Original Article

Therapeutic effects of mouse bone marrow-derived clonal mesenchymal stem cells in a mouse model of inflammatory bowel disease

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Mouse bone marrow-derived clonal mesenchymal stem cells (mcMSCs), which were originated from a single cell by a subfractionation culturing method, are recognized as new paradigm for stem cell therapy featured with its homogenous cell population. Next to proven therapeutic effects against pancreatitis, in the current study we demonstrated that mcMSCs showed significant therapeutic effects in dextran sulfate sodium (DSS)-induced experimental colitis model supported with anti-inflammatory and restorative activities. mcMSCs significantly reduced the disease activity index (DAI) score, including weight loss, stool consistency, and intestinal bleeding and significantly increased survival rates. The pathological scores were also significantly improved with mcMSC. We have demonstrated that especial mucosal regeneration activity accompanied with significantly lowered level of apoptosis as beneficiary actions of mcMSCs in UC models. The levels of inflammatory cytokines including TNF-α, IFN-γ, IL-1β, IL-6, and IL-17 were all significantly concurrent with significantly repressed NF-κB activation compared to the control group and significantly decreased infiltrations of responsible macrophage and neutrophil. Conclusively, our findings provide the rationale that mcMSCs are applicable as a potential source of cell-based therapy in inflammatory bowel diseases, especially contributing either to prevent relapse or to accelerate healing as solution to unmet medical needs in IBD therapy.

***Key Words*: clonal MSCs, inflammatory bowel disease, colitis, anti-inflammation, mucosal regeneration**

Inflammatory bowel disease (IBD) is a chronic disorder related to dysfunctions of innate and adaptive immunity.[1–4] Depending on differences in histopathological features as well as clinical characteristics, IBD includes largely two forms, Crohn's disease (CD) and ulcerative colitis (UC). CD occurs in the whole gastrointestinal (GI) tract from the mouth to anus, whereas UC is limited to the colon and terminal ileum in some.[1,4] Patients with IBD have an increased risk for colorectal cancer and associations with other chronic inflammatory disorders such as psoriasis, uveitis, and primary sclerosing cholangitis, etc.[5–7] Since IBD is known to be caused by a variety of factors, including genetic backgrounds, environmental factors, and food habits,[3,4] the exact mechanisms that lead to the IBD development are not clearly understood. However, recent studies indicate that IBD is mainly due to overwhelmed inflammation of the GI tract by uncontrolled immune responses and increased abnormal T cell activities coupled with an intolerance to gut flora.[8,9]. Furthermore, considerable amounts of reports have emphasized the essential roles of macrophages and neutrophils during IBD relapse as evidenced that depletion of neutrophils by anti-neutrophil antibodies attenuated dextran sulfate sodium (DSS) and 2,4,6-trinitronitrobenzene sulfonic acid (TNBS)-induced colitis in rats.[10] In addition, it has long been known that macrophage-derived inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) are significantly increased in human IBD[11–13] as well as animal models of colitis.[14,15] All of these backgrounds strongly suggest that activated macrophages and neutrophils are recruited into inflamed colon tissues and increased expressions of pro-inflammatory cytokines eventually contributed to the initiation of mucosal injuries as well as the exacerbation of IBD symptoms.[16–18]

Although a number of studies had revealed molecular and physiological clues regarding the onset and progress of IBD, effective drug development to quit IBD initiation and progression is still challenging. To date, conventional IBD treatment has focused on regulating the abnormal immune responses and excessive inflammation. For example, 5-aminosalicylic acid (5-ASA) and corticosteroids have been used to reduce inflammation and other drugs such as 6-mercaptopurines, cyclosporine, and anti-TNF-α antibody have been applied for immunomodulation.[19,20] However, the efficacy of these drugs appears to be dependent on the severity and location of the disease as well as individual variations, and some drugs trigger serious complication.[19] Thus, new treatment strategies to either accelerate therapeutic effects for IBD or prevent disease relapse are urgently prerequisite.

Recently, cell-based therapy using mesenchymal stem cells (MSCs) have recently attracted attention as a potential therapeutic strategy against refractory to conventional treatment and to solve complicated condition such as anal fistula. Based on multipotential properties of stem cells such as differentiation into cell types such as osteocytes, chondrocytes, and adipocytes,[21,22] and impartment of potent immune modulating activity,[23] great concerning of stem cell therapy has been paid to IBD, along with previous achievements that MSCs improved several inflammatory diseases such as graft-versus-host gradient, arthritis, and acute pancreatitis.[24–26] Although MSCs are generally isolated by conventional gradient centrifugation method, however, a heterogeneous population of MSCs sometimes cause conflicting and controversial results in both animal experiments and clinical trials.[27]

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Our research group successfully established human clonal MSCs (hcMSCs) and mouse clonal MSCs (mcMSC) lines through subfractionation culturing method (SCM) to guarantee consistent therapeutic effects of stem cells.[28,29] These mcMSCs were reported to express stem cell markers and show the effects of immunomodulation in several disease models[26,30–32], one of which was the efficacy of mcMSC against necrotizing pancreatitis.[26] In the current study, we investigated the therapeutic potential of mcMSCs in an animal model of IBD and found that our mcMSC showed utmost therapeutic effects featured with excellent regenerative and anti-inflammatory outcomes.

