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Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice

Zexian Chen [1], Xiaowen He [1], Xiaosheng He, Xiuting Chen, Xutao Lin, Yifeng Zou, Xiaojian Wu, Ping Lan *

Department of Colorectal and Anal Surgery, The Sixth Affiliated Hospital, Sun Yat-sen University, Guangzhou City, Guangdong Province, PR China

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ABSTRACT

Background and Aims: Bone marrow-derived mesenchymal stem cell (MSC) is widely studied in inflammatory bowel disease (IBD) in basic and clinical research. However, patients with IBD have higher risk of developing colorectal cancer and MSC has dual effect on tumorigenesis. This study aims to evaluate the role of MSC on tumorigenesis of IBD.
Methods: MSCs were isolated from the bone marrow of allogenic mice and identified by flow cytometry. Mice in the model of colitis-associated tumorigenesis induced by azoxymethane and dextran sulfate sodium were injected with MSCs. Colon length, spleen size and tumors formation were assessed macroscopically. Pro-inflammatory cytokines and STAT3 phosphorylation in colon tissues were analyzed.
Results: MSCs ameliorated the severity of colitis associated tumorigenesis compared with PBS control, with attenuated weight loss, longer colons and smaller spleens. Tumor number and tumor load were significantly less in the MSC group while tumor size remained comparable. Histological assessment indicated MSCs could reduce histological damage of the colon tissue. Decreased expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), and down-regulation of STAT3 phosphorylation in colon tissue were found after MSC treatment.
Conclusion: MSCs might ameliorate the tumorigenesis of inflammatory bowel disease by suppression of expression of pro-inflammatory cytokines and STAT3 activation.

1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease is a chronic, idiopathic, relapsing form of inflammatory disorder in the digestive tract [1]. Chronic inflammation is believed to have decisive roles in the pathogenesis of cancer [2,3]. The shift from IBD to colorectal cancer (CRC) is one salient example of the link between chronic inflammation and tumorigenesis, which is characterized by an "inflammation-dysplasia-cancer" sequence [4]. As the most serious complication of IBD, CRC tends to develop in such patients, with a significantly increased risk compared with general population. Eaden et al. found cumulative risks of CRC in patients with ulcerative colitis were 2%, 8%, 18% after 10, 20 and 30 years of disease duration, respectively [5]; while Jess et al. showed standardized incidence ratio of 1.9 for CRC in Crohn's disease [6].

Mesenchymal stem cells (MSCs) are non-hematopoietic stem cells with the capacity of self-renewal and pluripotent differentiation into osteogenic, chondrogenic and adipogenic lineages [7,8]. Moreover, MSCs display profound immunomodulatory especially immunosuppressive properties by inhibiting proliferation and function of several major immune cells such as T and B lymphocytes, dendritic cells and natural killer cells [9,10]. Due to all these capacities, MSCs become promising therapeutic candidate in tissue engineering, regenerative medicine and autoimmune disease. Actually, MSCs have been widely studied and even tried clinically in cartilage and meniscus repair, myocardial infarcts, graft-versus-host disease and IBD [11,12]. For instance, in a randomized controlled trial conducted by Tan et al. [13], MSC treatment after renal transplantation resulted in lower incidence of acute rejection, decreased risk of opportunistic infection and better estimated renal function. In a prospective, randomized controlled clinical trial by Wong et al. [14], intra-articular injection of MSCs was proved effective in patients undergoing high tibial osteotomy and microfracture for varus knees with cartilage defects. Previously, we also discovered the anti-inflammatory effect of allogenic bone marrow-derived MSCs in an experimental mouse colitis model [15].

* Corresponding author. Address: The Sixth Affiliated Hospital, Sun Yat-Sen University, 26 Yuancun Erheng Rd, Guangzhou, Guangdong 510655, PR China. Fax: +86 020 38254159.
E-mail address: sumslp@163.com (P. Lan).
[1] Zexian Chen and Xiaowen He contributed equally to this work.

