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REGULAR ARTICLE

Early injection of human adipose tissue-derived mesenchymal stem cell after inflammation ameliorates dextran sulfate sodium-induced colitis in mice through the induction of M2 macrophages and regulatory T cells

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Abstract

Inflammatory bowel diseases (IBDs) are sometimes refractory to current therapy or associated with severe adverse events during immunosuppressive therapy; thus, new therapies are urgently needed. Recently, mesenchymal stem cells (MSCs) have attracted attention based on their multitude of functions including anti-inflammatory effects. However, proper timing of MSC therapy and the mechanisms underlying the therapeutic effects of MSCs on colitis are not fully elucidated. Human adipose tissue-derived mesenchymal stem cells (hAdMSCs; 1×10^6) were administrated via the tail vein on day 3 (early) or 11 (delayed) using a 7-day dextran sulfate sodium (DSS)-induced mouse model of colitis. The effects were evaluated based on colon length, disease activity index (DAI) and histological score. Cytokine-encoding mRNA levels T cells and macrophages were evaluated by real-time PCR and flow cytometry. Regarding the timing of administration, early (day 3) injection significantly ameliorated DSS-induced colitis in terms of both DAI and histological score, compared to those parameters with delayed (day 11) injection. With early cell injection, the tissue mRNA levels of anti-inflammatory cytokine genes (*Il10*, *Tgfb*) increased, whereas those of inflammatory cytokine genes (*Il6*, *Tnfa* and *Il17a*) decreased significantly. Regarding the associated mechanism, hAdMSCs suppressed T cell proliferation and activation in vitro, increased the number of regulatory T cells in vivo and changed the polarity of macrophages (into the anti-inflammatory M2 phenotype) in vitro. Timing of injection is critical for the effective therapeutic effects of hAdMSCs. Furthermore, part of the associated mechanism includes T cell activation and expansion and altered macrophage polarization.

Keywords Mesenchymal stem cells · Regulatory T cells · Macrophages · Adipose tissue · Dextran sulfate sodium

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Introduction

Inflammatory bowel diseases (IBDs) comprise chronic inflammatory disorders and include ulcerative colitis (UC) and Crohn's disease (CD). The pathogenesis of IBDs is thought to be very complex owing to several factors such as environmental, genetic predisposition and inflammatory abnormalities. In the USA, the prevalence of IBDs is increasing (Kappelman et al. 2013). In particular, among the commercially insured population, the overall incidences of CD and UC in the adult population in 2009 were 241 and 263 per 100,000 persons, respectively and these rates are presently increasing. In Japan, the age-standardized prevalence of UC in 2005 was 63.6 per 100,000 persons and that of CD was 21.2 (Asakura et al. 2009). The occurrence of IBDs in Japan is still much lower than that in Western countries, although the incidence of IBDs in Japan is also increasing. Thus, countermeasures against IBDs are urgently needed for this important global problem.

UC is characterized by inflammation of the mucosal membrane of the colon, which continues to the rectum. Type 2 T helper cell cytokine profiles have been associated with the pathogenesis of this disease. CD constitutes a segmental, transluminal disorder that can arise in the entire gastrointestinal tract from the mouth to the anus. Type 1 T helper cells have been associated with its pathogenesis. Furthermore, a recent report showed that Th17 cells are present during both UC and CD. Accordingly, mucosal CD4+ T cells are key mediators driving this response. Whereas the mechanisms of inflammation are different between UC and CD, regulatory T cells (Tregs) are associated with the pathogenesis of IBDs and play an important role in maintaining homeostasis of intestinal immunity. Changes in the balance between Tregs and effector T cells (Th1, Th2 and Th17) are thought to be involved in the onset of IBDs (Yamada et al. 2016).

In addition to T cells, macrophages have attracted recent attention regarding their relationship with the pathogenesis of IBDs. Macrophages exhibit polarity and can typically be divided into two phenotypes, namely "classically activated macrophages" (M1 macrophages) and "alternatively activated macrophages" (M2 macrophages). When injury occurs, M1 macrophages contribute to the induction of inflammation. In contrast, when injury is stabilized, M2 macrophages contribute to the inactivation of inflammation (Terai and Tsuchiya 2017). However, macrophage polarization during IBDs and the effect of mesenchymal stem cell (MSC) therapy on this process have not been elucidated.

Dietary treatment and drugs such as 5-aminosalicylic acid, steroids and immunosuppressive agents are representative therapies for IBDs. Recently, the therapeutic effect was found to dramatically increase with the appearance of anti-TNFα antibodies. However, some patients experience weak or no effects using this therapy and some suffer adverse events such as serious infection during therapy; thus, further development of new treatments is awaited. New candidate drugs such as antibodies against IL-6 and IL-12/23, small molecules including Janus kinase inhibitors, antisense oligonucleotide against *SMAD7* mRNA and inhibitors of leukocyte trafficking to intestinal sites of inflammation are now in development (Monteleone and Pallone 2015). Furthermore, treatment using fecal microbiota transplantation or the administration of Lgr5+ intestinal stem cells is also anticipated, as is the use of MSCs (Cho et al. 2013; de la Portilla et al. 2013; Duijvestein et al. 2010; Forbes et al. 2014; Hu et al. 2016; Lee et al. 2013; Molendijk et al. 2015; Panes et al. 2016; Yui et al. 2012)

MSCs have recently received worldwide attention for cell therapy owing to the associated ease of expansion and their wide range of functions. MSCs can be obtained from bone marrow as well as medical waste such as adipose tissue, umbilical tissue and dental pulp. MSCs are positive for the common markers CD73, CD90 and CD105; however, they are negative for the endothelial marker CD31 and the hematopoietic marker CD45. MSCs have low antigenicity and exert multiple effects such as anti-inflammation, immunoregulatory and tissue repair, through the production of cytokines, chemokines and exosomes (Tsuchiya et al. 2017). Thus, many clinical studies including the use of fistulas for CD and luminal treatment for CD and UC are ongoing using autogenic and allogeneic MSCs. Favorable therapeutic effects through local fistula injection or intravenous or intra-arterial injections have been reported.

However, there is still some ambiguity regarding MSC-based therapies, such as the proper timing of administration and the mechanisms associated with the therapeutic effect, especially in the field of inflammatory colorectal diseases. In addition, although MSCs are considered to be poorly antigenic, whether the administration of human MSCs is safe and effective for mouse experimental systems has not been sufficiently studied. In this study, we investigate the efficacy of human adipose tissue-derived MSCs (hAdMSCs) using a dextran sulfate sodium (DSS)-induced mouse model of colorectal inflammation, a representative colitis model and clarify the mechanism associated with the therapeutic effect of stem cells, in particular by analyzing the effect on T cells and macrophages.

Materials and methods

Mice We purchased 10- to 12-week-old C57BL/6 male mice from Charles River Laboratories International Inc. (Yokohama, Japan). Animals were housed in a specific pathogen-free environment and kept under standard conditions with a 12-h day/night cycle and access to food and water ad libitum.

MSC preparation hAdMSCs (PT-5006, passage 2) were purchased from Lonza (Basel, Swiss Confederation), cultured and passaged using Rhoto MSC growth medium (ROHTO Pharmaceutical Co., Ltd., Osaka, Japan) twice by InterStem Co., Ltd. (Osaka, Japan). These expanded cells were a kind gift from InterStem Co., Ltd. Expanded cells were frozen and delivered to Niigata University. After thawing, the cells were immediately washed, counted, suspended in PBS and administered to mice.

hAdMSC culture in the presence of serum Serum was obtained from mice that received DSS for 3 days. hAdMSCs were cultured with the serum (final concentration; 10%) from DSS-colitis mice and compared to hAdMSCs cultured with added serum (final concentration; 10%) from normal mice. After 72 h, cells were harvested and mRNA expression levels were analyzed by real-time PCR. The results were obtained for at least three or more separate samples.

Induction of experimental colitis and study design Colitis was induced through the administration of 2.5% DSS (molecular weight 36,000–50,000; MP Biomedicals, Santa Ana, CA, USA) via drinking water ad libitum for 7 days. Mice were divided into three groups as follows: (1) mice were injected intravenously with 1×10^6 hAdMSCs or PBS on day 3 and were sacrificed on day 11 after the start of the experiment (day 3 injection, day 11 sacrifice group); (2) mice were injected intravenously with 1×10^6 hAdMSCs or PBS on day 3 and were sacrificed on day 21 after the start of the experiment (day 3 injection, day 21 sacrifice group); (3) mice were injected intravenously with 1×10^6 hAdMSCs or PBS on day 11 and were sacrificed on day 21 after the start of the experiment (day 11 injection, day 21 sacrifice group) (Fig. 1). hAdMSCs were injected after thawing, without culture, on day 3 (early point) or day 11 (delayed point), because we detected weight loss and bloody stool on day 3; in addition, signs of disease began to improve naturally by day 11 after stopping DSS on day 7. To confirm appropriate timing and route, additional mice were injected intraperitoneally with 1×10^6 hAdMSCs or PBS on day 3 and sacrificed on day 21 after the start of the experiment; alternately, mice were injected intravenously with 1×10^6 hAdMSCs or PBS on day 7 and sacrificed on day 21 after the start of the experiment.

