Original Article
Mesenteric injection of adipose-derived mesenchymal stem cells relieves experimentally-induced colitis in rats by regulating Th17/Treg cell balance

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Abstract: Efficient delivery routes are critical for the effectiveness of adipose-derived mesenchymal stem cells (ADMSCs) in treating inflammatory bowel disease (IBD). Conventional ADMSC delivery routes include local, intravenous and intraperitoneal injection. Whether mesenteric injection has potential in IBD treatment remains unknown. In the present study, we investigated the therapeutic effects of mesenteric injection of ADMSCs in a trinitrobenzene sulfonic acid-induced rat IBD model and explored whether this treatment affected T helper 17 (Th17)/regulatory T (Treg) cell ratio. The results showed that mesenteric injection of ADMSCs markedly reduced signs of colitis, colon shortening, weight loss and pathological damage. The treatment also decreased serum tumor necrosis factor alpha concentration, increased serum tumor necrosis factor alpha-stimulated gene protein 6 concentration, and augmented repair via proliferation (assessed by evaluating Ki-67 levels) in colonic tissue. Moreover, mesenteric injection of ADMSCs reduced interleukin (IL)-17A and IL-6 mRNA expression, and increased IL-10 and transforming growth factor-beta mRNA expression in colonic tissue. Protein analyses indicated that mesenteric injection of ADMSCs was associated with increased expression of forkhead box P3$^+$ and IL-10 as well as decreased expression of retinoid-related orphan receptor λt and IL-17. Additionally, the treatment inhibited phosphorylation of signal transducer and activator of transcription (STAT) 3 and activated phosphorylation of STAT5. Taken together, these results suggest that mesenteric injection of ADMSCs is a promising approach to treating trinitrobenzene sulfonic acid-induced IBD, and achieves its therapeutic effect by regulating the pro/anti-inflammatory Th17/Treg cell balance.

Keywords: Inflammatory bowel disease, adipose-derived mesenchymal stem cells, Th17/Treg cells, mesenteric injection

Introduction

Inflammatory bowel diseases (IBDs), which include Crohn's disease and ulcerative colitis, share manifestations such as abdominal pain, chronic inflammation, diarrhea and visceral hypersensitivity [1, 2]. Inflammatory bowel disease prevalence and mortality have been increasing globally during recent decades, including in areas of Asia that previously had low-incidence [3, 4]. Severe manifestations may be associated with life-threatening complications [5].

The causes of IBD are unclear. Available treatments for IBD are mainly based on non-specific immunosuppression. These therapies are either harmful or unable to sustain remission [6].

About 30% of patients with Crohn's disease first undergo bowel resection surgery within 7 years of diagnosis and many require secondary surgery [7]. There is therefore an increasingly strong impetus to search for more effective IBD treatment approaches.

Adipose-derived mesenchymal stem cells (ADMSCs) display strong plasticity and are easily isolated from adipose tissues [8]. Furthermore, ADMSCs migrate towards inflammatory sites and can be transplanted between different individuals without species restriction [9-11], because they are immunogenic and have some immunosuppressive capability [12]. After engraftment, ADMSCs suppress inflammation through immunomodulation and play a regenerative role by promoting secretion of trophic

Table 1. Disease activity index score (DAI)

Weight loss (%)	Occult blood	Stool consistency	Score
< 1	Negative	Normal	0
1-5	Haemoccult positive	Soft stool	1
5-10		Loose stool	2
10-15		Muddy stool	3
> 20	Gross bleeding	Diarrhea	4

Five grades of weight loss and stool consistency and three grades of occult blood.

and pro-angiogenic factors [13, 14]. Recently, ADMSCs have emerged as a novel cell-based therapy strategy [15, 16].

Hematopoietic stem cells and mesenchymal stem cells (MSCs) have shown promise for treating IBD. However, most experimental and clinical studies have focused on the therapeutic effects of the cells and their effect on T helper 1 (Th1)/Th2 cell balance. Few studies have investigated injection routes or the effect of the therapy on Th17/regulatory T (Treg) cell balance, which may have a significant impact on the therapeutic efficiency of ADMSCs in IBD [17].

This study aimed to investigate whether mesenteric injection of ADMSCs could relieve trinitrobenzene sulfonic acid (TNBS)-induced IBD in rats, and to evaluate the potential effects of the treatment on the balance of Th17/Treg cells.

Materials and methods

Animals

Eight-week-old male Sprague-Dawley rats (each weighing between 250 g and 280 g) were procured from the Shanghai Laboratory Animal Center and kept in a specific-pathogen-free environment at the Animal Experiment Center of Tongji University School of Medicine. The study protocol was approved by the Animal Welfare and Ethics Committee of the Shanghai East Hospital (affiliated with Tongji University School of Medicine).

Isolation, cultivation and identification of rat ADMSCs

Epididymal fat was obtained aseptically from rats and the phanic blood vessels were removed. The fat was then washed three times

with phosphate-buffered saline (PBS), cut into 1-2 mm^3 pieces, and digested with collagenase type I (Sigma, St. Louis, MO, United States) in culture medium (Dulbecco's modified Eagle medium [DMEM]/F12 [1:1]; Gibco, Invitrogen Inc., Carlsbad, CA, USA) at 37°C for 60 min. Cell suspensions were obtained by filtering the digested mixture through a 100-μm filter (BD Biosciences, Franklin Lakes, NJ, USA). The cell suspensions were then centrifuged at 760 × g for 15 min to yield cell pellets, which were then re-suspended and cultivated in Dulbecco's modified Eagle medium/F12 medium supplemented with 10% fetal bovine serum, 20 ng/μl transforming growth factor and 1% penicillin/streptomycin (Gibco) at 37°C. Non-adherent cells were removed from the cell culture after 72 h. Adherent cells at 50%-80% confluence were disassociated from the culture surface using 2.5 mL/L trypsin-EDTA solution (Gibco) and seeded into 6-well plates at 4000 cells/cm^2 for sub-culture. Cells at passage 4-5 were used in experiments.

