\text{m}$ .

mucosal integrity and, consequently, affects the barrier function of the colonic mucosa. TUNEL staining was performed to assess the effect of T $\beta_4$  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-T $\beta_4$  administration significantly attenuated these changes. The AI of the AAV-T $\beta_4$  groups was markedly lower than that of the PBS or AAV-LacZ groups.

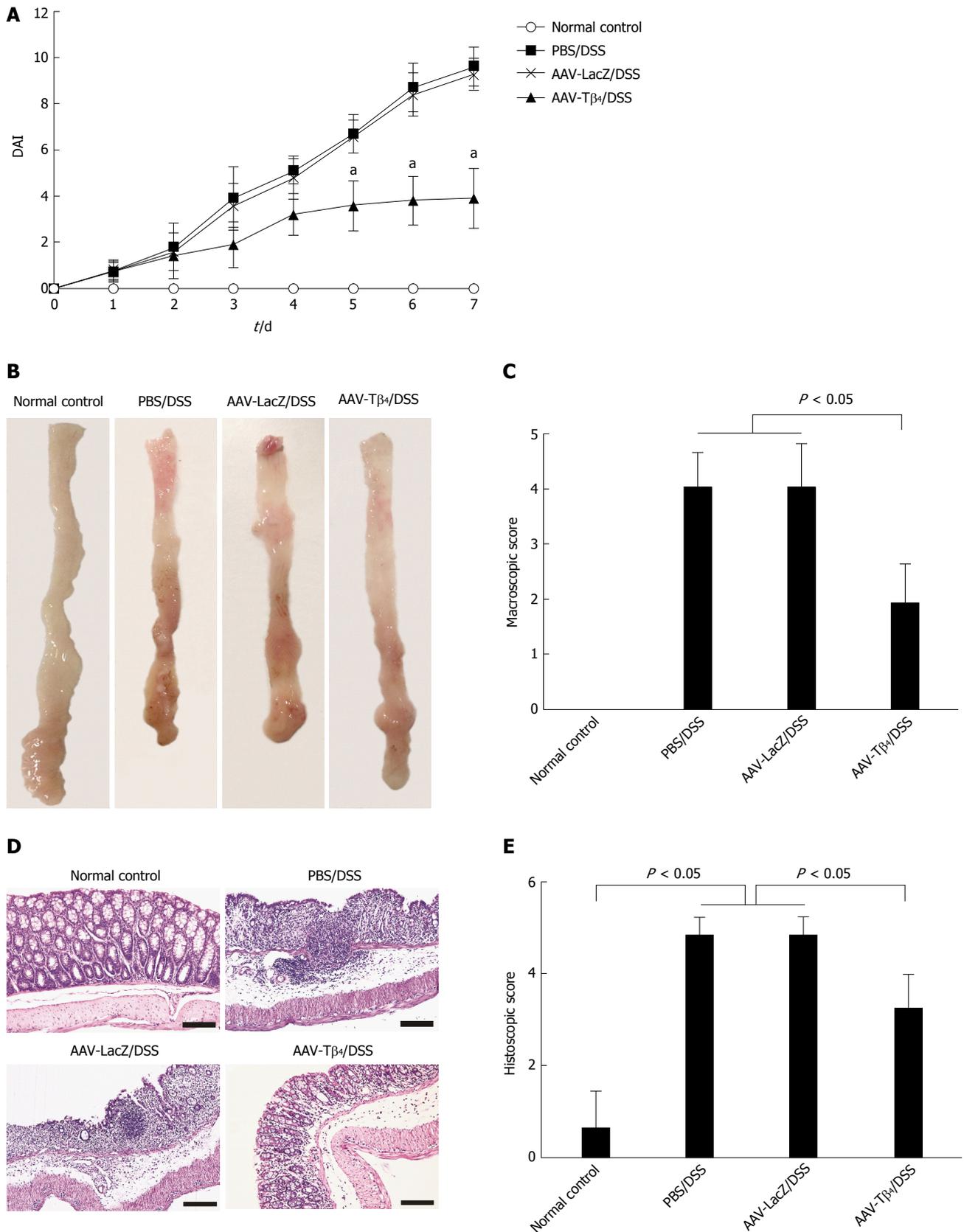
#### T $\beta_4$ reduces oxidative damage in colonic tissues