Evaluation of therapeutic effect To evaluate the therapeutic effect of hAdMSCs, the disease activity index (DAI), colon length and histological score were analyzed. DAI was calculated by the combined scores of weight loss, stool consistency and bleeding, as described previously (Cooper et al. 1993). Colon lengths were measured from the anus to the cecum soon after harvesting the colon. Histological score was calculated as follows. The colon was excised, fixed in 10% formalin, embedded in paraffin wax and sliced into 4-μm sections. After hematoxylin and eosin (H&E) staining, histological evaluation was performed in a blinded fashion according to a previously published scoring system (Williams et al. 2001). Briefly, total colitis score was the sum of the three sub-scores (inflammation severity 0–3 points, inflammation extent 0–3 points and crypt damage 0–4 points), which were multiplied by the degree of inflammation involvement as follows: × 1: 1–25%; × 2: 26–50%; × 3: 51–75%; × 4: 76–100%. The specimen with a high score was shown to have severe histological damage. We evaluated the histological score in the medial colon, because it is an appropriate location to evaluate such lesions; inflammation of the distal colon was too severe and inflammation of the proximal colon was too mild.

id-BMM preparation For induced bone marrow-derived macrophage (id-BMM) culture, 10- to 12-week-old male C57BL/6 mice were sacrificed by cervical dislocation and the limbs were removed. Bone marrow precursors were flushed with Dulbecco's modified Eagle medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) from the medullary cavities of tibias and femurs using a 25G needle. id-BMMs were cultured at 37 °C, with 5% CO_2 in specific dishes (ultra-low attachment flasks; Corning, Armonk, NY, USA) with medium (DMEM/F12 medium; Thermo Fisher Scientific Inc.) containing 20 ng/mL M-CSF (CSF-1) (recombinant murine M-CSF; Peprotech, Rocky Hill, NJ, USA). id-BMMs were cultured for 7 days with the medium changed twice weekly.

T cell preparation For T cell isolation, 10- to 12-week-old male C57BL/6 mice were sacrificed by cervical dislocation and the spleens were removed. Spleen tissue was mashed up with a 40-μm Cell Strainer (Thermo Fisher Scientific Inc.). Thereafter, erythrocytes were removed from the collected cells with ACK lysing buffer (Thermo Fisher Scientific Inc.) and lymphocytes were collected. To collect T cells from the lymphocytes, the Pan T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) was employed, as described in the manufacturer's instructions.

Co-culture of hAdMSCs and id-BMMs For co-culture of id-BMMs and MSCs, id-BMMs were cultured with or without MSCs at 37 °C, with 5% CO_2 in specific dishes (Transwell 6-well plates; Corning) for 72 h. Subsequently, id-BMMs were harvested and the mRNA levels of Tnfα, Il-6, iNos, Il-10, Tgfβ, Ym-1, Fizz-1, Cd206, Hgf and Vegf were compared to those of controls.

Co-culture of hAdMSCs and T cells To analyze the effect of MSCs on T cell proliferation and activation, the following experiments were performed. Before analyzing T cell proliferation, they were stained using a CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific Inc.). Then, MSCs and stained T cells were co-cultured with medium

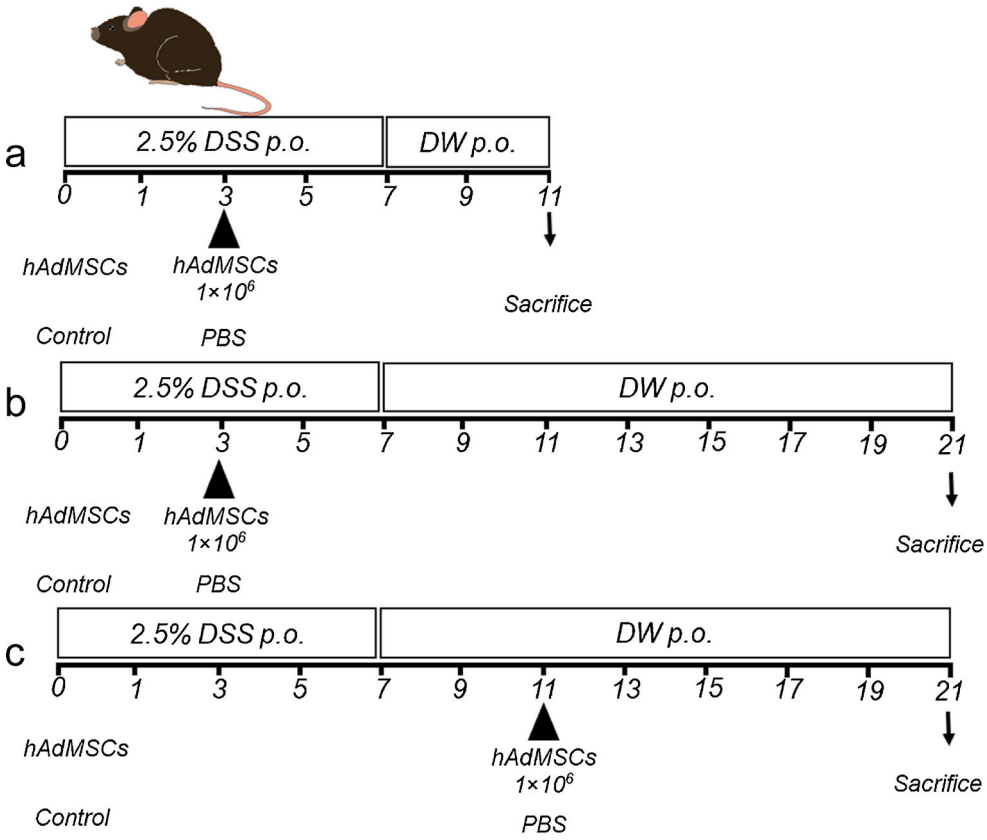


Fig. 1 Experimental design of the study. Colitis was induced through the administration of 2.5% dextran sulfate sodium (DSS) in drinking water (DW) ad libitum for 7 days. Mice were divided into three groups. (**a**) Group 1, mice were injected intravenously with 1×10^6 human adipose tissue-derived mesenchymal stem cells (hAdMSCs) or PBS on day 3 and were sacrificed on day 11. (**b**) Group 2, mice were injected intravenously with 1×10^6 hAdMSCs or PBS on day 3 and were sacrificed on day 21. (**c**) Group 3, mice were injected intravenously with 1×10^6 hAdMSCs or PBS on day 11 and were sacrificed on day 21. p.o., per o.s

containing Dynabeads Mouse T-Activator CD3/CD28 (VERITAS Corporation., Tokyo, Japan) (for activation of T cells) at 37 °C, with 5% CO_2, in 24-well microplates (AGC Techno Glass Co., Shizuoka, Japan) for 72 h. Subsequently, T cells were harvested and carboxyfluorescein diacetate succinimidyl ester (CFSE) expression was compared to that of controls using a FACS Caliber (Becton Dickinson, Franklin Lakes, NJ, USA). T cell activation was confirmed by the expression of the T cell activation markers CD25 and CD69 using flow cytometry.

Real-time PCR Total RNA was reverse transcribed using the QuantiTech Reverse Transcription kit (Qiagen, Hilden, Germany). Gene expression analysis was performed using pre-validated QuantiTech primers (Supplemental Table 1) with QuantiTech SYBR reagent (Qiagen). Real-time PCR was conducted using a Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The results were obtained using at least three separate samples and *Gapdh* was used as the housekeeping gene. Fold change in relative gene expression, compared to that of the control, was calculated using the ΔΔCT method with pooled control samples as the calibrator.

Immunostaining For immunohistochemistry, tissues were fixed in 10% formalin and cut into 4-μm sections. Where necessary, sections were subjected to antigen retrieval by heating the sample in the appropriate buffer (10 mM sodium citrate buffer at pH 6.0 or 1 mM EDTA buffer at pH 8.0). For 3,3-diaminobenzidine (DAB) staining, sections were blocked using 3% hydrogen peroxide for 10 min in room temperature (RT). Primary antibodies (Supplemental Table 2) were incubated with the samples overnight at 4 °C. Following this, species-specific biotinylated anti-IgG antibodies were used for detection. Slides were then stained using the Vectastain® ABC kit (Vector Laboratories, Inc. Burlingame, CA, USA) and the DAB substrate (Muto Pure Chemicals, Tokyo, Japan). Nuclei were stained using hematoxylin solution (Vector Laboratories, Inc.). Photographs were taken using a Nikon Eclipse e600 microscope and camera (DXM1200F; Nikon, Tokyo, Japan).

Isolation of LPLs and macrophages from the colon For lamina propria lymphocytes (LPL) isolation from the colon, 10- to 12-week-old male C57BL6 mice were sacrificed by cervical dislocation and the colons were removed. Colon tissue was agitated at 37 °C for 20 min at 160 times/min in PBS containing 5 mM EDTA-2Na (Sigma-Aldrich, St. Louis, MO, USA) to remove the intestinal epithelium, which was followed by incubation with PBS containing 250 U/mL collagenase (Wako, Osaka, Japan) at 37 °C for 90 min with shaking. The digested tissues were meshed and the resulting cells were

centrifuged for 10 min at 2000 rpm with 35% Percoll plus (GE Healthcare, Buckinghamshire, UK) to isolate LPLs. Collected LPLs and macrophages were analyzed by flow cytometry.

Flow cytometry Cells were incubated with an anti-mouse CD16/32 antibody (Becton Dickinson) for 10 min on ice to block non-specific binding. Cells were then incubated with antibodies (described in Supplemental Table 3) for 30 min on ice. After washing, cells were analyzed using a FACS Caliber (Becton Dickinson). Results of flow cytometry were obtained from at least three separate samples and analyzed using FlowJo v10 (Tomy Digital Biology, Tokyo, Japan).