The identity of the isolated ADMSCs was confirmed before they were used in the study by analysis with a MoFlo High-Speed Cell Sorter (DAKO-Beckman Coulter, Carpinteria, CA, USA) using phycoerythrin- or fluorescein isothiocyanate-conjugated antibodies against CD29, CD90, CD11b, CD34, CD45, CD106 or isotype-matched irrelevant monoclonal antibodies (BD-Pharmingen, San Jose, CA, USA). Data analysis was performed using Summit 4.3 software (DAKO-Beckman Coulter). The isolated ADMSCs were also characterized by osteogenic and adipogenic differentiation experiments.

Induction of colitis in rats by TNBS treatment

The rat IBD model was generated as previously described [18]. Briefly, rats were anesthetized intraperitoneally with 3% pentobarbital sodium and colitis was induced by intracolonic infusion of 1.2 mL of 50% alcohol containing 30 mg TNBS (Sigma) through an 8-cm, 4-French catheter positioned 8 cm from the anal verge. The rats were held vertically for 1 min to allow the TNBS solution to disperse throughout the colon cavity. The animals were then given standard water and chow ad libitum.

Experimental groups

The 21 rats were allowed to acclimatize to the environment for 1 week, then were randomly

Table 2. Sequences of primer pairs used in quantitative real-time PCR

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
IL-17A	AAGCACAGAAAGCATGATCCG	GAGTCCAGGGTGAAGTGGA
IL-10	CCTTCCTCCGTGTGGTTTGA	TTCTTGAGGGCCACTTCGTA
IL-6	GCCAGATAGAGTCGTTGCCC	AGCTAGTTGCCGTGTGTCTG
TGF-β	CCCACATGAGATCAGCCTCC	GGAGTTGGGTGGCAAGAGTT
GAPDH	TCAATGAAGGGGTCGTTGAT	CGTCCCGTAGACAAAATGGT

assigned to three paralleled groups. The rats in the TNBS + ADMSC group were treated with TNBS on day 0 and induction of IBD was confirmed by surgical inspection at day 1, before surgical mesenteric injection of ADMSCs (2 × 10^6 cells in 0.6 mL PBS per rat). The rats in the TNBS + PBS group were subjected to IBD induction by the same method but were treated with PBS instead of ADMSCs at the same time points. Rats in the control group underwent sham IBD induction with PBS only and were treated with PBS only. During the experiment, disease activity index (DAI) score was determined every day as described previously (**Table 1**) [19]. All assays were repeated at least three times. On day 6, all rats were sacrificed by cervical dislocation, and blood and tissue samples were collected.

Histopathological analysis

Isolated colon tissues were immediately washed in PBS, then fixed with 4% paraformaldehyde and mounted in paraffin wax. Tissue sections (5 μm thick) were collected on coated slides and stained with hematoxylin and eosin (H&E; Wako Pure Chemical Industries, Osaka, Japan). Histopathology scores were determined in a blinded fashion as described by Obermeier et al. [20]. Inflammatory bowel disease severity scores were determined by macroscopic evaluation according to a previous method [21]. Eight sections from each animal were assessed.

Fecal occult blood test

Occult blood tests were performed with a fecal occult blood kit (Baso Diagnostics Inc., Zhuhai, China). Tests were scored on a scale of 0 to 5 according to the color indicators provided by the manufacturer.

Myeloperoxidase (MPO) activity assay

Measurement of MPO activity is used to monitor neutrophil infiltration [22], as reported by

Krawise JE [23]. Myeloperoxidase activity levels in the colonic tissues were determined using a MPO activity kit (Jiancheng Institute of Biological Engineering, Nanjing, China) according to the manufacturer's protocol. The MPO activity per gram of wet tissue was calculated as follows: MPO activity (U/g wet tissue) = optical density in measuring tube - optical density in control tube)/11.3 × tissue weight (g). The coefficient 11.3 was the reciprocal of the slope.

Cytokine measurement and analysis of serum levels of tumor necrosis factor alpha-stimulated gene protein 6 (TSG-6)