Figure 6 shows that MDA content was distinctly

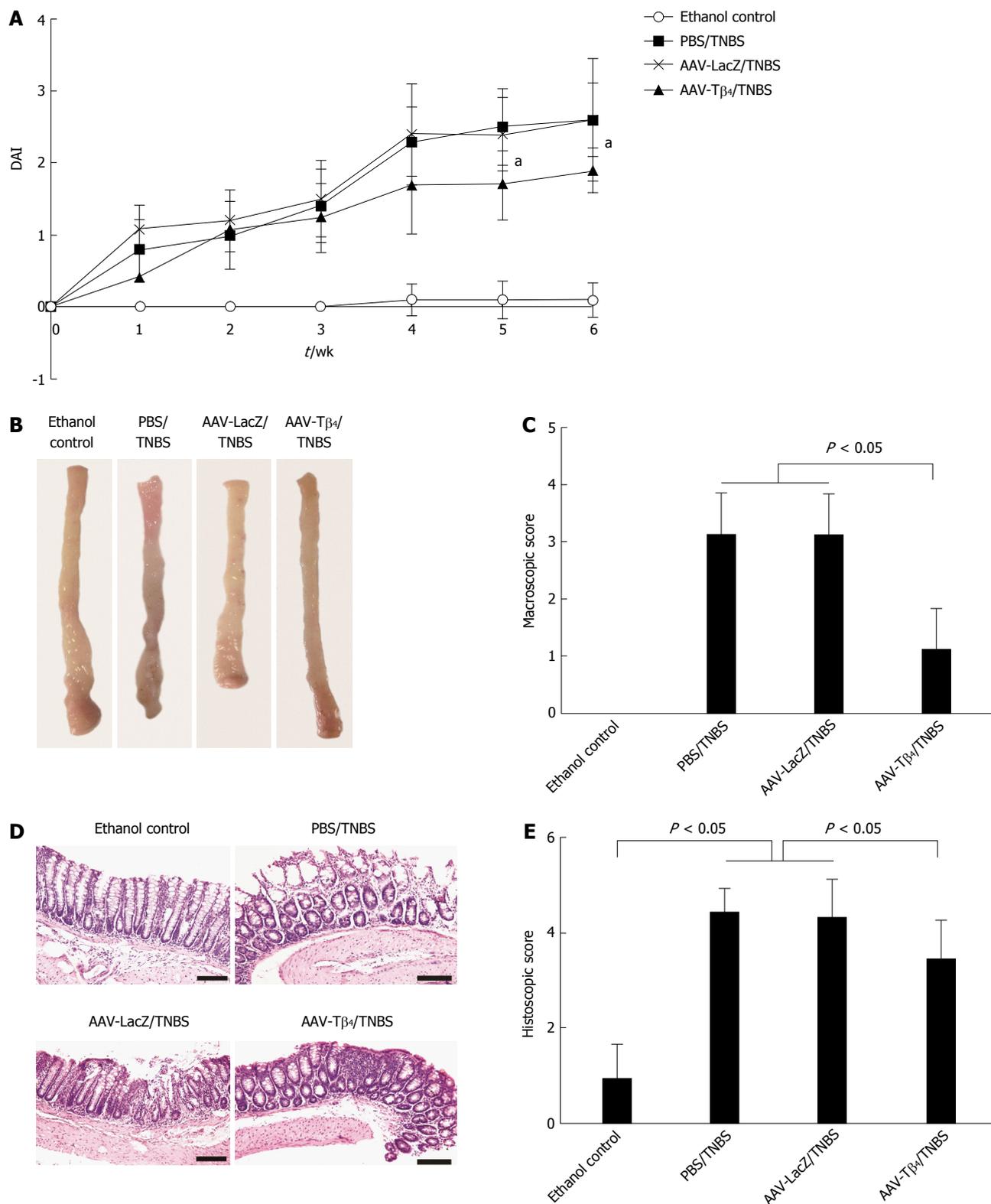
increased after DSS treatment, while it was mildly increased in the TNBS model. AAV-T $\beta_4$  markedly decreased the colonic MDA levels in both DSS- and TNBS-treated mice. DSS and TNBS treatments decreased the colonic SOD activities similarly, while AAV-T $\beta_4$  reversed these changes. These results demonstrate the efficacy of T $\beta_4$  in the alleviation of oxidative stress in colitis.