Statistics Statistical analyses were performed using GraphPad Prism6J software (GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Data are presented as the means ± SD. Results were assessed using a Mann-Whitney *U* test and Student's *t* test. Differences were considered significant when the *P* value was less than 0.05.

Ethical considerations All animal experiments were conducted in full compliance with the regulations of and were approved by the Institutional Animal Care and Committee at the Niigata University (Permit number 27-83-9).

Results

Characteristics of hAdMSCs and changes after serum stimulation

First, we analyzed the characteristics of hAdMSCs in simple culture and after the addition of serum to mimic the environment after administration. hAdMSCs in simple culture produced mRNAs of Il-6, Tnfα, Tgfβ, Il-4, Vegf, Hgf, Egf, Pge2, Tsg6 and Ido, whereas after adding serum from mice with DSS-induced colitis, the characteristics of hAdMSCs changed. Specifically, mRNA levels of Il-6, Il-4, Vegf, Hgf, Pge2 and Tsg6 increased and those of Tnfα, Tgfβ, Egf and Ido decreased (Fig. 2). These results suggested that the effect of hAdMSCs could be altered depending on the extracellular context.

Early injection of hAdMSCs after inflammation ameliorates DSS-induced colitis We next determined the therapeutic effect of hAdMSCs at an early point after DSS-induced colitis. Mice injected with hAdMSCs on day 3 after DSS administration showed significantly improved DAI (DAI: hAdMSC injection group vs control group, 7.75 ± 1.64 vs 10.6 ± 1.2, respectively, $P = 0.044$, with mice sacrificed on day 11; DAI: hAdMSC injection group vs control group, 3.57 ± 1.18 vs 5.66 ± 1.17, respectively, $P = 0.014$, with mice sacrificed on day 21; Fig. 3c, f). Especially, after day 10, mice injected with hAdMSCs exhibited improvement in terms of loose stool or diarrhea and bloody stools. Shortening of the colon was also significantly alleviated (hAdMSC injection group vs control group, 6.75 ± 0.109 vs 5.82 ± 0.700 cm, respectively, $P = 0.0008$, with mice sacrificed on day 11; hAdMSC injection group vs control group, 7.74 ± 0.544 vs 6.53 ± 0.701 cm, respectively, $P = 0.014$, with mice sacrificed on day 21; Fig. 3a, b, d, e). Histological score also improved significantly in the group treated with hAdMSCs (hAdMSC injection group vs control group, 8.5 ± 7.26 vs 19.2 ± 4.49, respectively, $P = 0.023$, with mice sacrificed on day 11; hAdMSC injection group vs control group, 7.00 ± 4.72 vs 12.3 ± 4.38, respectively, $P = 0.032$, with mice sacrificed on day 21; Fig. 4a–f).

Next, colon tissues were analyzed by real-time PCR on day 11 and 21 after DSS induction. At day 11, mRNA encoding inflammatory cytokines (Il-6, Il-17a and Tnfα) decreased, whereas that for the anti-inflammatory cytokines (Tsg6, Il-10 and TGFβ) increased significantly, compared to those in the control group (Fig. 4g). At day 21, mRNAs encoding inflammatory cytokines (Il-6, Il-17a and Tnfα) decreased and those for growth factors (Hgf, Vegf and Egf) increased significantly, whereas there was no significant difference in the mRNA of anti-inflammatory cytokines (Il-10 and Tgfβ) (Fig. 4h). At both days 11 and 21, mRNAs encoding the M2 macrophage marker *Cd206* and the M2 macrophage-inducing factor *Pge2* were highly expressed, compared to levels in control tissue (Fig. 4g, h). These data suggested that colitis severity was alleviated in mice injected with hAdMSCs on day 3 after DSS administration on day 11 and day 21. Despite xenogeneic cell injection, mice injected with hAdMSCs on day 3 improved and did not show any specific adverse events during the 21-day observation period, revealing that hAdMSCs could be employed for mouse experiments.

To evaluate the effect of MSC-based cell therapy from an alternative route, 1×10^6 cells were injected at day 3 intraperitoneally and DAI, colon length, histological score and real-time PCR were assessed at day 21. We could detect a significant therapeutic effect in DAI and colon length compared to that of the control (DAI: hAdMSC injection group vs control group, 4.10 ± 1.22 vs 5.37 ± 0.99, respectively, $P = 0.038$. Colon length: hAdMSC injection group vs control group, 8.42 ± 0.40 vs 7.78 ± 0.62 cm, respectively, $P = 0.025$). However, we could not observe a significant difference in histological score (hAdMSC injection group vs control group, 6.38 ± 4.71 vs 9.88 ± 7.46, respectively, $P = 0.171$), whereas real-time PCR revealed that a significant decrease of mRNAs for pro-inflammatory factors Il-6, Il-17a and TNFα and increase of the mRNAs for M2 macrophage-related markers Cd206 and Pge2 and growth factors Vegf and Hgf could be detected. From these results, we concluded that intraperitoneal injection may have therapeutic effects, albeit to a lesser degree compared to that of MSC intravenous injection therapy (Supplemental Fig. 1).

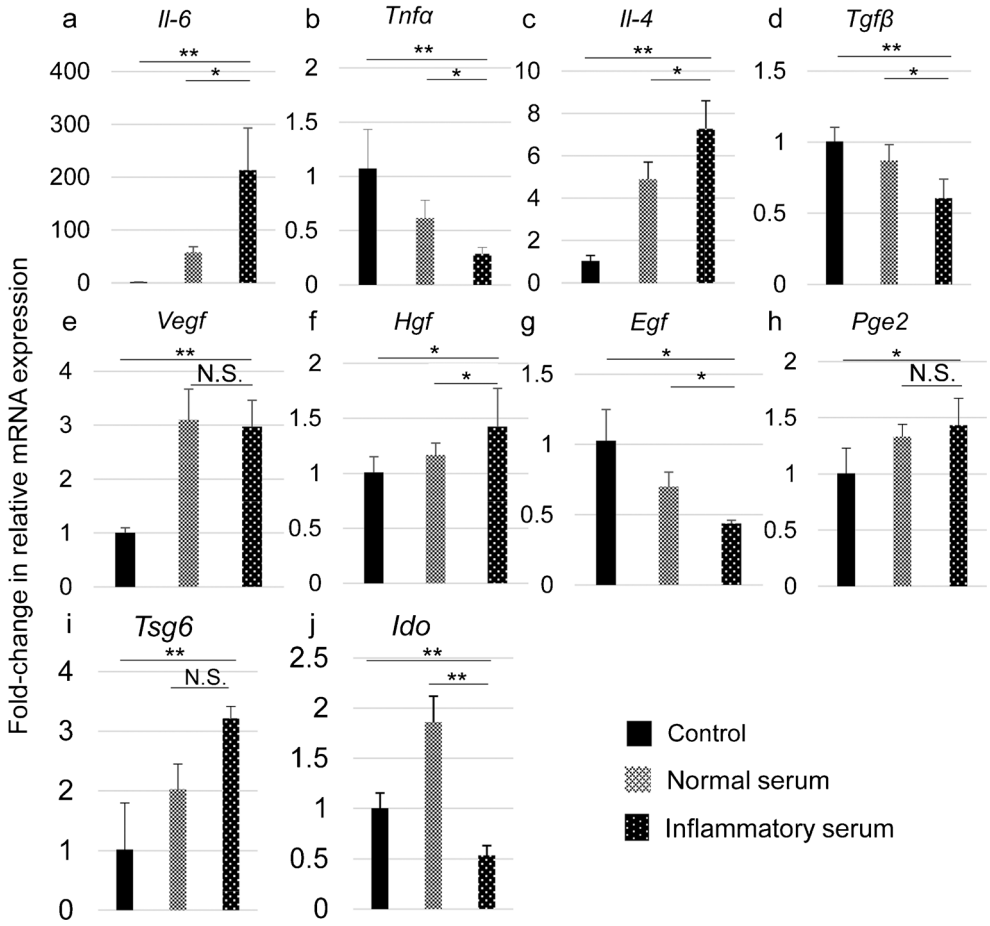


Fig. 2 Analysis of mRNA expression changes in human adipose tissue-derived mesenchymal stem cells (hAdMSCs) with or without serum. No serum was added (control), left bar; normal mouse-derived serum was added, middle bar; serum derived from dextran sulfate sodium (DSS)-treated mice serum (inflammatory serum) was added, right bar. mRNA changes were analyzed 48 h after adding serum. $^{*}P < 0.05$; $^{**}P < 0.01$

Delayed hAdMSC administration does not effectively ameliorate DSS-induced colitis To evaluate the timing of hAdMSC administration, cells were administered on day 11 (delayed time-point) after DSS administration, when body weight loss and diarrhea were spontaneously diminished and the results were compared to those at day 3 of hAdMSC administration (early time-point). In the day 3 group, DAI was improved, shortening of the colon was significantly alleviated and the histological score improved on day 21, as described above. In contrast, in the day 11 group, DAI, shortening of the colon and histological score did not significantly change on day 21 (DAI: hAdMSC injection group at day 11 vs control group, 5.00 ± 1.26 vs 5.00 ± 1.22, respectively, $P = 0.9579$; shortening of colon length: hAdMSC injection group at day 11 vs control group, 6.48 ± 0.43 vs 6.30 ± 0.55, respectively, $P = 0.6508$; histological score: hAdMSC injection group at day 11 vs control group, 10.0 ± 3.35 vs 11.5 ± 4.09, respectively, $P = 0.6508$). Thus, there was no improvement after injecting hAdMSCs on day 11 (Fig. 5a–f). To confirm these results, real-time PCR was performed. This revealed that whereas the mRNAs for inflammatory cytokines (Il-6, Tnfα and Il-17a) were decreased and those for growth factors (Hgf, Vegf and Egf) were elevated in the group injected on day 3 and sacrificed on day 21, as described above, the mRNAs for inflammatory cytokines (Il-6, Tnfα and Il-17a) and growth factors (Hgf, Vegf and Egf) were not significantly changed in the group injected on day 11 and sacrificed on day 21 (Fig. 5g). However, M2 macrophage-related factor mRNAs such as those encoding Cd206 and Pge2 were increased in the latter group.