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However, the role of MSCs in tumorigenesis and tumor growth remains controversial. In some certain kinds of tumors, MSCs could suppress tumor growth both in vitro and in vivo, such as hepatoma, lung cancer and gliomas [16–18]. On contrary, MSCs intervention could serve tumor-promoting functions in a wide range of cancer models, such as breast cancer, prostate cancer and colon cancer [19,20]. Notably particularly, all the tumor models mentioned above were mainly performed by injecting cancer cell lines into immunodeficient nude mice. Such models may be defective as tumors can be induced by chronic inflammation [2,3].

Knowledge about what role MSCs play on tumor initiation remains limited. In this study, we aim to investigate the role of MSCs on tumorigenesis of IBD in a mouse model.

2. Materials and methods

2.1. Mice

Female C57BL/6 mice were obtained from the Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China. Mice were kept in special pathogen free facility with free access to drinking water and a pellet-based diet and were quarantined for 7 days before experiment. The experimental protocol was approved by the Ethical Committee of Sun Yat-sen University. All animal studies were conducted with the approval of the Institutional Animal Care and Use Committee of Sun Yat-sen University.

2.2. Culture and identification of MSCs

MSCs were isolated from 3–4 weeks old female C57BL/6 mice as described previously [21]. Briefly, mice were killed by cervical dislocation and hind limbs were excised carefully. After removing the muscle and connective tissue from the femurs and tibias, bone marrow was flushed out from the marrow cavity with complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco, New York, USA), 10% fetal bovine serum (FBS, Gibco, New York, USA) and 1% penicillin/streptomycin (Gibco, New York, USA), using a 0.45 mm syringe needle until the bones became pale. Cell suspension was filtered through a 70 mm filter mesh and then centrifuged at 600*g* for 3 min. After removal of the supernatant, cells were resuspended and viability and yield of cells were determined by Trypan blue exclusion and counting on a hemocytometer. Cells were then cultured in plastic culture dishes (Nest, Shanghai, China) at a density of 25×10^6 cells per ml in complete culture medium mentioned above. Medium was regularly replaced every 2 days to remove the non-adherent cells. When the culture reached over 80%, cells were digested with 0.25% trypsin for 2 min at room temperature and subcultured. For all experiments, cells at passage 3 or 4 were used.

In order to identify the cultured MSCs achieved above, we performed flow cytometric analysis. Briefly, cells were freshly retrieved after digestion and washed with cold PBS. Then cells were incubated with phycoerythrin (PE) conjugated anti-mouse Sca1, CD11b, CD34, CD 44, CD 45 and CD 105 (BD Biosciences, New Jersey, USA) in dark at 4 °C for 30 min. After washing with phosphate buffer saline (PBS) twice, cells were resuspended and examined using flow cytometer (BD FACSCanto™, BD Biosciences, New Jersey, USA). A total of 10,000 viable events were collected and analyzed.

2.3. Animal model induction and treatment

Eighteen female C57BL/6 mice (aged 6–8 weeks old, about 20 g) were divided into 3 groups (6 mice per group), including the experimental group (MSC group, receiving AOM/DSS modeling and MSC treatment), the control group (PBS group, receiving AOM/DSS modeling and PBS treatment) and the negative control group (NC group, receiving no AOM/DSS modeling or MSC).

Azoxymethane (AOM, Sigma–Aldrich, Saint Louis, USA) and dextran sulfate sodium (DSS, 36–50 kDa, MP Biomedical, California, USA) were used to induced colitis associated cancer in mice [22]. Briefly, mice were injected intraperitoneally with a single dose (10 mg/kg) of AMO, followed by 3 cycles of DSS, with each cycle consisting of 1 week of 2% DSS in the drinking water and 2 weeks of normal drinking water. On days 4, 14 and 24, mice in the MSC group were injected with MSCs (10^6 cells in 0.3 ml PBS) via the tail vein. Instead, mice in the PBS group received 0.3 ml PBS without MSCs.