#### T $\beta_4$ modulates TNF- $\alpha$ , IL-1 $\beta$ , 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



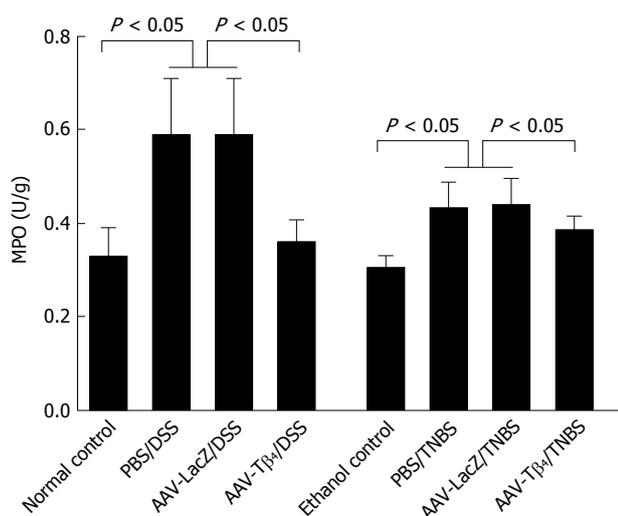
**Figure 2** Adeno-associated virus carrying thymosin  $\beta_4$  ameliorated dextran sulfate sodium-induced colitis in mice. **A:** Dynamic changes in DAI [the asterisk indicates  $^aP < 0.05$  vs PBS/dextran sulfate sodium (DSS) or adeno-associated virus (AAV)-LacZ/DSS group]; **B** and **C:** AAV-thymosin  $\beta_4$  (T $\beta_4$ ) 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  $\times 200$ ) revealed the mice in AAV-T $\beta_4$ /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  $\mu\text{m}$ . DAI: Disease activity index.



**Figure 3** Adeno-associated virus carrying thymosin  $\beta_4$  improved 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice. **A:** Dynamic changes in DAI [the asterisk indicates  $^*P < 0.05$  vs PBS/2,4,6-trinitrobenzene sulfonic acid (TNBS) or adeno-associated virus (AAV)-LacZ/TNBS group]; **B:** TNBS treatment resulted in hyperemia, edema and ulceration, which were attenuated by T $\beta_4$ ; **C:** Macroscopic score of AAV-T $\beta_4$ /TNBS group was significantly lower than those of the other two TNBS groups; **D** and **E:** Histological observation of colonic tissue (HE staining, magnification  $\times 200$ ) showed that AAV-T $\beta_4$  relieved TNBS-induced colonic mucosal lesions and reduced the histological score. Error bars indicate the SD. Scale bars = 50  $\mu\text{m}$ . DAI: Disease activity index.

effects of T $\beta_4$ . As shown in Figure 7, DSS treatment resulted in distinct increases in colonic TNF- $\alpha$ , IL-1 $\beta$  and IL-10 levels, while ectopically expressed T $\beta_4$

significantly inhibited these increases. Repeated TNBS enemas increased colonic TNF- $\alpha$  levels and decreased IL-10 levels, while AAV-T $\beta_4$  administration attenuated



**Figure 4** Adeno-associated virus carrying thymosin  $\beta_4$  suppressed dextran sulfate sodium - and 2,4,6-trinitrobenzene sulfonic acid-induced inflammation in colonic tissue, as indicated by myeloperoxidase activity assessment. Error bars indicate the SD. AAV: Adeno-associated virus; T $\beta_4$ : Thymosin  $\beta_4$ ; DSS: Dextran sulfate sodium; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

these alterations (Figure 7A and C). Unlike the findings in the DSS model, neither TNBS treatment nor ic AAV-T $\beta_4$  significantly altered colonic IL-1 $\beta$  levels (Figure 7B,  $P > 0.05$ ). Collectively, these data indicate that T $\beta_4$  could modulate the production of inflammatory cytokines in inflamed colons.

#### **T $\beta_4$ 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 T $\beta_4$  mediated by AAV significantly suppressed these increases (Figure 8), indicating that T $\beta_4$  alleviated the acceleration of cell proliferation in the inflamed colons.

## **DISCUSSION**

Although increased levels of serum T $\beta_4$  were reported in IBD patients<sup>[20]</sup>, the role of T $\beta_4$  in the development of IBDs is still elusive. In this study, the colonic expression of T $\beta_4$  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 T $\beta_4$  might be helpful to predict and assess the severity of IBDs. The present study also showed that AAV-mediated overexpression of T $\beta_4$  in mouse colons alleviated both DSS- and TNBS-induced colitis. Collectively, these data suggest a dose-dependent protective effect of T $\beta_4$  in inflamed colons. The increased production of local T $\beta_4$  in mice with severe colitis reflected an adaptive response of these mice to colonic damages, while the increased expression of endogenous T $\beta_4$  might not be