To confirm the effect at another time point, 1×10^6 MSCs were injected at day 7 using the tail vein and DAI, colon length, histological score and real-time PCR were assessed at day 21. We could detect a significant difference in colon length at day 21 (colon length: hAdMSC injection group vs control group, 7.60 ± 0.23 vs 7.11 ± 0.36 cm, respectively, $P = 0.022$). However, we could not detect a significant difference in DAI and histological score (DAI: hAdMSC injection group vs control group, 5.14 ± 0.92 vs 6.00 ± 1.00, respectively, $P = 0.182$; histological score: hAdMSC injection group vs control group, 9.57 ± 5.23 vs 12.50 ± 4.75, respectively, $P = 0.355$). Real-time PCR revealed that a significant decrease of mRNAs for pro-inflammatory factors Il-17a and TNFα and increase of mRNA for growth factor Egf could be detected. These results revealed that the therapeutic effect at day 3 is better than that at day 7 (Supplemental Fig. 2). These results suggested that the timing of hAdMSC administration is very important, as efficacy was established when cells were

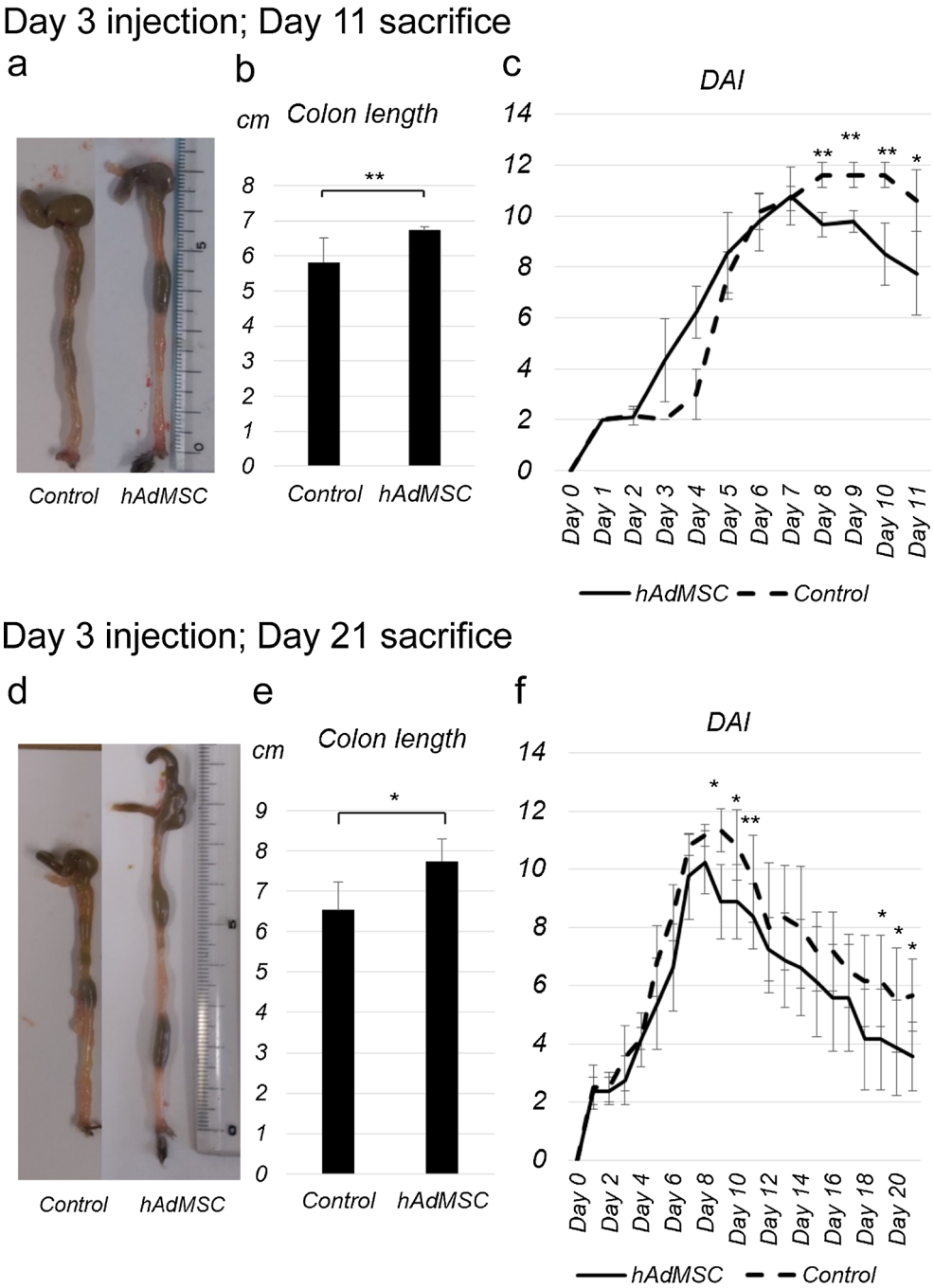


Fig. 3 Evaluation of the therapeutic effect of human adipose tissue-derived mesenchymal stem cells (hAdMSCs) in a mouse model of dextran sulfate sodium-induced colitis, after administration on day 3 after initiation. (**a**–**c**) Prevention of colon length shortening and improved disease activity index (DAI) scores were observed in the group administered hAdMSCs on day 3 and sacrificed on day 11 ($N = 12$ mice; control, 5 mice, injected, 7 mice). (**d**–**f**) Prevention of colon length shortening and improved DAI scores were also observed in the group administered hAdMSCs on day 3 and sacrifice on day 21 ($N = 12$ mice; control, 6 mice, injected, 7 mice) (*$P < 0.05$; **$P < 0.01$)

injected early during inflammation; however, with delayed injection, the effects of hAdMSCs were diminished and restricted.

Injection of hAdMSCs suppresses T cell proliferation and activation To elucidate the mechanism of improved colitis with hAdMSCs, we focused on T cells and macrophages. The effect of hAdMSCs on T cell proliferation and activation, as well as the induction of regulatory T cells, was evaluated. To evaluate T cell proliferation, T cells were extracted from mouse spleens and activated; cell division was then evaluated by flow cytometry with or without hAdMSC exposure. The T cell to hAdMSC proportions were adjusted to 10:1, 5:1, 2:1 and 1:1 to effectively evaluate this effect. The percentage of T cells that divided more than four times was 88.93 ± 1.17% (T cell:hAdMSC ratio = 10:1), 83.92 ± 1.79% (5:1), 71.38 ± 2.59% (2:1) and 62.8 ± 1.77% (1:1). These results revealed that hAdMSCs could suppress T cell proliferation in a concentration-dependent manner (Fig. 6a–e). To evaluate the effect of hAdMSCs on T cell activation, extracted T cells were exposed to hAdMSCs and the expression of the associated markers CD25 and CD69 were evaluated by flow cytometry. In the co-culture group, the mean