Mice were monitored twice one week for the body weight, stool consistency and the presence of blood in the excreta. At the end of week 12, mice were sacrificed by cervical dislocation. Colon length (from the ileocecal junction to the anal verge) and spleen size were measured. Then colon was incised longitudinally and macroscopic tumors were counted and measured with a caliper. Segments of the distal colon were fixed in 10% neutral buffered formalin for subsequent paraffine embedding, or kept in RNA stabilization solution (RNA later, Ambion, California, USA) as tissue sample for further analysis.

2.4. Histopathological evaluation

Four micrometer-thick sections of formalin-fixed paraffin-embedded tissues were stained with hematoxylin and eosin (HE) to evaluate the inflammation severity. Colitis was scored in a blind fashion as previously published, with a combined score for tissue injury (score, 0–3) and infiltration of inflammatory cells (score, 0–3) [23]. Briefly, for tissue injury, 0 = normal colonic mucosa; 1 = discrete lymphoepithelial lesions; 2 = surface mucosal erosion or focal ulceration; 3 = extensive mucosal damage and extension into deeper layers. And for infiltration of inflammatory cells, 0 = occasional presentation of inflammatory cells in the lamina propria; 1 = increasing number of inflammatory cells in the lamina propria; 2 = inflammatory cells extending into the submucosa; 3 = transmural extension of the infiltration. The histological score was defined as the sum of the two parameters above (from 0 to 6).

2.5. Quantitative Real-time Polymerase Chain Reaction (PCR)

Total RNA was extracted from colon segments using trizol reagent (Ambion, California, USA) and then quality and concentration were assessed. RNA (1 μg) was then reverse transcribed using the ReverTra Ace qPCR RT kit (FSQ-101, Toyobo, Osaka, Japan) according to the manufacturer's protocol. Quantitative Real-time PCR was performed with SYBR Green Realtime PCR Master Mix (QPK-201, Toyobo, Osaka, Japan) on Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, California, USA). Tumor necrosis factor (TNF)-α, interferon (IL)-1β, IL-6 were measured. All reactions were performed in triplicate, with 4 samples from different groups. The quantification of target mRNA was normalized by glyceraldehydes phosphate dehydrogenase (GAPDH), an internal control gene. The relative expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$. Primer sequences were as follows, AGCACAG AAAGCATGATCCG (forward primer of TNF-α), CTGATGAGAGGG AGGCCATT (reverse primer of TNF-α); ACCTGCTGGTGTGTGACGTT (forward primer of IL-1β), TCGTTGCTTGGTTCTCCTTG (reverse primer of IL-1β); GAGGATACCACTCCCAACAGACC (forward primer of IL-6), AAGTGCATCATCGTTGTTCATACA (reverse primer of IL-6); TCAATGAAGGGGTCGTTGAT (forward primer of GAPDH), CGTCCCG TAGACAAAATGGT (reverse primer of GAPDH).

2.6. Western blot

Protein was extracted using RIPA buffer with a cocktail of protease and phosphatase inhibitors. After detecting concentration using BCA assay with a microplate spectrophotometer (Thermo, Massachusetts, USA), protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Millipore, Massachusetts, USA). Membranes were probed with primary antibody anti p-STAT3 (Tyr 705, 1:1000, CST, Boston, USA), STAT3 (1:1000, CST, Boston, USA) and GAPDH at 4 °C overnight followed by secondary antibody. Finally, immunoblot detection was performed by Odyssey Imaging System (Li-COR Biosciences, Nebraska, USA).

2.7. Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissues were serially cut into 4 μm sections on silanized glass slides for IHC staining against p-STAT3 using standard protocol. Briefly, slides were deparaffinized with dimethylbenzene and rehydrated through graded alcohols before retrieving antigen by incubation in sodium citrate buffer. Endogenous peroxidase was blocked with hydrogen peroxide solution and then incubated with anti p-STAT3 (Tyr 705, 1:200, CST, Boston, USA) at 4 °C overnight. Slides were stained with diaminobenzidine in an Envision System (Dako, Denmark) and counterstained hematoxylin.