Blood samples were collected from the celiac artery blood on day 6 and stored in sodium citrate-treated Eppendorf tubes. Plasma was obtained via centrifugation at 1000 × g for 15 minutes, and stored at -80°C until TSG-6 and tumor necrosis factor alpha (TNF-α) analysis. Levels of TSG-6 and TNF-α were determined by ELISA assay (CUSABIO Life Science, Wuhan, China). To analyze cytokines in colon mucosa, protein was extracted from colonic segments homogenized using a Polytron® System PT-1200E (Kinematica AG, Luzern, Switzerland). Samples were centrifuged at 30000 × g for 15 mins. Cytokines were then evaluated using ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from colon tissues with TRIzol reagent (Ambion, Carlsbad, California, USA), and concentration and quality were assessed. The RNA (2 μg) was then reverse-transcribed using a FastQuant RT kit (kr14-0818, Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. Quantitative real-time PCR was conducted using SYBR Green SuperReal PreMix Plus (FP151203, Tiangen Biotech Co., Ltd) and a 7500 Real-time PCR system (Applied Biosystems, Foster City, California, USA). All reactions were conducted in triplicate. Gene expression levels were determined using the comparative threshold cycle (ΔΔCt) method, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as the internal control. Primer sets for target genes
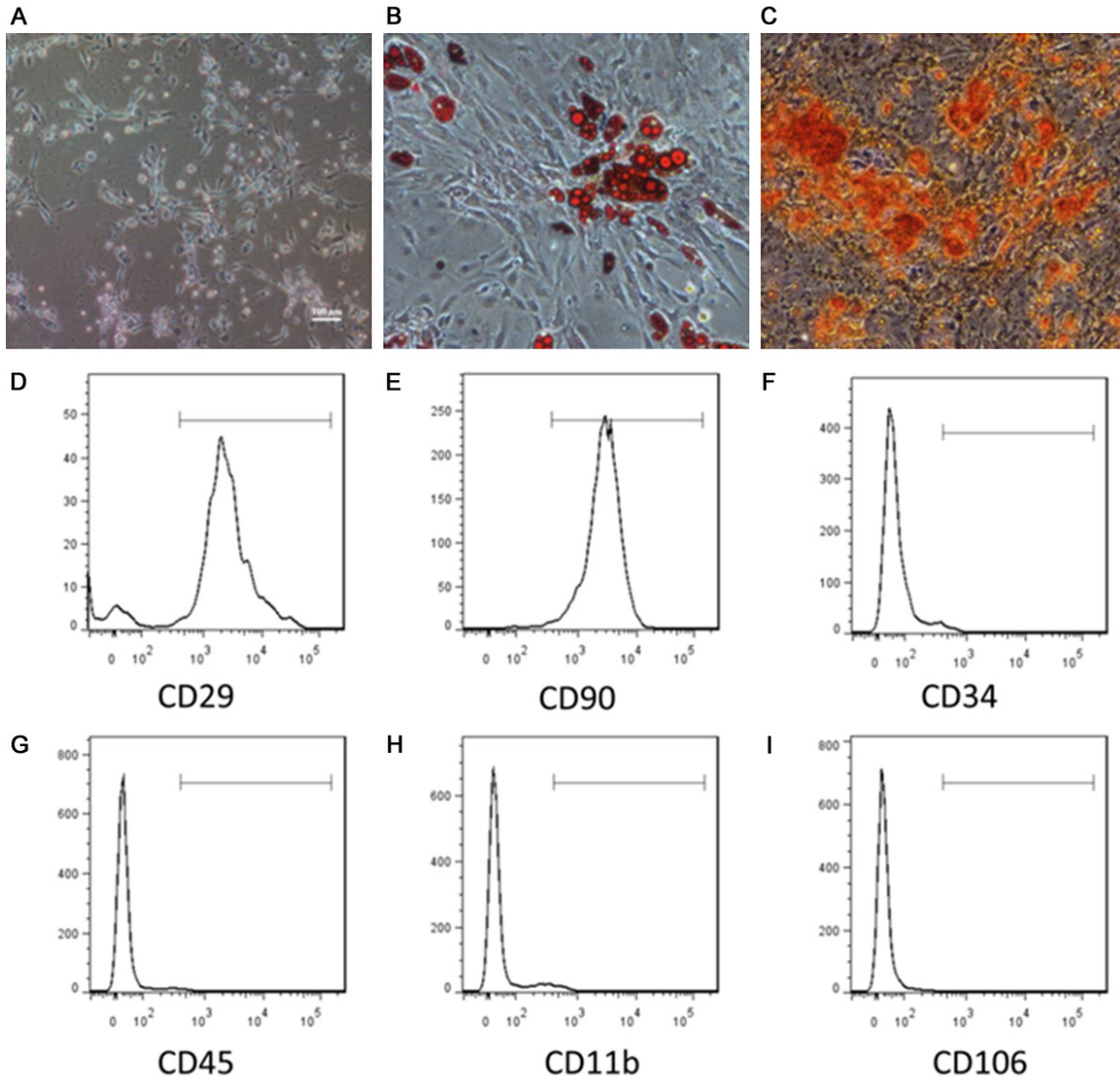
Figure 1. The isolated adipose-derived mesenchymal stem cells (ADMSCs) exhibit biological properties typical of MSCs. A. Representative field of ADMSC primary culture. The cells exhibit a classic spindle-shape morphology. Magnification, 10 ×; scale bar, 100 μm. B. Adipogenic differentiation of ADMSCs. Differentiation into adipocytes was confirmed by the presence of lipid vesicles stained with Oil Red O. Magnification, 10 ×; scale bars, 100 μm. C. Osteogenic differentiation of ADMSCs. Differentiation into osteocytes was confirmed by the presence of mineral nodule deposition stained with alizarin red S. Magnification, 10 ×; scale bar, 100 μm. D-I. Flow cytometric analysis of ADMSCs. Phenotypic analysis of ADMSCs, which was carried out by flow cytometry at passage 3, revealed that ADMSCs expressed the cell markers CD29 and CD90, but did not express the lineage markers CD34, CD45, CD11b or CD106.

are shown in **Table 2**. Data were analyzed using Sequence Detection Systems software (Applied Biosystems).