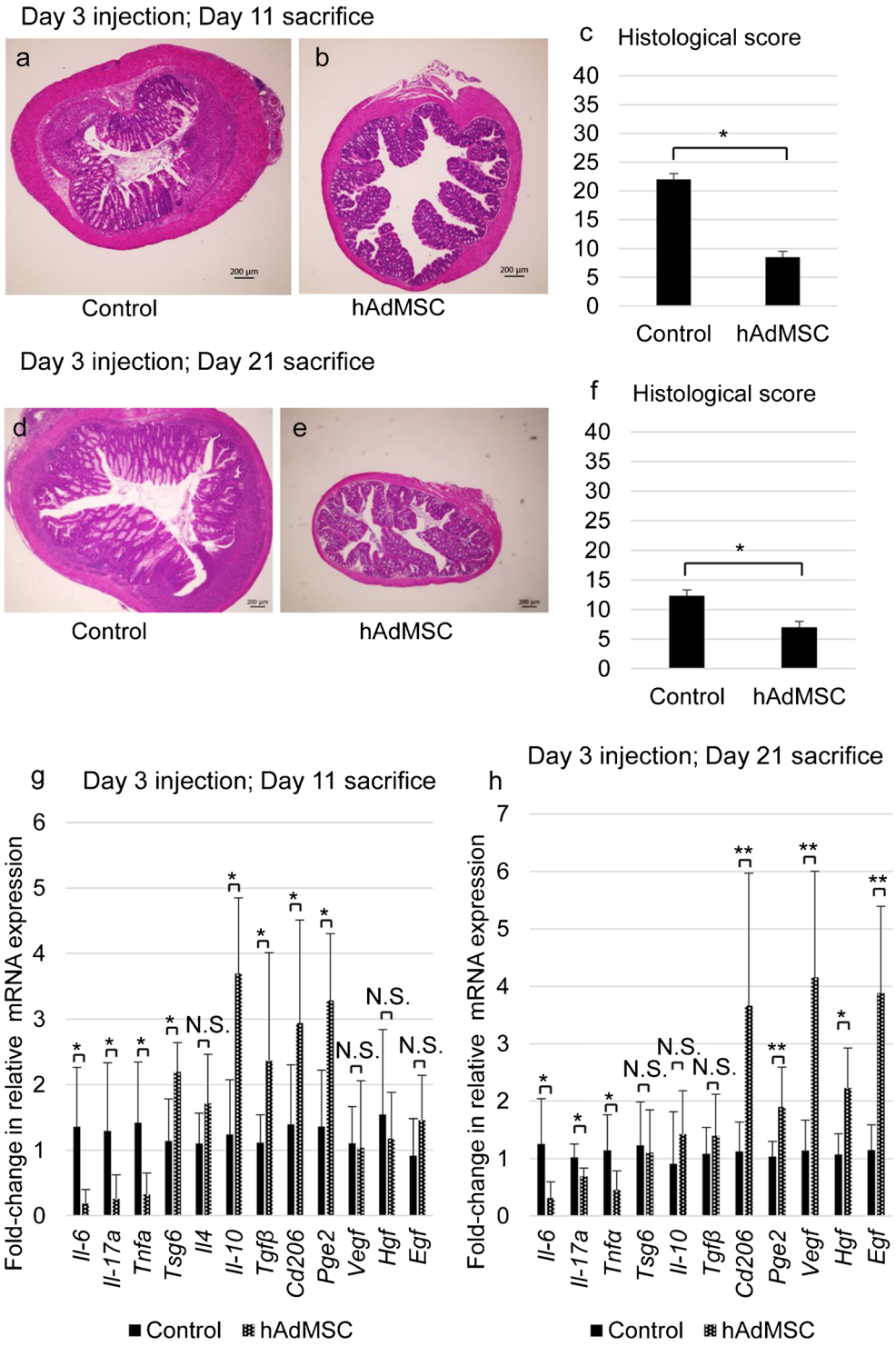


Fig. 4 Evaluation of histological scores and real-time PCR after human adipose tissue-derived mesenchymal stem cell (hAdMSC) administration on day 3 after dextran sulfate sodium treatment of mice. (**a**–**f**) Histological analysis. Improvement in histology was observed in the group administered hAdMSCs on day 3 and sacrificed on day 11 and in the group administered hAdMSCs on day 3 and sacrificed on day 21. **g**–**h** Analysis of mRNA expression changes, compared to control levels (as assessed by real-time PCR), in the middle colon of the group administered hAdMSCs on day 3 and sacrificed on day 11 (**g**) and in the group administered hAdMSCs on day 3 and sacrificed on day 21 (**h**). $^{*}P < 0.05$; $^{**}P < 0.01$. (Scale bar 200 μm)

fluorescence intensity (MFI) of CD25 and CD69 decreased compared to that in the non-co-cultured group (MFI of CD25: co-culture group vs non-co-culture group, 76.7 ± 4.76 vs 450 ± 132.47, respectively, $P = 0.0163$; MFI of CD69: co-culture group vs non-co-culture group, 70.7 ± 8.01 vs 294.7 ± 62.1, respectively, $P = 0.0072$). This revealed that hAdMSCs suppress T cell activation (Fig. 6f–i). Furthermore, to confirm the suppression of T cell proliferation and activation in vivo, the infiltration of CD3 T cells was assessed. When hAdMSCs were administered on day 3 and mice were sacrificed on day 11, immunostaining of the colon tissue showed that infiltration of CD3-positive cells significantly decreased (hAdMSC-administered group vs control group, 20.725 ± 7.008 vs 30.463 ± 9.850 cells/field, respectively, $P = 0.0478$; Fig. 7a, b, e). These in vitro and in vivo results

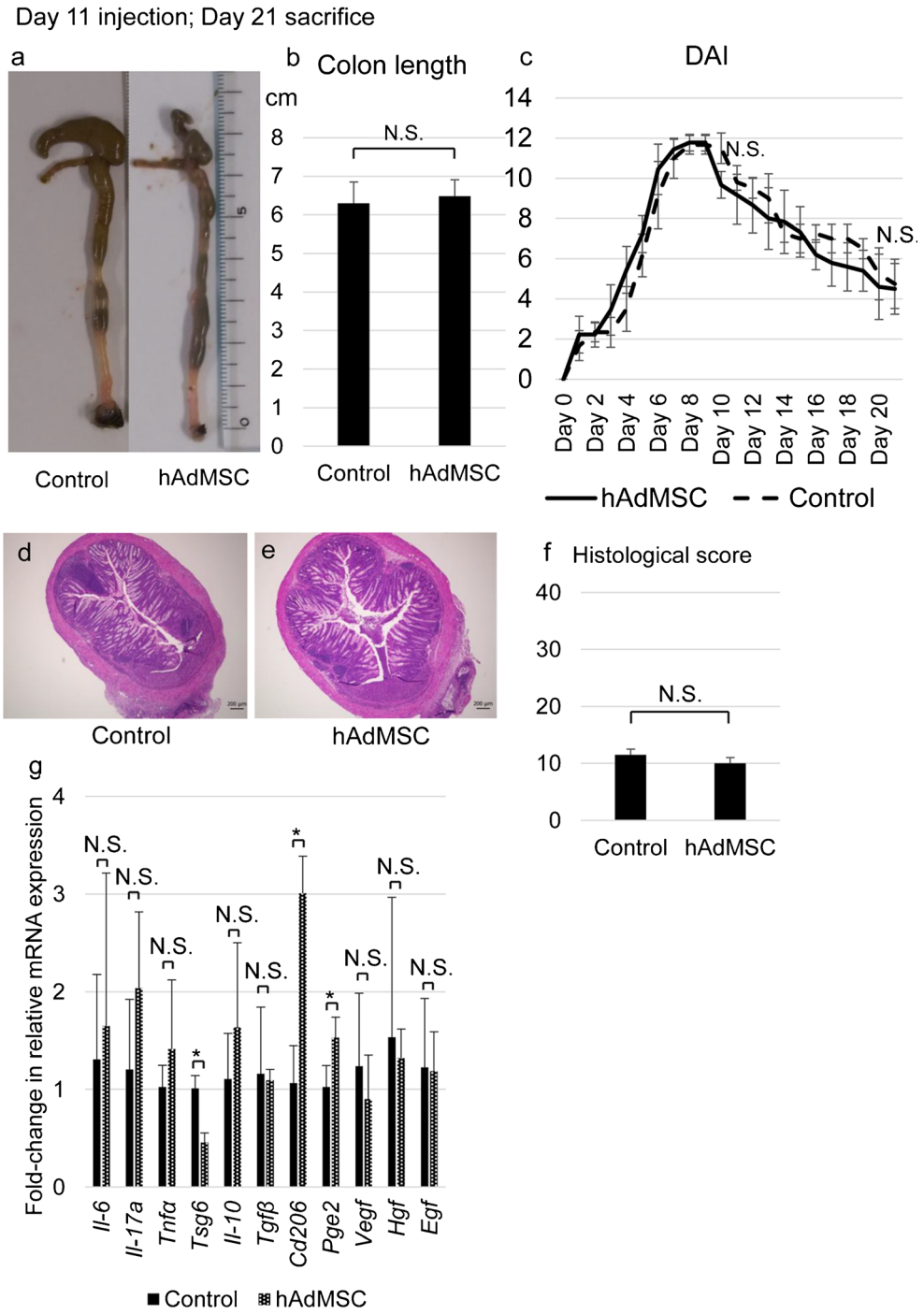
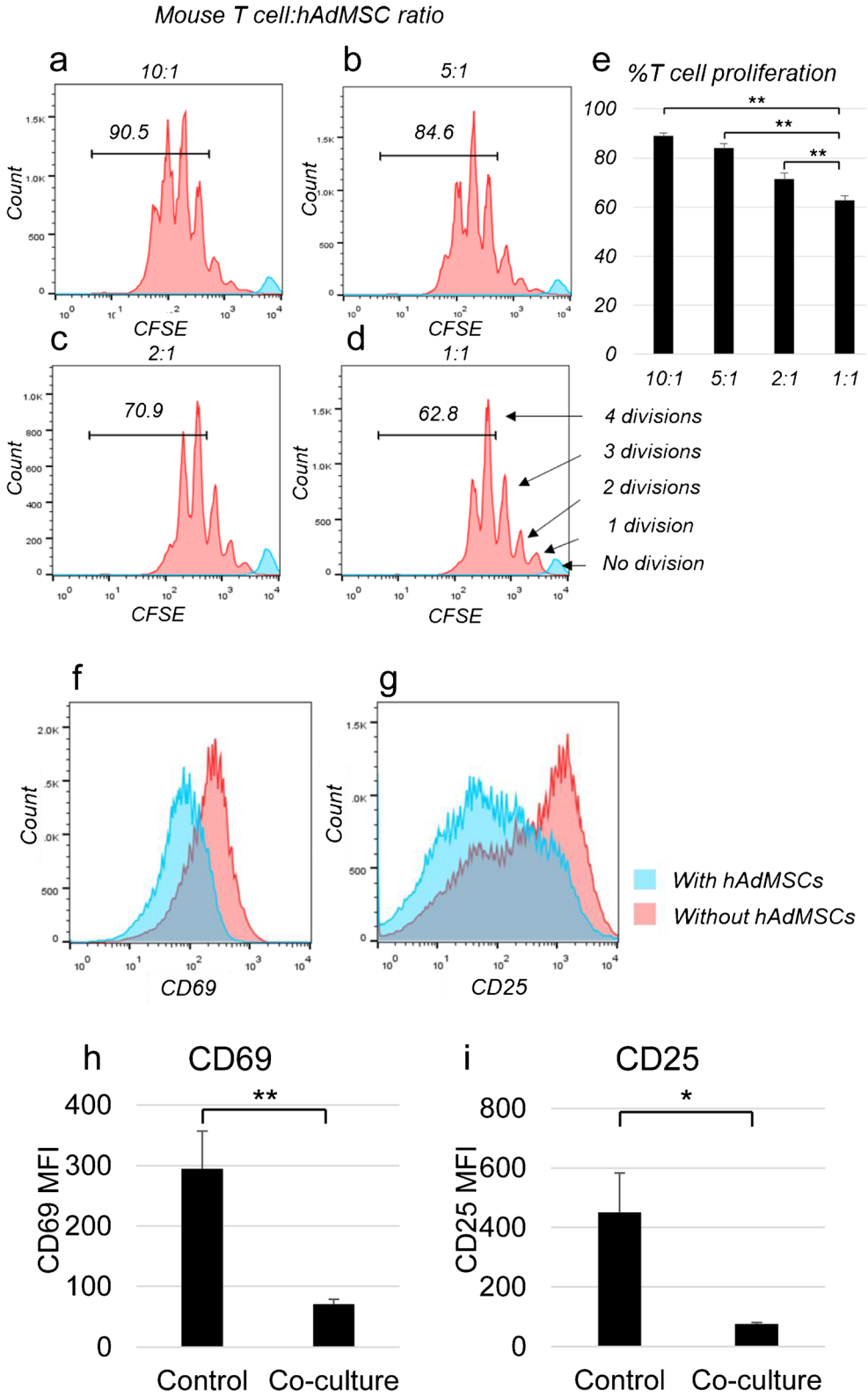