2.8. Statistics analysis

Values were expressed as mean ± standard error mean (SEM) and analyzed with the Student's *t*-test using SPSS version 16. Differences with p values <0.05 were considered statistically significant.

3. Results

3.1. Characterization of the isolated MSCs

Spindle-shaped cells appeared and gradually predominated in the primary culture. Flow cytometric analysis of surface markers was performed at passage 3 or 4, showing that the isolated MSCs were positive for Sca1, CD44 and CD105, but negative for CD11b, CD34 and CD 45 (Fig. 1).

3.2. MSC attenuated AOM/DSS-induced colitis associated tumorigenesis

To investigate the role of MSCs on colitis associated tumorigenesis, mice model induced by AOM/DSS received MSC treatment. Experimental procedure is showed in Fig. 2A. All the mice survived during the period of experiment, but developed clear clinical signs such as weight loss and bloody diarrhea. As shown in Fig. 2B, weight loss was significantly more obvious in the PBS group ($p < 0.001$).

At the end of week 12, mice were sacrificed and colon length was measured without tension. Shortening and swelling of the colon was observed in both groups receiving AOM/DSS treatment compared with the NC group. However, MSC treatment reduced the extent of such shortening. Colons in the MSC group were much longer than that in the PBS group (73.88 mm in MSC group versus 65.05 mm in PBS group, $p = 0.004$, Fig. 2C and D). What's more, size of spleen in the MSC group was smaller than the PBS group (275.52 mm^3 in MSC group versus 468.70 mm^3 in PBS group, $p = 0.01$, Fig. 2E and F).

After incising the colons open, tumors were observed between the mid colon and the distal rectum in all mice in the MSC group and the PBS group while no tumor was found in NC group. Tumors were more scattered after MSCs injection, whereas mice in PBS