Western blotting

Proteins were extracted using radioimmuno-precipitation assay lysis buffer (Beyotime Biotechnology, Shanghai, China). Protein concentration was measured using a bicinchoninic acid assay kit (Beyotime Biotechnology). Protein samples were then subjected to SDS-PAGE using a PAGE gel rapid preparation kit (EpiZyme Biotechnology Co, Ltd, Shanghai, China) and transferred to polyvinylidene fluoride membranes (Billerica MA 0182, Millipore Co., Billerica, Massachusetts, USA). Membranes were incubated with primary antibodies against forkhead box P^{3+} (FoxP^{3+}), retinoid-related orphan receptor λt (RORλt), signal transducer and acti-
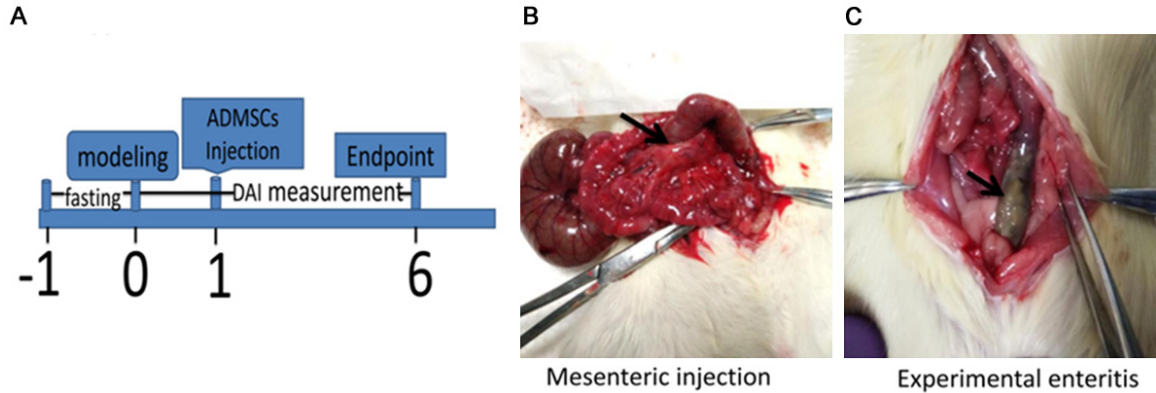
Figure 2. Experimental protocol for 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model generation. A. Experimental protocol. Rats were fasted for 24 h then received TNBS enemas on day 0; adipose-derived mesenchymal stem cells (ADMSCs) were injected into the mesentery 24 h later. Disease activity index (DAI) score was determined every day from day 0 to day 6. Rats were sacrificed and samples were obtained on day 6. B. ADMSCs (2 × 10^6 cells in 0.6 mL PBS per rat) were injected into the mesentery via a sterile surgical procedure. C. TNBS-induced inflammatory bowel disease (IBD) model. The induced IBD was confirmed by surgical inspection at day 1. The distal colon was congested and edematous and there were multiple ulcers in the colonic mucosa.
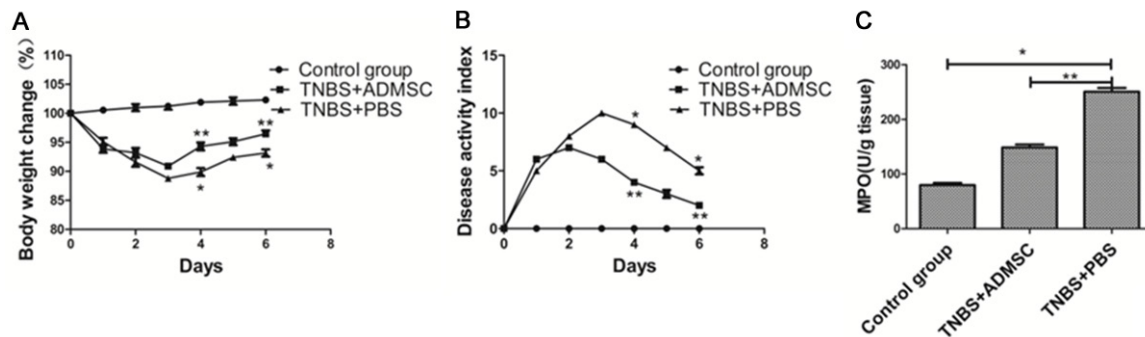

Figure 3. Mesenteric injection of adipose-derived mesenchymal stem cells (ADMSCs) protects against 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. A. Percentage body weight change over time. B. Disease activity index (DAI). The DAI score was determined by an investigator blinded to the protocol. Animals were observed daily for weight loss, stool consistency and presence of blood in the feces and anus. A score from 0 to 4 was assigned for each feature assessed, with the total score therefore ranging from 0 to 12. C. Myeloperoxidase activity. Values are expressed as means ± standard deviation (n = 7 rats/group). Data were compared by one-way analysis of variance followed by the Newman-Keuls test. *$P < 0.01$ vs. control group; **$P < 0.05$ vs. treatment group (TNBS + ADMSCs).

vator of transcription-3 (STAT3) and STAT5 at 4°C overnight and then with horseradish peroxidase-conjugated secondary antibody. Target proteins were visualized and analyzed using an Odyssey Imaging System (Li-COR Biosciences, Lincoln, Nebraska, USA).

Immunofluorescence staining

Immunofluorescence staining was performed to identify Ki-67-positive colon cells according to published procedures [24]. Briefly, tissue samples were embedded in resin and snap-frozen in isopentane in a liquid nitrogen bath, and stored at -80°C until use. The samples