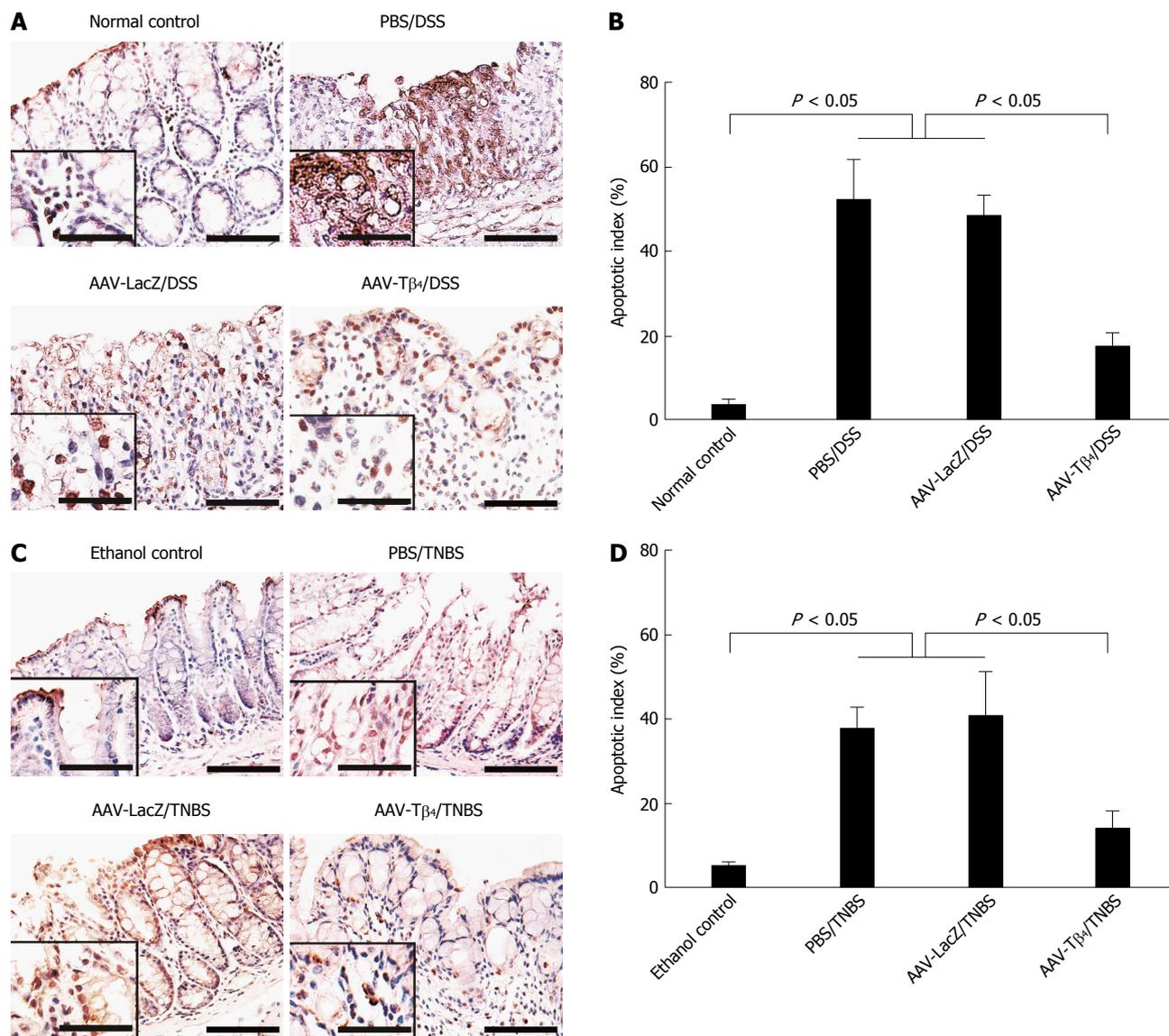
sufficient to withstand the colonic injury and present the protective effect. Only a dramatic increase in the local T $\beta_4$  content as in the present study achieved by AAV delivery, can distinctly attenuate the colitic manifestations in mice. Furthermore, our subsequent results indicated that the protective effect of T $\beta_4$  may involve suppressing oxidant damage and inflammatory cell infiltrations, regulating colonic 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 T $\beta_4$  treatment reduced the infiltration of MPO-positive neutrophils into the infarct zone after myocardial infarction<sup>[25]</sup>, and reduced the MPO activity in bleomycin-intoxicated lungs in mice<sup>[26]</sup>. In this study, MPO activity was distinctly decreased after AAV-T $\beta_4$  administration in both DSS- and TNBS-treated mice, enhancing our understanding of the inflammation-inhibitory efficacy of T $\beta_4$ .

TNF- $\alpha$  is the most potent pro-inflammatory cytokine involved in IBDs, which was verified by the successful application of TNF- $\alpha$  antibodies for the treatment of IBDs<sup>[27-29]</sup>. Consistent with the previous study in which systemic administration of T $\beta_4$  decreased the TNF- $\alpha$  level in mice with sepsis<sup>[9]</sup>, our results show that AAV-T $\beta_4$  markedly alleviated the upregulated expression of TNF- $\alpha$  in the injured colonic tissues, suggesting that suppressing TNF- $\alpha$  expression is an essential mechanism for the T $\beta_4$ -mediated alleviation of colitis.