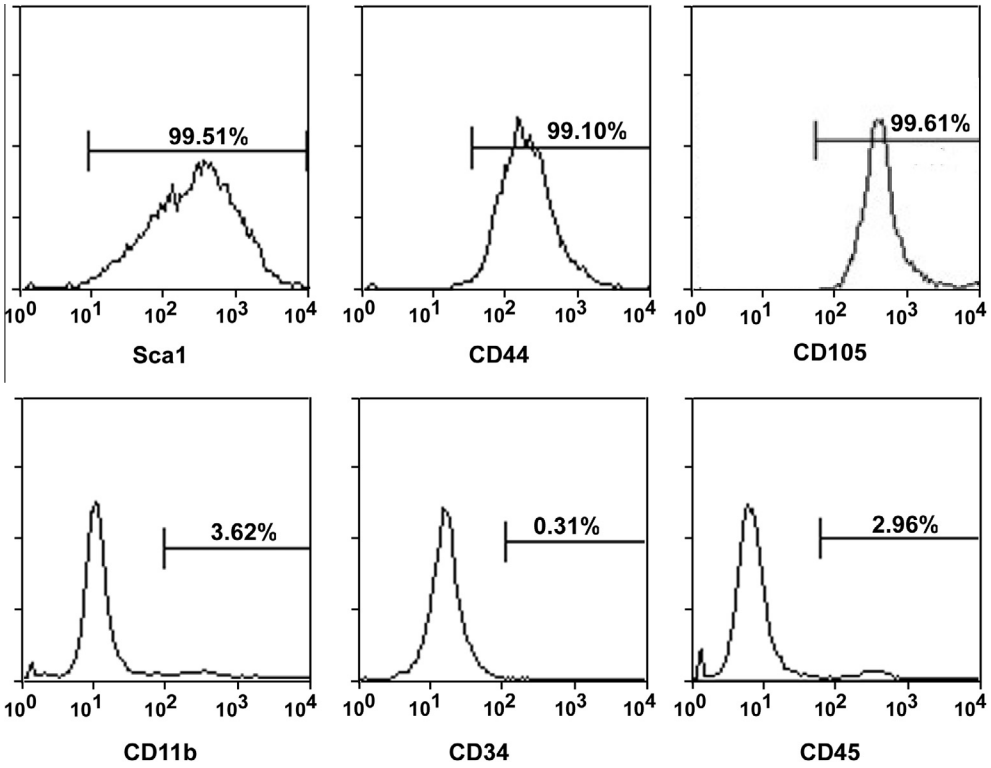

Fig. 1. Flow cytometric analysis of surface antigens of bone marrow-derived MSCs from C57BL/6 mice, shows that they are positive for Sca1, CD44, CD105 and negative for CD11b, CD34, CD45.