Fig. 5 Evaluation of the therapeutic effect of human adipose tissue-derived mesenchymal stem cell (hAdMSC) administration on day 11 after initiation of dextran sulfate sodium treatment in mice. (**a**–**c**) Significant improvements in the disease activity index (DAI) scores and significant prevention of colon shortening were not observed in mice administered hAdMSCs on day 11 and sacrificed on day 21 ($N = 10$; control, 5, injected, 5). (**d**–**f**) Histological analysis. Significant improvement in histological sores was also not observed in this group. (Scale bar 200 μm). (**g**) Analysis of mRNA expression changes in the middle colon in this group, compared to levels in the untreated group (*$P < 0.05$)

revealed that hAdMSCs ameliorate DSS-induced colitis by suppressing the proliferation and activation of T cells.

Injection of hAdMSCs suppresses inflammation by inducing Treg cells To elucidate whether hAdMSCs induce Treg cells, we immunostained colon tissue of mice (injected on day 3 and sacrificed on day 11) with CD3 and Foxp3 and the number of positive cells and the frequency of Foxp3-positive cells in the CD3-positive population were evaluated. There was no significant difference in the number of Foxp3-positive cells whereas the frequency of Foxp3-positive cells in the CD3-positive population increased significantly (hAdMSC injection group vs control group, 25.03 ± 6.31% vs 11.63 ± 3.24, respectively, $P = 0.0013$) (Fig. 7a–g). To confirm this result, LPLs from the intestinal tract of DSS-induced mice (injected on day 3 and sacrificed on day 11) and corresponding control mice were analyzed by flow cytometry using the markers



CD4, CD25 and Foxp3. In the hAdMSC-injected group, the frequency of CD4 + CD25 + Foxp3+ cells significantly increased compared to that in control mice (hAdMSC-injected group vs control group, 2.30 ± 0.37% vs 1.27 ± 0.27, respectively, $P = 0.0173$; Fig. 7h–l). These results revealed that hAdMSCs improve DSS-induced colitis by increasing the frequency of Treg cells and suppressing T cell expansion and activation.

◀ **Fig. 6** Effect of human adipose tissue-derived mesenchymal stem cells (hAdMSCs) on mouse T cell proliferation and activation. **a**–**d** Mouse T cells were activated and induced to proliferate using T-activator CD3/CD28 for 72 h and these cells were cultured with hAdMSCs at proportions adjusted to 10:1, 5:1, 2:1 and 1:1 to evaluate the effect of hAdMSCs on T cell proliferation by flow cytometry. The percentage of T cells that divided more than four times was quantitated (**e**). hAdMSCs had the ability to suppress T cell proliferation in a concentration-dependent manner (**$P < 0.01$). **f**–**i** Activated T cells were cultured with or without hAdMSCs and the expression of activated T cell markers CD25 and CD69 was evaluated by flow cytometry (**$P < 0.01$). CFSE carboxyfluorescein diacetate succinimidyl ester; MFI mean fluorescence intensity

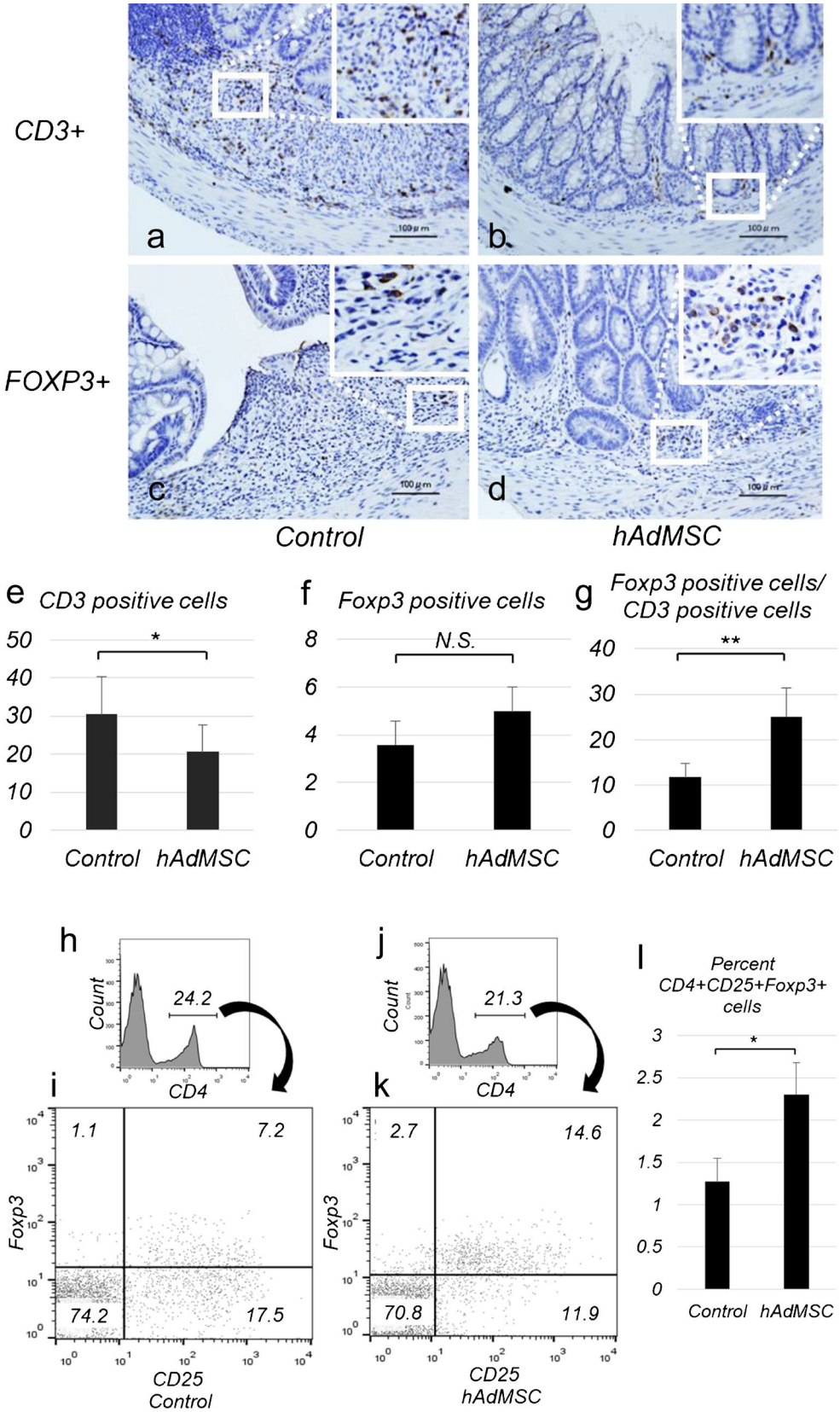
hAdMSCs shift the polarity of macrophages to the anti-inflammatory M2 phenotype It was recently reported that the polarization of macrophages is affected by interactions with MSCs. When MSCs are stimulated with various cytokines and chemokines that are produced in inflammatory environments, the secretion of PGE2 and TSG-6, which can shift macrophage phenotypes to the anti-inflammatory M2 type, increases and contributes to tissue repair (Mittal et al. 2016; Wang et al. 2014). To confirm the effect of MSCs on macrophages, bone marrow macrophages (id-BMMs), which were induced by 7 days of floating culture with M-CSF, were co-cultured with AdMSCs for 72 h and mRNA expression related to the polarity of macrophages was analyzed by real-time PCR. This revealed that whereas the mRNAs encoding anti-inflammatory markers Il-10 and Tgfβ, as well as those encoding M2 macrophage markers Cd206 and Ym-1, increased, the mRNAs for M1 macrophage markers iNos and TNFα decreased (Fig. 8). In addition, as mentioned above, the mRNA expression of the M2 macrophage marker Cd206 was found to be increased in colon tissue after hAdMSC administration (Figs. 4g, h and 5g). Flow cytometry analysis using colon tissue revealed that the frequency of CD206-positive cells/F4/80-positive cells significantly increased on day 11 in mice injected with hAdMSCs on day 3 after DSS administration (Supplemental Fig. 3). These results suggested that hAdMSCs could alter the polarity of macrophages to the anti-inflammatory type, which could contribute to improved DSS-induced colitis.