IL-1 $\beta$  is an important mediator of inflammation, and its level is often increased during the development of UC. However, reports regarding alterations of IL-1 $\beta$  levels in CD are controversial<sup>[28,30-32]</sup>. Distinct upregulation of IL-1 $\beta$  was found in the colons of DSS-treated mice, while the IL-1 $\beta$  content was hardly changed in the TNBS model. T $\beta_4$  reversed the DSS-induced increase in colonic IL-1 $\beta$  expression, which suggests that the suppression of IL-1 $\beta$  expression contributes to the protective effect of T $\beta_4$  on colitis.

IL-10, a potent anti-inflammation cytokine, is considered a negative regulator in IBDs<sup>[33,34]</sup>. 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<sup>[27,35]</sup>, but normal<sup>[28]</sup>, decreased<sup>[36,37]</sup> and elevated<sup>[38,39]</sup> 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<sup>[40]</sup>. In our study, AAV-T $\beta_4$  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



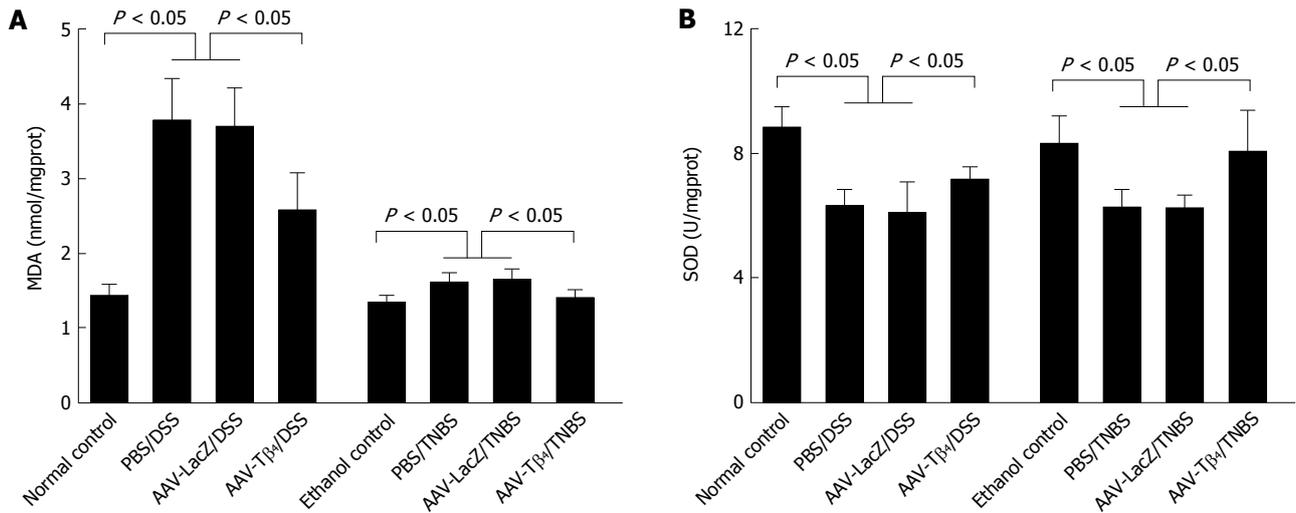
**Figure 5** Adeno-associated virus carrying thymosin  $\beta_4$  alleviated dextran sulfate sodium- and 2,4,6-trinitrobenzene sulfonic acid-induced epithelial cell apoptosis in the colonic mucosa. TUNEL staining showed that intracolonic adeno-associated virus (AAV)-thymosin  $\beta_4$  (T $\beta_4$ ) 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  $\mu$ m and 25  $\mu$ m (insets).

colitis is characterized by a predominant Th2 response (increase in IL-10). A previous study reported that T $\beta_4$  had inhibitory effects on human colonic lamina propria lymphocytes<sup>[18]</sup>. Thus, we speculate that the disparate effects of T $\beta_4$  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 T $\beta_4$  in attenuating colitis.