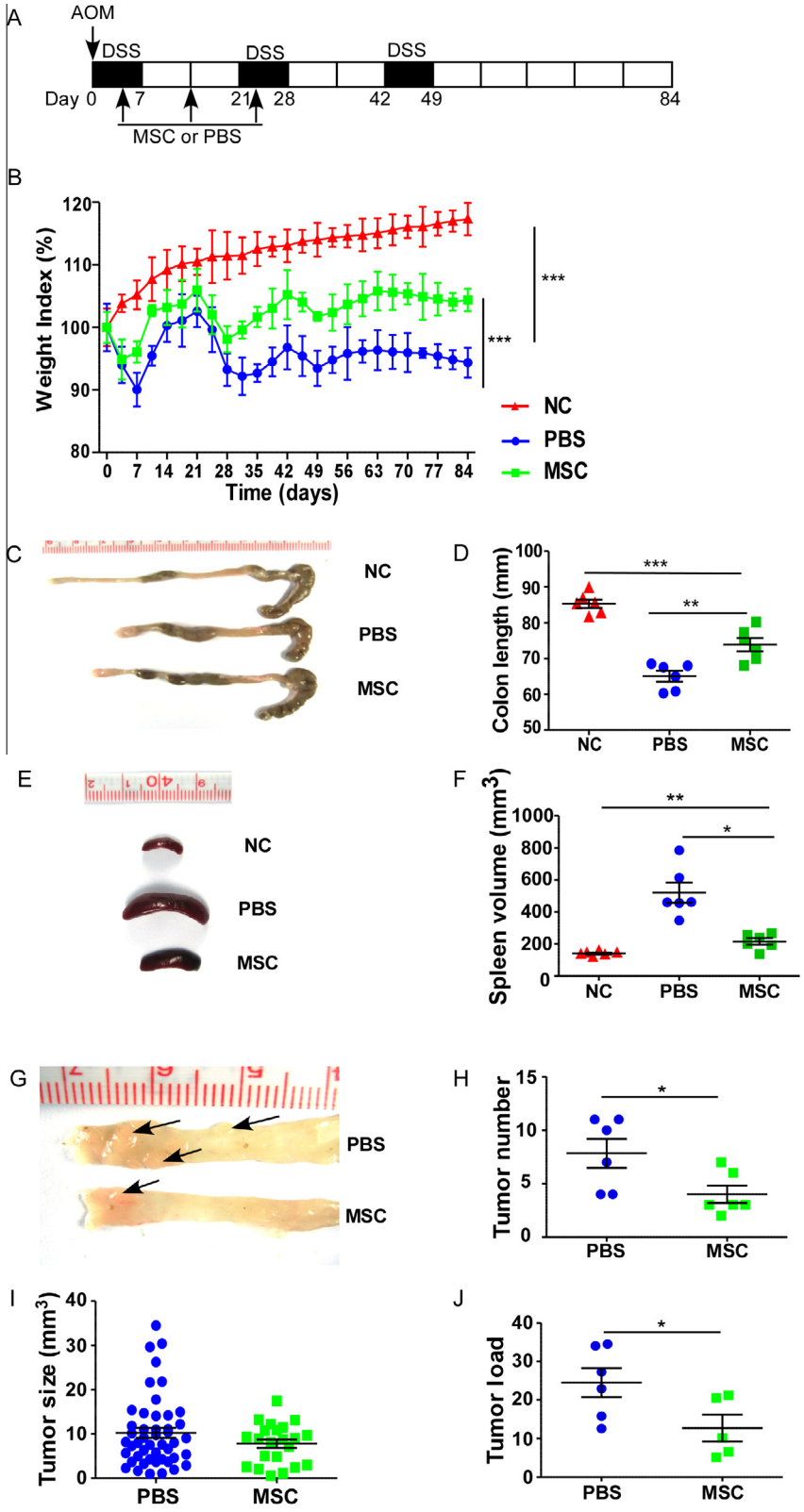

Fig. 2. MSCs attenuate AOM/DSS-induced colitis associated tumorigenesis. (A) Schematic overview of MSC administration during colitis- associated tumorigenesis induction by AOM/DSS. MSC or PBS is administered by injection via the tail vein on days 4, 14 and 24. Mice were sacrificed on day 84. (B) Changes in body weight. MSC treatment ameliorates weight loss. (C and D) Comparison of colon length. MSC treatment ameliorates colon shortening. (E and F) Comparison of spleen size. Size of spleen is smaller after MSC treatment. (G–J) Comparison of tumor number, size and load. Arrows in G indicate the tumors. MSC treatment attenuates tumorigenesis with less tumor number and load. Values are mean ± SEM ($n = 6$ mice per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

group developed numerous and confluent tumoral masses (Fig. 2G). MSCs treatment resulted in a remarkable reduction in the number of tumors per mouse compared with PBS-treated control (4.00 in MSC group versus 8.33 in PBS group, $p = 0.041$, Fig. 2H). Interestingly, the average size of the existed tumors was smaller after MSC treatment but did not reach statistic significance

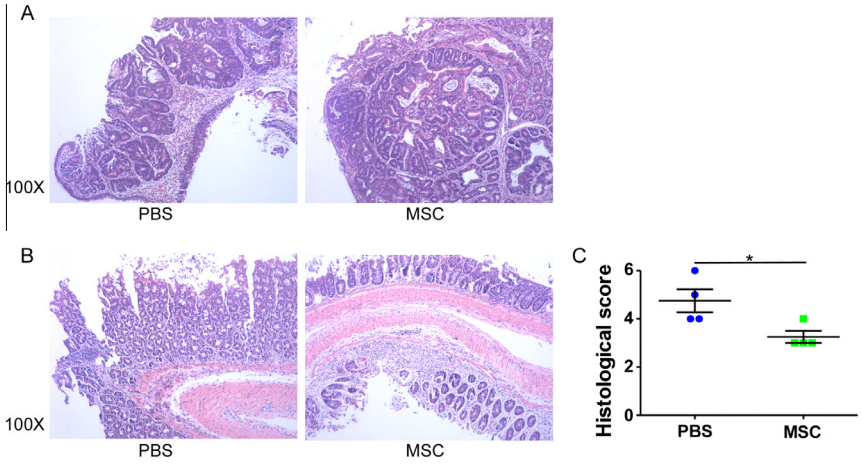

Fig. 3. MSCs reduce histological damage of the colon tissue. (A) Representative HE staining of tumor tissues from PBS and MSC treated mice. All tumors are adenomas with high-grade dysplasia. (B and C) Representative HE staining and histological score comparison of inflammatory tissues from PBS and MSC treated mice. Values are mean ± SEM ($n = 4$ mice per group). $^{*}p < 0.05$.