Discussion

In this study, we demonstrated that the injection of hAdMSCs is effective against DSS-induced colitis and showed that the timing of administration is very important. Although many researchers have reported that experimental colitis such as DSS- and trinitrobenzenesulfonic acid (TNBS)-induced colitis are improved through the administration of MSCs with anti-inflammatory ability, we provided a detailed analysis of the associated therapeutic mechanisms by analyzing the effect on T cell expansion and activation, as well as macrophage polarization. We also found that MSC injection early in inflammation exerts more powerful effects than delayed injection. Furthermore, the present study clearly showed that the effects of MSCs, such as improved DAI score, colon length and histological score, can be induced in other species without any particular adverse events. Gonzalez-Rey et al. reported the treatment of DSS-induced colitis in C57Bl/6 mice by intraperitoneally administering 1×10^5 to 5×10^6 human adipose-derived MSCs (Gonzalez-Rey et al. 2009). In addition, Kim et al. reported the treatment of TNBS-induced colitis in BALB/c mice by intraperitoneally administering 2×10^6 human umbilical cord blood MSCs, without particular adverse events (Kim et al. 2013). The present study supported these findings, specifically that MSCs have low immunogenicity and cause no significant adverse events, even with xenogeneic administration.

Next, to elucidate the appropriate timing of cell injection, hAdMSCs were injected on day 3 (early phase) or day 11 (delayed phase) during the 21-day experiments, which consisted of 7 days of DSS induction and 14 days of recovery. We found that whereas the colitis of mice in the day 3 hAdMSC-administered group improved significantly compared to that in the controls, injection on day 11 did not result in a significant therapeutic effect. These results clearly showed that the timing of hAdMSC injection is very important and that the early phase (associated with marked inflammation) is more susceptible to the therapeutic effect than the delayed inflammation or remission phase. Ren et al. reported that pro-inflammatory cytokines such as IL-17, TNF-α and IFN-γ mediate the immunosuppressive effect of MSCs (Ren et al. 2008). Our results, indicating that the anti-inflammatory effect of hAdMSCs is strong when colon inflammation begins and diminishes when colon inflammation starts to improve naturally, support their results. In addition, the therapeutic effects of MSCs are often discussed in the context of combination with immunosuppressants. Chen et al. reported that in models of liver fibrosis, administration of MSCs in combination with dexamethasone decreased the immunosuppressive effects of MSCs and disease regression (Chen et al. 2014). This suggests that MSCs cannot exert an anti-inflammatory effect without an inflammatory environment, which supports our results indicating that a stronger effect occurs with enhanced inflammation. To the best of our knowledge, this is the first study to evaluate anti-inflammatory effects based on the timing of MSC injections, in a single experiment. Further discussion is necessary regarding the timing of MSC administration and inflammatory conditions that can obtain the best therapeutic effect.

A variety of mechanisms has been reported to explain the therapeutic effects of MSCs, which include anti-inflammatory effects, angiogenesis, anti-apoptosis and anti-fibrosis. One of the most representative mechanisms of MSCs is the anti-inflammatory effect. From our results, we consider that the



anti-inflammatory and/or immunomodulatory functions of MSCs were important; thus, we further analyzed the effect of MSCs on T cells and macrophages, key inflammatory cells. Regarding T cells, we analyzed the effect of hAdMSCs on

◀ **Fig. 7** Effect of human adipose tissue-derived mesenchymal stem cells (hAdMSCs) on regulatory T cells. **a**–**g** Evaluation of regulatory T cells by immunohistochemistry. In the hAdMSC-injected group, immunostaining of the middle colon showed a significant decrease in CD3-positive cells and an increase in the Foxp3-positive/CD3-positive cell ratio (*$P < 0.05$; **$P < 0.01$) (Scale bar 100 μm.). h–l Evaluation of regulatory T cells by flow cytometry. Regulatory T cells, characterized by CD4+ Foxp3+ CD25+ cells, were increased in the hAdMSC-injected group (*$P < 0.05$)

mouse T cell proliferation and activation, as well as regulatory T cell induction. We found that hAdMSCs suppress T cell proliferation and activation and increase the proportion of Tregs. Kim et al. reported that intraperitoneal administration of human umbilical cord blood-derived MSCs to DSS-induced mice increases Treg infiltration into the colon, whereas Gonzalez et al. reported that intraperitoneal administration of human adipose-derived MSCs to TNBS-induced animals increased Tregs in the mesenteric lymph nodes (Gonzalez et al. 2009; Kim et al. 2013). We consider that our results, together with these results, indicate that the key mechanisms of MSC-mediated improved colitis include suppression of T cell proliferation and activation and the induction of Tregs.

We also analyzed the effect of hAdMSCs on the polarity of macrophages. Through co-culturing analysis, we revealed that hAdMSCs can alter the polarity of cultured bone marrow-derived macrophages, inducing a shift to anti-inflammatory M2 macrophages. It has been reported that IL-4, TSG-6 and

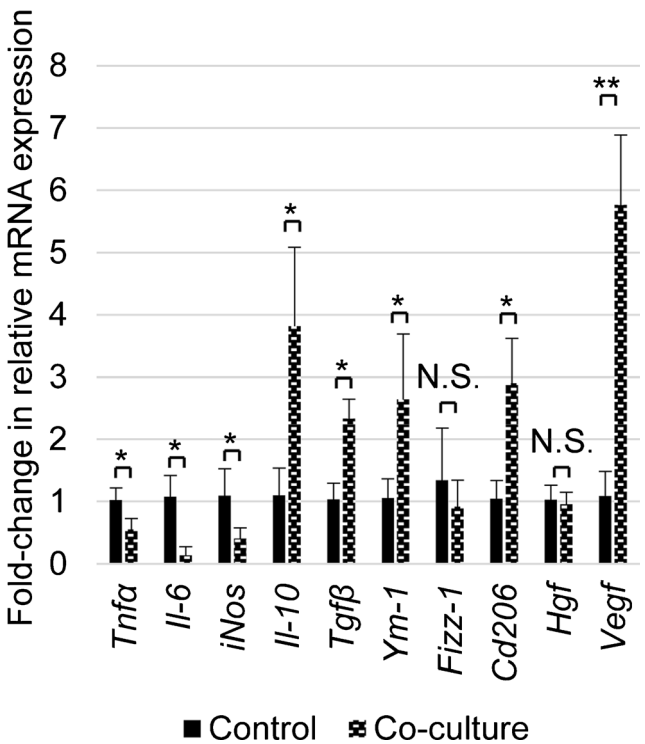


Fig. 8 The influence of human adipose tissue-derived mesenchymal stem cells (hAdMSCs) on macrophage phenotypes. Analysis of changes in mRNA expression in macrophages after co-culture with hAdMSCs for 72 h (*$P < 0.05$; **$P < 0.01$), as assessed by real-time PCR

PGE2, among others, induce a shift in macrophage polarity toward the M2 phenotype. Stein et al. reported that IL-4 changes shift polarity in the M2 direction (Stein et al. 1992). In addition, Mittal et al. reported that in TSG-6 knockout mice, lipopolysaccharide-induced lung injury is improved through the intra-tracheal administration of recombinant TSG-6 and concluded that the mechanism was changing the polarity of macrophages toward the M2 phenotype (Mittal et al. 2016). In addition, Sala et al. reported that DSS- or TNBS-induced colitis was improved through the intra-peritoneal administration of mouse bone marrow-derived MSCs and that this occurred through TSG-6-meditated changes in macrophage polarity to the M2 phenotype and by increasing Tregs (Sala et al. 2015). Furthermore, Dave et al. reported that DSS-induced colitis was improved through the intra-peritoneal administration of murine stem cells for interstitial cells of Cajal through increased expression of PGE2. In our hAdMSCs, after adding serum from DSS-treated mice, mRNA encoding *Pge2* slightly increased and that encoding *Tsg6* and *Il4* significantly increased (Dave et al. 2015). We further detected a significant increase in *Pge2* mRNA in DSS-damaged colon tissue. We consider that these facts suggest that hAdMSCs affect the polarity of macrophages, shifting these cells toward the M2 phenotype. Although there may be other anti-inflammatory and/or immunomodulatory effects of MSCs, we consider that these mechanisms are central to the anti-inflammatory and/or immunomodulatory functions observed in DSS-induced colitis models.

According to ClinicalTrials.gov (https://clinicaltrials.gov/), there are more than 700 clinical trials using autologous and allogeneic MSCs from bone marrow, adipose tissues, cord tissues and dental pulps, among others, for the treatment of disorders related to the bone, neurons, lung, heart, liver and the gastro-intestine, as well as diabetes mellitus. It is generally understood that MSCs have low antigenicity owing to low expression of MHC and co-stimulatory molecules; however, our experiments showed that MSCs elicit an anti-inflammatory effect even if the animal species differs. Thus, not only autologous but also allogeneic MSCs may be employed as cell sources for treatment. For different therapies, allogenic MSC injection has several advantages compared to autologous MSCs. Specifically, these cells can be obtained from medical waste, expanded cells are of high quality and expanded cells can be available on demand for more patients and at a lower cost.