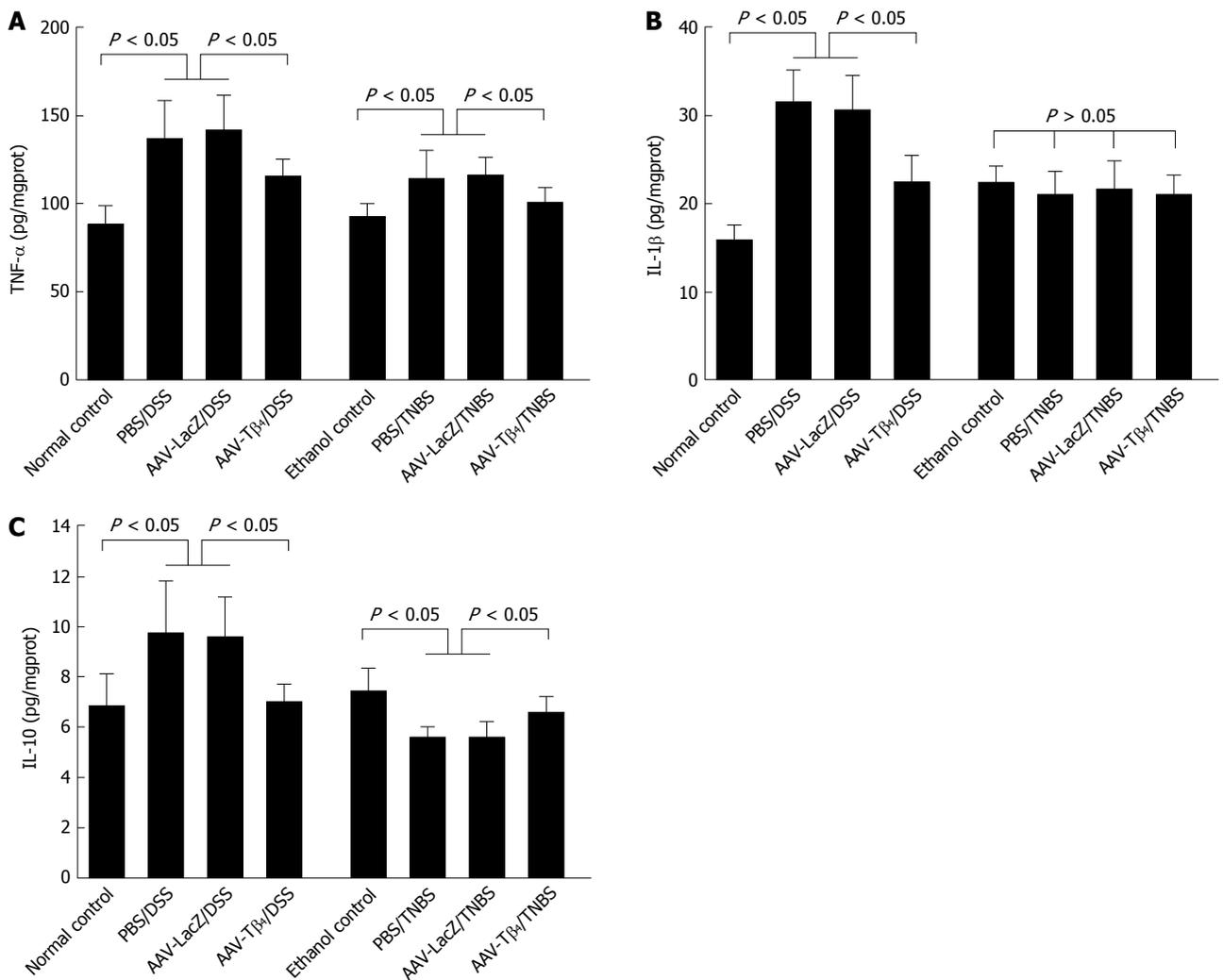
Oxidant/antioxidant imbalance plays an essential role in the pathogenesis of IBDs. The present study demonstrates that AAV-T $\beta_4$  administration decreased MDA production and increased SOD activity in the inflamed colons, which illustrates the efficacy of T $\beta_4$  in scavenging ROS and boosting anti-oxidative defense during colitis. ROS mainly originate from

inflammatory cells. Therefore, the mechanism of T $\beta_4$  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 T $\beta_4$  reduced ROS levels in corneal epithelial cells, cardiac fibroblasts and cardiomyocytes *via* the upregulation of the expression of antioxidant enzyme SOD and catalase<sup>[41-43]</sup>. A direct enhancement of SOD expression by T $\beta_4$  might also contribute to the protection of oxidative damage.