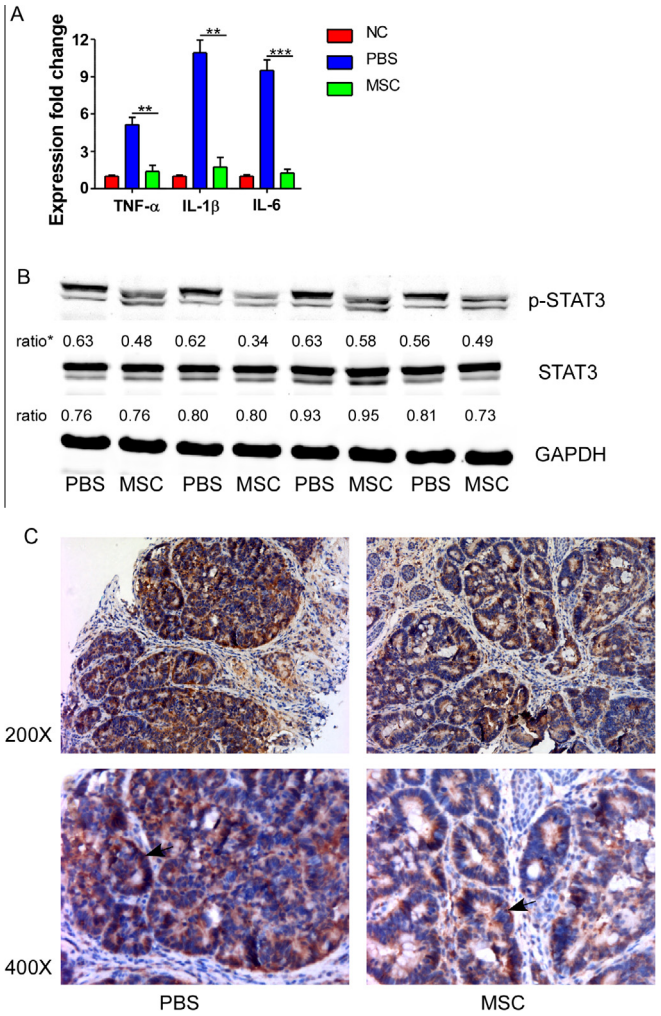

Fig. 4. MSCs decrease inflammatory cytokines and down-regulates STAT3 phosphorylation in colon tissue. (A) RT-PCR shows decreased expression of inflammatory cytokines in colon tissue after MSC treatment. Values are mean ± SEM ($n = 4$ mice per group). $^{**}p < 0.01$, $^{***}p < 0.001$. (B) Western blot shows down-regulation of STAT3 phosphorylation in colon tissue after MSC treatment. Ratio means the relative expression normalized by GAPDH. $^{*}p < 0.05$. (C) IHC shows down-regulation of STAT3 phosphorylation in colon tissue after MSC treatment. Arrows indicate positively stained area.

($p = 0.444$, Fig. 2I), suggesting that MSC acts to limit tumor initiation rather than to restrict tumor progression. However, as we performed MSC injection in the relatively early stage of the animal modeling, the effects of MSC on the progression of tumor might have been missed. Tumor load, defined as the sum of all tumor diameters for a given mouse, was significantly decrease in the MSC group compared with the PBS group ($p = 0.025$, Fig. 2J).

3.3. MSC reduced histological damage of the colon tissue

After general observation, colon tissue was stained with HE. All tumors from both MSC and PBS groups were confirmed as adenomas with high-grade dysplasia (Fig. 3A). To assess the severity of inflammation, tissue injury and infiltration of inflammatory cells in the non-tumoral areas were evaluated by microscopic examination. Consistent with the macroscopic findings mentioned above, MSC treatment effectively reduced inflammatory severity by comparing histological score (3.25 in MSC group versus 4.75 in PBS group, $p = 0.032$, Fig. 3B and C).