were cut into 6-mm sections using a -20°C cryostat. The sections were air-dried and immersed for 5 min in Tissue-Tek OCT compound (CA9051, Sakura Finetek Inc., Torrance, California, USA). Antigen retrieval was conducted by incubation at 96°C for 15 min. The sections were then washed with PBS three times and blocked with normal non-immune donkey serum for 60 min, then incubated with anti-Ki-67 antibody (1:200; Santa Cruz, Delaware, California, USA) overnight at room temperature. The sections were incubated for a further 2 h with Cy3-conjugated goat anti-mouse antibody at room temperature, then washed with PBS and fixed in anti-fading medium containing DAPI.
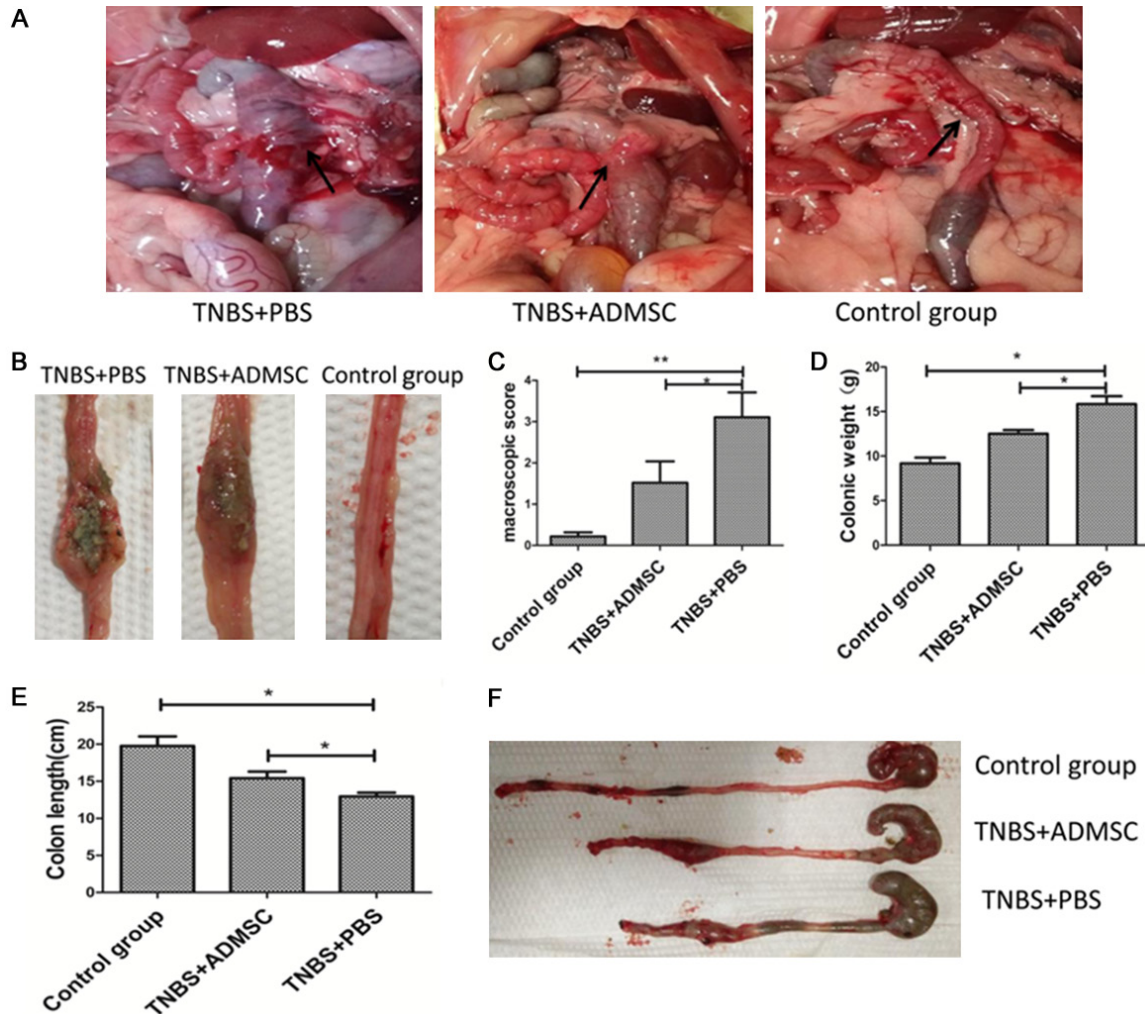
Figure 4. Effect of adipose-derived mesenchymal stem cells (ADMSCs) on macroscopic disease, colon weight and colon length in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. A. Effect of ADMSCs on colon congestion and swelling in rats with TNBS-induced colitis. The TNBS + ADMSC group showed reduction of congestion and swelling of colon compared with the TNBS + PBS group. B. Macroscopic damage to the colonic mucosa. The TNBS + ADMSC group showed a reduction in colonic mucosal ulceration and edema compared with the TNBS + PBS group. C. Macroscopic damage score. D. Change in colon weight in rats with TNBS-induced colitis. E, F. Change in colon length in rats with TNBS-induced colitis. The TNBS + PBS group exhibited reduced colonic length compared with the TNBS + ADMSC group. Colons were removed from the cecum to the anus. Samples were measured, weighed and observed as an indirect assessment of inflammation. Macroscopic assessment of colitis was scored according to the range of ulceration and inflammation; a score from 0 to 5 was assigned for each measured characteristic. Data are expressed as mean ± standard deviation (n = 7 rats/group). Data were compared by one-way analysis of variance followed by the Newman-Keuls test. *P < 0.05; **P < 0.01.

The sections were examined with an inverted fluorescence microscope (VS120-S, Olympus, Shinjuku-ku, Tokyo, Japan).

Statistical methods

Data are presented as mean ± standard deviation. Data were compared between three groups by one-way analysis of variance followed by the Newman-Keuls test. *P* values < 0.05

were considered statistically significant. All statistical analyses were conducted using SPSS 17.0 (SPSS, Chicago, IL, USA).

Results

ADMSC phenotype identification

The cells extracted from epididymal fat exhibited the spindle-shaped morphology typical of
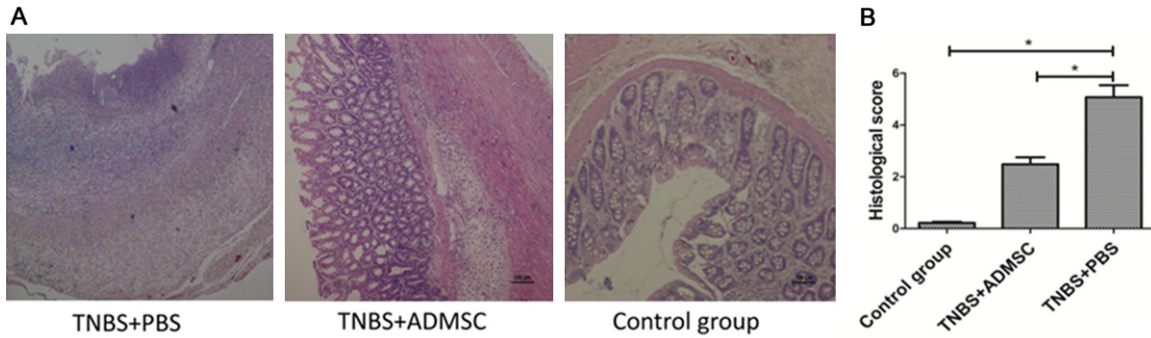
Figure 5. Mesenteric injection of adipose-derived mesenchymal stem cells (ADMSCs) promotes histological improvement. A. Hematoxylin and eosin (HE) staining. Colon histology demonstrated that mesenteric injection of ADMSCs reduced the extent of the inflamed area, crypt damage and infiltration of inflammatory cells. Magnification, 10 ×; scale bar: 100 μm. B. Colitis histological score. Histopathological scores were determined in a blinded fashion according to the grade of epithelium damage and infiltration. A score from 0 to 4 was assigned for each feature assessed, and the total score was defined as the sum of the scores for the two features. Rats were scored individually; eight sections for each animal were evaluated. Each group score represents the mean of at least six rats in each group. Data are expressed as mean ± standard deviation (n = 7 rats/group). Data were compared by one-way analysis of variance followed by the Newman-Keuls test. *$P < 0.05$.

ADMSCs (**Figure 1A**) and were capable of adipogenic and osteogenic differentiation (**Figure 1B** and **1C**). As expected of ADMSCs [25], most of the cells were positive for CD29 and CD90 (**Figure 1D** and **1E**) and had low expression levels of CD34, CD35, CD11b and CD106 (**Figure 1F-I**). These results demonstrate that ADMSCs were successfully established.