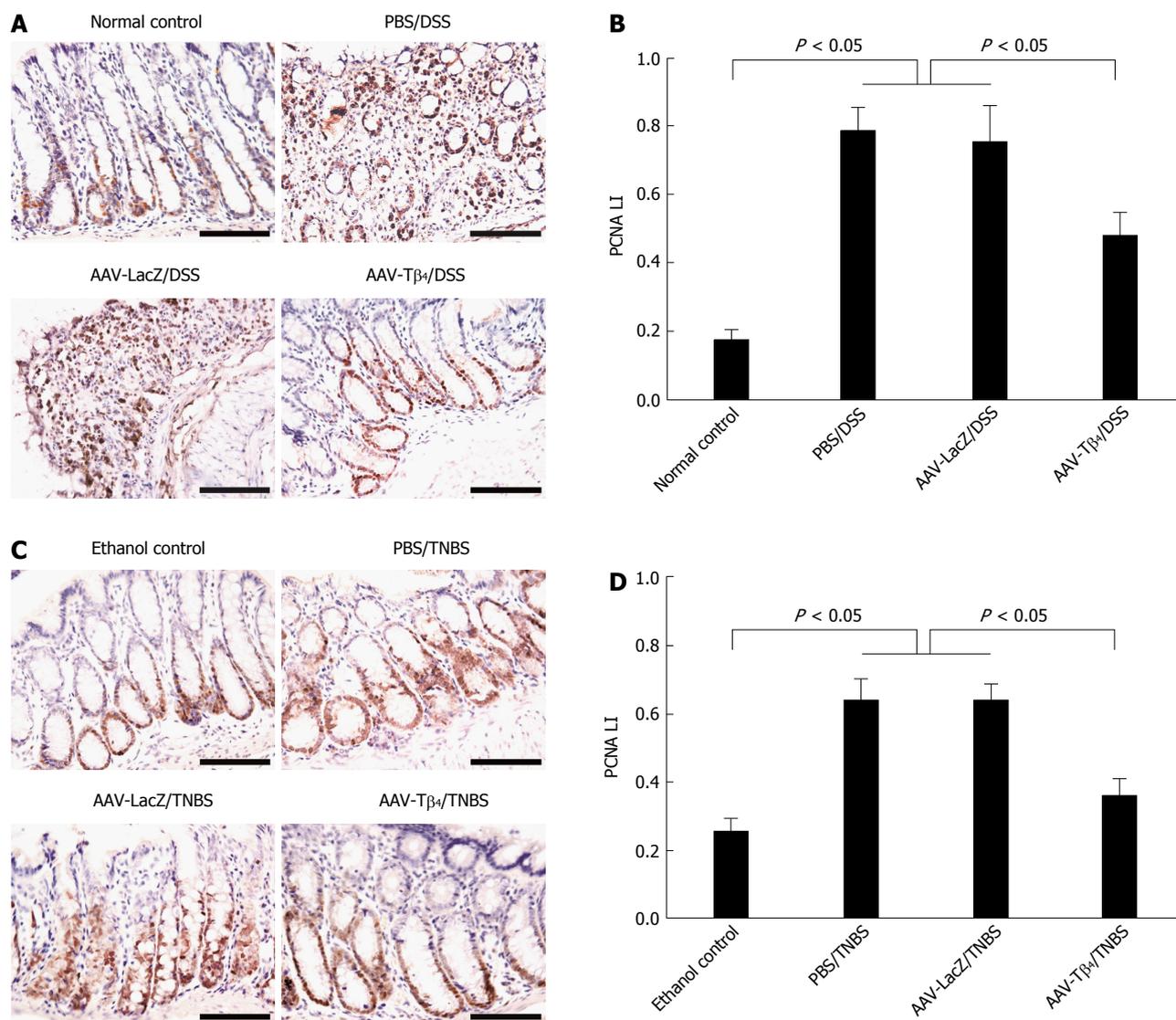
Intestinal epithelial apoptosis is a representative indicator of mucosal damage in IBDs. In the present study, the attenuation of colonic cell apoptosis by T $\beta_4$  should be mainly due to its inhibitory effects on inflammation and oxidative reaction, which protect mucosal epithelia against death-inducing factors. Moreover, there is substantial evidence that T $\beta_4$  suppresses



**Figure 6** Adeno-associated virus carrying thymosin  $\beta_4$  relieved colonic oxidant damage in the mice with colitis. MDA content (A) and SOD activity (B) were determined using biochemical assays. Error bars indicate the SD. AAV: Adeno-associated virus; T $\beta_4$ : Thymosin  $\beta_4$ .



**Figure 7** Adeno-associated virus carrying thymosin  $\beta_4$  modulated colonic TNF- $\alpha$ , IL-1 $\beta$  and IL-10 levels in dextran sulfate sodium and 2,4,6-trinitrobenzene sulfonic acid models. Colonic TNF- $\alpha$  (A), IL-1 $\beta$  (B) and IL-10 (C) levels were determined by ELISA. Error bars indicate the SD. AAV: Adeno-associated virus; T $\beta_4$ : Thymosin  $\beta_4$ ; DSS: Dextran sulfate sodium; TNBS: 2,4,6-trinitrobenzene sulfonic acid.