3.4. MSC decreased inflammatory cytokines in colon tissue

Inflammatory cytokines not only reflect the degree of inflammation, but also play important part in tumorigenesis. Thus, we further performed quantitative real-time PCR to detect the gene expression level of several inflammatory cytokines in colon tissues including TNF-α, IL-1β and IL-6. As shown in Fig. 4A, MSC treatment significantly decreased the expression of TNF-α, IL-1β, IL-6, ($p = 0.004$, 0.001 and <0.001, respectively).

3.5. MSC down-regulated STAT3 phosphorylation in colon tissue

Western blotting of tissue lysate showed down-regulation of p-STAT3 in colon tissue after MSC treatment ($p = 0.037$, Fig. 4B). From the result of immunohistochemistry for tumor tissue, down-regulation of p-STAT3 was also observed (Fig. 4C).

4. Discussion

In clinic studies, MSC has been used to treat patients with fistulas in CD by intrafistular injection or patients with resfractory IBD by systemic infusion. Garcia-Olmo et al. conducted a phase I [24] and a phase II [25] clinical trials of the treatment of Crohn's fistula by MSC intralesional injection and found it an effective and safe

treatment for complex perianal fistula and appeared to achieve higher rates of healing. Moreover, in a multicenter phase I/IIa clinical trial, de la Portilla et al. [26] showed that intralesionally injection of MSCs was a simple, safe and beneficial therapy for perianal fistula in Crohn's disease patients, with 69.2% of 24 included patients received a reduction in the number of draining fistulas. As to the systemic use of MSC, in a phase 2 study by Forbes et al. [27], patients with refractory Crohn's disease received intravenous infusion of MSC and clear signals of efficacy (clinical remission and endoscopic improvement) were observed. All these clinical trials showed promising therapeutic effect of MSC for IBD treatment. However, patients with IBD have higher risk of developing colorectal cancer in the long term [5,6] and MSC has dual effect on tumorigenesis [28]. Thus, what role would MSC play on the tumorigenesis of IBD should be considered discreetly.

In this study, bone marrow-derived MSCs were used to interfere the mice model of colitis associated cancer induced by AOM/DSS in immunocompetent mice. This rodent model mimics the pathological process of human IBD associated cancer, with DSS inducing chronic inflammation in the colon while AOM trigging tumorigenesis. Tumor initiation usually began after week 5 in this model [22,29], so MSCs were injected before this time point in order to evaluate whether MSCs could prevent initiation of tumorigenesis. Our results showed that bone marrow-derived MSCs could inhibit colitis associated tumorigenesis probably by decreased inflammatory status and suppression of STAT3 phosphorylation.

Chronic inflammation could function as a driving force in the journey to tumorigenesis by inducing gene mutations, enhancing proliferation and resisting apoptosis, among which inflammatory cytokines plays important roles [30]. TNF-α, IL-1β and IL-6 are important pro-inflammatory cytokines and can prompt tumorigenesis [31]. In our study, we macroscopically observed that weight loss, shortening of colons and largening of spleen in the AOM/DSS model were all attenuated after MSC treatment, indicating that MSC could relief systemic and local inflammation and thus ameliorate tumorigenesis. Real-time PCR demonstrated that inflammatory cytokines including TNF-α, IL-1β and IL-6 were significantly decreased.

IL-6, as a proinflammatory cytokine, binds to gp130 and leads to the phosphorylation of its downstream effector STAT3, regulating the gene expression that mediates cell proliferation and apoptosis suppression in colitis associated tumorigenesis [32,33]. Aberrant STAT3 activation up-regulates the survival factors such as Bcl-2 and Bcl-xL, leading to the promotion of proliferation and inhibition of apoptosis [34]. Our study found that phosphorylation of STAT3 was suppressed after MSC treatment, which to some extent, could be a potent mechanism for the observations of this study.

In summary, MSC could inhibit colitis-associated tumorigenesis in a mice model. However, the pathogenesis of IBD-associated cancer in human is much more complex than the animal model and the exact detailed mechanism of MSC on this pathogenesis remains unclear. More studies on animals and clinical observations on human are needed to confirm this result.

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