Until now, there have been more clinical trials for CD than UC. A total of eight CD trials and one UC trial are registered in ClinicalTrias.gov (Cho et al. 2013; de la Portilla et al. 2013; Duijvestein et al. 2010; Forbes et al. 2014; Hu et al. 2016; Lee et al. 2013; Molendijk et al. 2015; Panes et al. 2016). For this, local administration of allogeneic MSCs has been primarily attempted. In addition, six papers describing clinical trials for CD report the treatment of perianal fistulas, whereas two trials

for luminal CD include the systemic administration of MSCs. Regarding the local administration of MSCs for CD, Molendijk et al. reported improved healing of refractory perianal fistulas using allogeneic bone marrow-derived MSCs. They administered these allogenic MSCs locally and concluded that injection of 3×10^7 MSCs promotes the healing of perianal fistulas (Molendijk et al. 2015). Regarding the systemic administration of MSCs for CD, Forbes et al. reported a phase II study using allogeneic bone marrow-derived MSCs for luminal CD that is refractory to therapy. They administered 2×10^6 cells/kg weekly for 4 weeks and found that allogenic MSCs reduced the CD activity index and CD endoscopic index of severity scores in patients with luminal CD refractory to therapy (Forbes et al. 2014). Regarding the systemic administration of MSCs for UC, Hu et al. reported a phase I/II study for severe UC using umbilical cord-derived allogeneic MSCs, combination injection through the peripheral blood and superior mesenteric artery with a 7-day interval. They confirmed the safety of MSCs and the alleviation of diffuse and deep ulcer formation and severe inflammation of the mucosa using this type of therapy (Hu et al. 2016). However, appropriate timing and MSC origin must be determined for the treatment of IBDs; based on our results, we consider that the anti-inflammatory and/or immunomodulatory effects of MSCs can be exploited for the treatment of these patients.

With respect to the behavior of MSCs after administration, it is thought that most MSCs migrate to the lungs after administration via the peripheral vein. Although we have not performed the cell tracking analysis of the colon, we previously generated the carbon tetrachloride (CCl4)-induced liver cirrhosis model and tracked the injected cells from the tail vein for 7 days, which revealed that the majority of the MSCs were trapped in the lung after cell injection and disappeared after 7 days from the lung, spleen and liver. Conversely, very few MSCs were detected in the liver at only day 1. From these results, we suspected that the majority of the MSCs do not migrate to the colon and that humoral factors or extracellular vesicles play an important role in the colon or liver. Some papers have reported that the main effects of MSCs were exhibited by cytokines, chemokines and exosomes that are produced by MSCs. Watanabe et al. reported that DSS- or TNBS-induced colitis in mice was improved through the administration of MSC-conditioned medium and the report suggested that the effects of MSCs are not only based on MSCs themselves but also on substances produced by these cells (Watanabe et al. 2014). It has also been reported that the administration of only exosomes, which are secreted from cells and include several factors such as microRNA, can improve various diseases (Katsuda et al. 2013). We suspect that in the future, novel studies will reveal differences between MSCs based on tissue origin and culture conditions. If MSC research progresses and similar effects can be obtained using specific cell-free substances, this option represents an ideal approach for future therapeutics.

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Compliance with ethical standards

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References

Asakura K, Nishiwaki Y, Inoue N, Hibi T, Watanabe M, Takebayashi T (2009) Prevalence of ulcerative colitis and Crohn's disease in Japan. J Gastroenterol 44:659–665

Chen X, Gan Y, Li W, Su J, Zhang Y, Huang Y, Roberts AI, Han Y, Li J, Wang Y et al (2014) The interaction between mesenchymal stem cells and steroids during inflammation. Cell Death Dis 5:e1009

Cho YB, Lee WY, Park KJ, Kim M, Yoo HW, Yu CS (2013) Autologous adipose tissue-derived stem cells for the treatment of Crohn's fistula: a phase I clinical study. Cell Transplant 22:279–285

Cooper HS, Murthy SN, Shah RS, Sedergran DJ (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 69:238–249

Dave M, Hayashi Y, Gajdos GB, Smyrk TC, Svingen PA, Kvasha SM, Lorincz A, Dong H, Faubion WA Jr, Ordog T (2015) Stem cells for murine interstitial cells of cajal suppress cellular immunity and colitis via prostaglandin E2 secretion. Gastroenterology 148:978–990

de la Portilla F, Alba F, Garcia-Olmo D, Herrerias JM, Gonzalez FX, Galindo A (2013) Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. Int J Color Dis 28:313–323

Duijvestein M, Vos AC, Roelofs H, Wildenberg ME, Wendrich BB, Verspaget HW, Kooy-Winkelaar EM, Koning F, Zwaginga JJ, Fidder HH et al (2010) Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. Gut 59:1662–1669

Forbes GM, Sturm MJ, Leong RW, Sparrow MP, Segarajasingam D, Cummins AG, Phillips M, Herrmann RP (2014) A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. Clin Gastroenterol Hepatol 12:64–71

Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M (2009) Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 136:978–989

Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M (2009) Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 58:929–939

Hu J, Zhao G, Zhang L, Qiao C, Di A, Gao H, Xu H (2016) Safety and therapeutic effect of mesenchymal stem cell infusion on moderate to severe ulcerative colitis. Exp Ther Med 12:2983–2989

Kappelman MD, Moore KR, Allen JK, Cook SF (2013) Recent trends in the prevalence of Crohn's disease and ulcerative colitis in a commercially insured US population. Dig Dis Sci 58:519–525

Katsuda T, Kosaka N, Takeshita F, Ochiya T (2013) The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles. Proteomics 13:1637–1653

Kim HS, Shin TH, Lee BC, Yu KR, Seo Y, Lee S, Seo MS, Hong IS, Choi SW, Seo KW et al (2013) Human umbilical cord blood mesenchymal stem cells reduce colitis in mice by activating NOD2 signaling to COX2. Gastroenterology 145(1392–1403):e1391–e1398

Lee WY, Park KJ, Cho YB, Yoon SN, Song KH, Kim DS, Jung SH, Kim M, Yoo HW, Kim I et al (2013) Autologous adipose tissue-derived stem cells treatment demonstrated favorable and sustainable therapeutic effect for Crohn's fistula. Stem Cells 31:2575–2581

Mittal M, Tiruppathi C, Nepal S, Zhao YY, Grzych D, Soni D, Prockop DJ, Malik AB (2016) TNFalpha-stimulated gene-6 (TSG6) activates macrophage phenotype transition to prevent inflammatory lung injury. Proc Natl Acad Sci U S A 113:E8151–E8158

Molendijk I, Bonsing BA, Roelofs H, Peeters KC, Wasser MN, Dijkstra G, van der Woude CJ, Duijvestein M, Veenendaal RA, Zwaginga JJ et al (2015) Allogeneic bone marrow-derived mesenchymal stromal cells promote healing of refractory perianal fistulas in patients with Crohn's disease. Gastroenterology 149(918–927):e916

Monteleone G, Pallone F (2015) Mongersen, an Oral SMAD7 antisense oligonucleotide, and Crohn's disease. N Engl J Med 372:2461

Panes J, Garcia-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, Dignass A, Nachury M, Ferrante M, Kazemi-Shirazi L et al (2016) Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. Lancet 388:1281–1290

Ren G, Zhang L, Zhao X, Xu G, Zhang Y, Roberts AI, Zhao RC, Shi Y (2008) Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2:141–150

Sala E, Genua M, Petti L, Anselmo A, Arena V, Cibella J, Zanotti L, D'Alessio S, Scaldaferri F, Luca G et al (2015) Mesenchymal stem cells reduce colitis in mice via release of TSG6, Independently of Their Localization to the Intestine. Gastroenterology 149:163–176.e20

Stein M, Keshav S, Harris N, Gordon S (1992) Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 176:287–292

Terai S, Tsuchiya A (2017) Status of and candidates for cell therapy in liver cirrhosis: overcoming the "point of no return" in advanced liver cirrhosis. J Gastroenterol 52:129–140

Tsuchiya A, Kojima Y, Ikarashi S, Seino S, Watanabe Y, Kawata Y, Terai S (2017) Clinical trials using mesenchymal stem cells in liver diseases and inflammatory bowel diseases. Inflamm Regen 37

Wang Y, Chen X, Cao W, Shi Y (2014) Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. Nat Immunol 15:1009–1016

Watanabe S, Arimura Y, Nagaishi K, Isshiki H, Onodera K, Nasuno M, Yamashita K, Idogawa M, Naishiro Y, Murata M et al (2014) Conditioned mesenchymal stem cells produce pleiotropic gut trophic factors. J Gastroenterol 49:270–282

Williams KL, Fuller CR, Dieleman LA, DaCosta CM, Haldeman KM, Sartor RB, Lund PK (2001) Enhanced survival and mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth hormone. Gastroenterology 120:925–937

Yamada A, Arakaki R, Saito M, Tsunematsu T, Kudo Y, Ishimaru N (2016) Role of regulatory T cell in the pathogenesis of inflammatory bowel disease. World J Gastroenterol 22:2195–2205

Yui S, Nakamura T, Sato T, Nemoto Y, Mizutani T, Zheng X, Ichinose S, Nagaishi T, Okamoto R, Tsuchiya K et al (2012) Functional engraftment of colon epithelium expanded in vitro from a single adult Lgr5(+) stem cell. Nat Med 18:618–623