Effects of mesenteric injection of ADMSCs on TNBS-induced IBD

We first studied the therapeutic impact of mesenteric injection of ADMSCs on TNBS-induced IBD using reported assessment criteria [26].

Figure 2A shows the time points of TNBS induction of IBD and ADMSC injection. ADMSCs were injected into the mesentery (**Figure 2B**) after experimental IBD was induced with TNBS (**Figure 2C**). To assess the severity of IBD, DAI and changes in body weight were recorded daily. Mesenteric injection of ADMSCs decreased the weight loss and DAI score, and also decreased MPO activity (**Figure 3A-C**). Moreover, mesenteric injection of ADMSCs relieved colitis (**Figure 4A**) and decreased macroscopic score (**Figure 4B** and **4C**), colon weight (**Figure 4D**) and colonic shortening (**Figure 4E** and **4F**). Intestinal ulceration and inflammation severity were further evaluated by H&E staining. Treatment with ADMSCs decreased histological score, inflammatory cell infiltration, and mucosal ulceration (**Figure 5A** and **5B**). Moreover, we compared

the Ki-67 expression among the three groups to assess mucosal repair via proliferation. More Ki-67-positive cells were present in the bottom of the crypts in the ADMSC-treated group (**Figure 6**) than in the other two groups. Furthermore, mesenteric injection of ADMSCs significantly increased serum TSG-6 protein levels (**Figure 7A**), compared with the other two treatments.

Mesenteric injection of ADMSCs reduced inflammatory responses in TNBS-induced IBD

We next investigated whether mesenteric injection of ADMSCs influenced the production of immunomodulatory cytokines involved in intestinal inflammation. As shown in **Figure 7B**, expression levels of TNF-α were significantly decreased after ADMSC treatment. Similarly, levels of IL-1β were also markedly reduced (**Figure 7C**). We also observed an obvious decrease in expression of the pro-inflammatory cytokines IL-17A and IL-6 (**Figure 8A** and **8B**) after mesenteric injection of ADMSCs, and an increase in anti-inflammatory IL-10 and transforming growth factor-beta (TGF-β) levels (**Figure 8C** and **8D**).

Mesenteric injection of ADMSCs corrected Th17/Treg cell imbalance in rats

As shown in **Figure 8A**, **8C** and **8E-G**, mesenteric injection of ADMSCs markedly increased levels of FoxP3+ and IL-10, but significantly
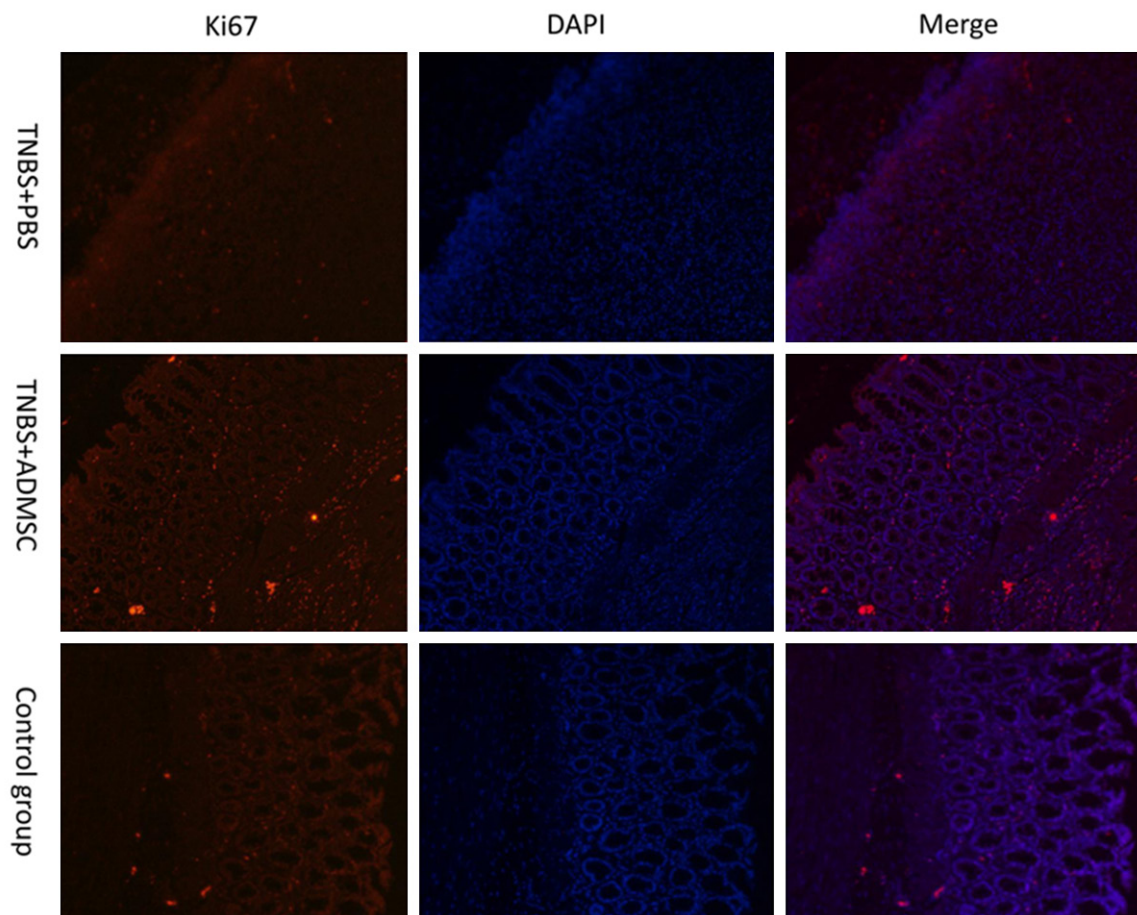
Figure 6. Colonic proliferation was analyzed on day 6. Ki-67-positive cells were detected at the inflamed colon. The adipose-derived mesenchymal stem cell (ADMSC)-treated group exhibited more Ki-67-positive cells in the lower part of the crypt than the other groups.

reduced levels of RORλt and IL-17A. Furthermore, mesenteric injection of ADMSCs inhibited STAT3 phosphorylation, but increased STAT5 phosphorylation (**Figure 8G**).