**Figure 8 Adeno-associated virus carrying thymosin  $\beta_4$  attenuated the acceleration of colonic mucosal cell proliferation following dextran sulfate sodium and 2,4,6-trinitrobenzene sulfonic acid treatments.** Colonic cell proliferation was determined by PCNA immunostaining in dextran sulfate sodium (DSS) (A) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) models (C). PCNA label indexes (PCNA LIs) of the DSS model (B) and TNBS model (D) were calculated. Error bars indicate the SD. Scale bars = 50  $\mu\text{m}$ . AAV: Adeno-associated virus; T $\beta_4$ : Thymosin  $\beta_4$ .

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<sup>[8,44-48]</sup>. Thus, a direct anti-apoptotic activity of T $\beta_4$  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 T $\beta_4$  for inhibiting colonic epithelial apoptosis.

T $\beta_4$  can promote the regeneration of the cornea, skin, heart, and intestine epithelial cells<sup>[1,46,47]</sup>; nevertheless, PCNA immunostaining in the current study revealed that T $\beta_4$  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 T $\beta_4$  reduces

the compensatory regeneration secondary to tissue damage, which surpasses the proliferation-promoting effect mediated by T $\beta_4$ .

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<sup>[49-51]</sup>. In the present study, we constructed AAV-T $\beta_4$  and confirmed its efficacy in transducing mouse colonic cells and mediating high expression of secretory T $\beta_4$ .

In conclusion, our study verifies that T $\beta_4$  is a protective molecule in murine colitis and suggests that ic

AAV-T $\beta_4$  could potentially be developed into a promising therapeutic approach for IBDs. To further clarify the importance of endogenous T $\beta_4$  in the maintenance of intestinal homeostasis and in the development of IBDs, tissue-specific ablation of T $\beta_4$  in mouse colons or neutralization of extracellular T $\beta_4$  by immunizing animals with a T $\beta_4$ -carrier protein conjugates should be carried out. Besides, the "treatment" effect of AAV-T $\beta_4$  on chronic colitis such as IL-10 knock-out mouse model is also under our consideration.

## COMMENTS

### Background

Although increased serum thymosin  $\beta_4$  (T $\beta_4$ ) level has been reported in inflammatory bowel diseases (IBDs) patients, its role in the development of IBDs is still unclear. Preclinical and clinical studies have confirmed that T $\beta_4$  can promote wound healing and attenuate inflammation, suggesting a possible beneficial role of T $\beta_4$  in colitis. Recombinant adeno-associated virus (AAV), when intracolonic (ic) administered, can efficiently infect the colonic mucosa and therefore it is a suitable vector to mediate ectopic expression of therapeutic genes for both research and therapeutic applications in IBDs.

### Research frontiers

This study is novel in demonstrating that T $\beta_4$  is a protective factor in colitis by relieving oxidant damage, attenuating epithelial apoptosis, reducing inflammatory cell infiltrations, and modulating tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-10 levels in the inflamed colonic mucosa of mice.

### Innovations and breakthroughs

To achieve persistent, secretory overexpression of T $\beta_4$  in mouse colons, the authors constructed a recombinant AAV carrying a fusion gene composed of the N-terminal 80 amino acid signal peptide of human neurotrophin-4 preproprotein fused to the N-terminus of the coding region of T $\beta_4$  and employed it via ic administration. Moreover, the authors tested the effects of ic AAV-T $\beta_4$  in both the dextran sulfate sodium colitis (ulcerative colitis-like) and TNBS (Crohn's disease-like) colitis models.

### Applications

The results of the present study suggest that intracolonic AAV-T $\beta_4$  is likely to be developed as an efficient and convenient therapeutic agent for IBDs.

### Terminology

T $\beta_4$ , a conserved 43-amino acid protein, is present in nearly all cells and in various body fluids. Despite the lack of a signal peptide, T $\beta_4$  can be released to extracellular spaces by either secretion, cell lysis or necrosis, while extracellular or exogenous T $\beta_4$  can be rapidly internalized by presently unknown mechanisms. T $\beta_4$  possesses many physiological activities, including promotion of cell migration, adhesion, angiogenesis, cell survival, stem cell maturation and down-regulation of inflammatory cytokines. There is evidence that the biological actions of T $\beta_4$  are mediated in part by activating integrin-linked kinase, increasing bcl-2 expression and decreasing caspase activation.

### Peer-review

This is a well-designed study. The role of T $\beta_4$  in colitis is novel and the data of the present study indicate that AAV-T $\beta_4$  may be explored as a promising therapeutic agent for IBDs.

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**P- Reviewer:** Gupta S **S- Editor:** Yu J **L- Editor:** A  
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ISSN 1007-9327



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