Discussion

The results of the present study show that mesenteric injection of ADMSCs ameliorated TNBS-induced IBD by promoting the functional activity of Treg cells while suppressing the Th17 cell response *in vivo*. Thus, mesenteric injection of ADMSCs may be a new delivery route option for stem cell therapy.

The administration route is important in stem cell therapy. Currently, intravenous injection is the most commonly used method for MSC delivery. However, intravenous injection has significant drawbacks, such as entrapment of injected cells in the lungs, a high rate of cell

death [27], and low efficiency (< 1%) of cell distribution to the target organ [28]. Intraperitoneal injection is also an important route of cell injection. Intraperitoneally injected MSCs pass through the intestinal wall and can engraft at the inflamed colon, where they exert anti-inflammatory effects [29-31]. Fluorescence-labeled peritoneal cells have been shown to migrate from the peritoneal cavity and were detected at intestinal villi [32]. The phenomenon may provide some hints. Our study demonstrated that mesenteric injection of ADMSCs significantly reduced signs of IBD and decreased bowel inflammation in rats with TNBS-induced IBD in comparison with control groups. Thus, mesenteric injection may be a feasible ADMSC delivery route. Mesenteric injection of ADMSCs exhibits some advantages compared with the other injection routes, although the exact explanations for these advantages are
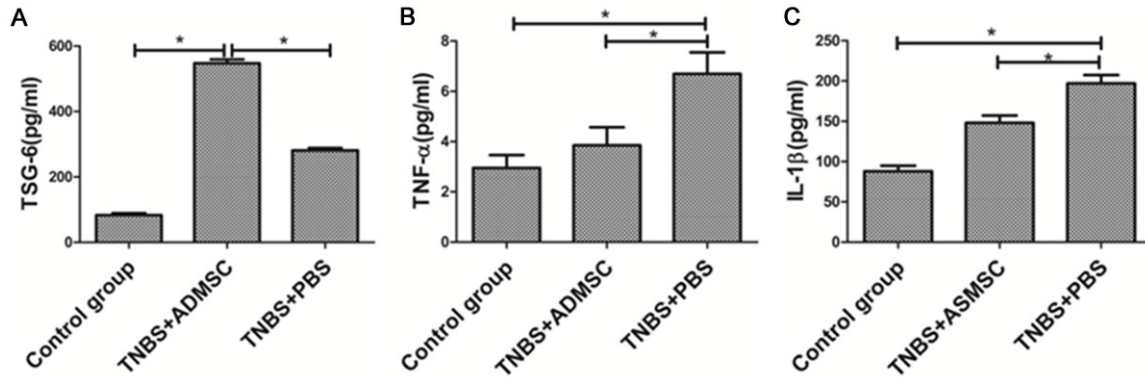
Figure 7. Mesenteric injection of adipose-derived mesenchymal stem cells (ADMSCs) decreased the systemic inflammatory response in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced inflammatory bowel disease (IBD) model. Plasma was obtained from blood taken from the celiac artery. A-C. Levels of cytokines (interleukin-1 beta [IL-1β], tumor necrosis factor alpha [TNFα] and TNFα-stimulated gene protein 6 [TSG-6]) were measured using ELISA Kits. Data are expressed as mean ± standard deviation (n = 7 rats/group). Data were compared by one-way analysis of variance followed by the Newman-Keuls test. *P < 0.05; **P < 0.01.
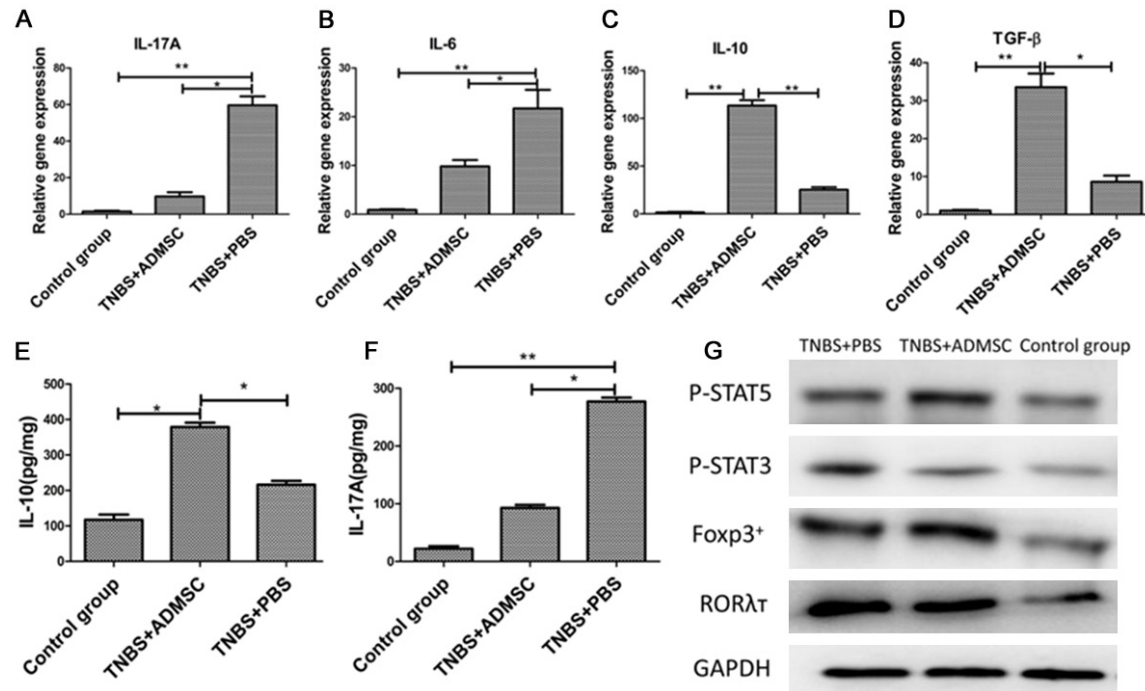

Figure 8. Mesenteric injection of adipose-derived mesenchymal stem cells (ADMSCs) corrected T helper 17 (Th17)/regulatory T (Treg) cell imbalance in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced inflammatory bowel disease (IBD) model. A-D. Effect on interleukin (IL)-17, IL-6, IL-10 and transforming growth factor-beta (TGF-β) expression, assessed by qRT-PCR. E, F. Effect on IL-10 and IL-17 expression, assessed by ELISA. G. Effect on STAT3 and STAT5 activation and on levels of the transcription factors FoxP3+ and RORλt, assessed by immunoblotting. The mesenterically injected ADMSCs increased expression of FoxP3+ and inhibited STAT3 phosphorylation, whereas they decreased expression of RORγt and increased STAT5 phosphorylation. All data are expressed as mean ± standard deviation (n = 7 rats/group). Data were compared by one-way analysis of variance followed by the Newman-Keuls test. *p < 0.05; **p < 0.01.

unclear. First, mesenteric injection is a more efficient and targeted delivery method, possibly because it may avoid the damage to the inject-

ed cells that would occur in the circulation and cell depletion by the lungs and spleen. Second, the mesentery may act as a temporary pool for

ADMSC storage and amplification. However, these possibilities require further validation *in vivo*.

The TNBS-induced IBD rat model is one of the most commonly used experimental IBD models, and has been demonstrated to be a reliable and reproducible animal model. Pathological analysis confirmed that IBD was successfully induced by TNBS treatment in rats in our study. We isolated ADMSCs from the subcutaneous adipose tissue of the rats. In keeping with previous reports, the ADMSCs exhibited multi-lineage differentiation potential and expressed mesenchymal markers [30].

Notably, our findings suggested involvement of multiple cytokines and biomarkers for IBD. MPO is found in neutrophils as a marker of inflammatory cell infiltration, which is an important characteristic of IBD [33]. Here, we observed inhibition of MPO activity after ADMSC injection, which suggests reduced neutrophil infiltration. We also demonstrated that mesenteric injection of ADMSCs was associated with increased serum TSG-6 levels, a result consistent with the findings of a study by Scaldaferri et al. [34]. TSG-6 is an anti-inflammatory factor released by neutrophils in secretory granules. It inhibits neutrophil migration and inflammatory responses [35]. TSG-6 secretion is induced by inflammatory cytokines and mediates the anti-inflammatory activities of MSCs. Upregulation of TSG-6 secretion after MSC transplantation is observed in IBD models [36]. Choi and colleagues reported that TSG-6 exerted an anti-inflammatory effect by regulating inflammatory cytokine expression [37]. Interestingly, we also observed that TSG-6 was significantly negatively associated with plasma TNF-α and IL-1β levels, in keeping with the results of previous studies [38]. TNF-α is a critical cytokine in IBD pathogenesis. The actions of anti-TNF-α agents strengthen the role of TNF-α in the pathogenesis of IBD [39]. In our study, the decrease in TNF-α levels was in keeping with the fact that MSCs display immunomodulatory effects [40]. Therefore, our results may help to explain why mesenteric injection of ADMSCs has a therapeutic effect in TNBS-induced IBD.

We also found that mesenteric injection of ADMSCs ameliorated TNBS-induced colitis by suppressing the inflammatory Th17 cell response and promoting Treg cell function *in vivo*.

Th-17 cells, which produce IL-17, are a pro-inflammatory sub-class of T cells that lead to autoimmunity and tissue damage when present in excessive numbers [41, 42]. Treg cells secret TGF-β and IL-10 and belong to a subset of CD4+ lymphocytes [43]. TGF-β and FoxP3^{+} are required for Treg cell differentiation. Treg cells exert an antagonistic effect against Th-17 cells. They are also responsible for self-antigen tolerance, protection against tissue injury, and inhibition of autoimmunity in infectious diseases [44]. Evidence shows that the relative phosphorylation level of STAT3 and STAT5 is an essential factor for mediation of Th17 cell differentiation [45]. STAT3 phosphorylation, along with increased IL-6 and TGF-β levels, initiates RORλt expression, leading to increased proinflammatory cytokines expression [46]. STAT5 phosphorylation suppresses differentiation of Th17 cells [47] and production of the cytokine IL-17 [48], and increases Treg cell differentiation and expression of Foxp3 [49]. However, STAT3 phosphorylation inhibits Treg cell differentiation. Thus, the balance of STAT3 and STAT5 phosphorylation is associated with the balance between Th17 and Treg cells. The Th17/Treg cell balance may therefore have a significant impact on the pathologic progress and outcome of IBD. Th17 and Treg cells have been reported to be key mediators of IBD. Previous studies found that Treg cell depletion worsened the disease in mice [50], while inflammation was ameliorated by infusing whole T cells [51]. New treatments targeting specific cytokines to address immune imbalances have emerged as therapeutic candidates for IBD. Tocilizumab and ustekinumab have been used in patients with IBD. Moreover, Hui Yin et al. demonstrated that sirolimus relieved TNBS-induced colitis by regulating the Th17/Treg cell balance [52]. In our study, we found that mesenteric injection of ADMSCs inhibited RORλt and IL-17 expression along with STAT3 phosphorylation in TNBS-induced colitis, and also induced FoxP3^{+} and IL-10 expression along with STAT5 phosphorylation. Our results suggest that mesenteric injection of ADMSCs restored the Th17/Treg cell balance in TNBS-induced IBD by regulating the STAT3/STAT5 signaling pathway. Our findings are consistent with the results of previous studies [53]. However, our study differed from previous experiments in that we compared the Th17/Treg cell balance by analyzing several specific cytokines and

transcription factors in colon tissues. This may be a better method because it reflects not only the balance of Th17/Treg cells, but also local changes in inflammatory factors in the colon.

In conclusion, our results demonstrate that mesenteric injection of ADMSCs can ameliorate TNBS-induced IBD by reversing Th17/Treg cell imbalance. Mesenteric injection of ADMSCs may be a new route for ADMSC administration. Future studies should focus on tracking the distribution of ADMSCs in colon tissues and comparing mesenteric injection with other delivery routes.

Acknowledgements

Disclosure of conflict of interest

None.

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