Materials and Methods

Ethics statements. Animal care and all experimental procedures for the isolation of mouse bone marrow-derived cMSCs were approved by the Inha University Medical School Institutional Review Board (Inha University School of Medicine, Korea) and conducted in accordance with the Guide for Animal Experiments published by the Korea Academy of Medical Sciences. Animal experiments for DSS-induced colitis were approved by the Institutional Animal Care and Use Committee at the Department of Biological Sciences, Sungkyunkwan University (Korea).

Isolation, culture, and characterization of mcMSCs. Isolation and culture of mcMSCs were performed by a modified SCM as previously described.[28,29] Briefly, whole bone marrow (BM) samples were harvested from tibiae and femurs of 5-week-old female C57/BL6 mice by flushing out the BM cavity with Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco-BRL, Grand Island, NY) supplemented with 2% antibiotic-antimycotic solution (Gibco-BRL). The isolation medium [DMEM-low glucose supplemented with 20% fetal bovine serum (FBS; Gibco-BRL) and 2% antibiotic-antimycotic solution] was added to the mixture. This mixture was then plated onto a 100 mm culture dish. After incubation for 2 h at 37°C in a humidified CO_2 incubator, only the supernatant was transferred to a new 100-mm dish. After a second incubation of 2 h, the supernatant was transferred to a new dish (D1) and incubated for another 2 h. The supernatant was then transferred from D1 to a new dish (D2), incubated for 24 h, then transferred from D2 to a new dish (D3) and incubated for 24 h. This process was repeated two more times with 24 h (D4)- and 48 h (D5)-incubation. After incubation for up to 14 days, the well-separated single-cell-derived colonies that appeared in the culture dishes were dissociated by trypsinization in cloning cylinders (CORNING, Corning, NY), transferred to a 6-well plate, and then to larger culture flasks where they continued to expand. Once the cells reached approximately 70% confluence, they were detached, replated at 2,000–4,000 cells/cm^2 and allowed to expand. Each cell line at passage 5 was subjected to characterization for MSC identification. Basic properties such as differentiation potential, *in vitro* immunosuppressive activity and cell surface marker expression were analyzed as previously described.[29] For the evaluation of differentiation potential, differentiation into three mesenchymal cell types were independently induced from each MSC and then assessed by cell type-specific cytostaining. Expressions of cell surface markers were examined by flow cytometry using specific antibodies (CD29, CD34, CD44, CD45, CD73, CD90, CD103, CD105, CD117, Sca-1, Nestin, and MHC Class II) and isotype control antibodies. All antibodies were purchased from BD Pharmingen (San Jose, CA). *In vitro* immunosuppressive activity was measured by ^{3}H-thymidine incorporation in two different conditions (in a condition stimulated by anti-CD3/CD28 and in a mixed lymphocyte reaction).

Animal studies. Eleven week male C57BL/6 mice were purchased from Daehan Biolink (Seoul, Korea). Mice were maintained on a 12-h light/12-h dark cycle and constant temperature of 22°C under specific pathogen free (SPF) conditions for 1 week in our animal chamber for adaptation. Mice were provided a standard diet and water until reaching 12 weeks of age, at which they were used to establish the DSS-induced colitis model. The protocol for DSS-induced colitis was based on a previous report[33] and thus we established the model with minor modifications. Briefly, mice were administered with drinking water with 2.5% DSS (MW: 36,000–50,000, MP Biomedicals, Santa Ana, CA) for 6 days ad libitum. We divided mice into three groups, the control group that did not drink DSS, 2.5% DSS plus PBS group, and 2.5% DSS plus mcMSCs group. The DSS plus mcMSC group was infused with mcMSCs (4×10^5 cells/50 μl PBS), which were in the passage 15 and 16 during cell culture, by intravenous (IV) injection through tail vein on days 1, 3 and 5. Also, DSS plus PBS group was injected with 50 μl PBS on days 1, 3 and 5 as a control group. The severity of disease was evaluated everyday by a sum of the disease activity index (DAI) that includes loss of body weight, stool condition, and fecal occult blood as previously described.[33–35] Mice were sacrificed on day 6 to isolate the colon. The entire colon length was measured and the tissue was fixed in 10% neutral buffered formalin (NBF, Noblebio, Hwasung, Korea) for histological evaluation. In addition, colon was homogenated for cytokine measurement. For survival analysis, 2.5% DSS was replaced by water after 6 day and mice were monitored once daily for 15 days.

Pathological assessment. Formalin-fixed colon tissues were embedded in a paraffin block. Distal parts of colons obtained from PBS-infused and mcMSCs-infused colitis mice at day 6 were cut into 5 μm slices horizontally and used for hematoxylin and eosin (H&E) staining. The histological severity of colitis was examined under a microscope. Histological scorings of H&E-stained tissue samples were based on the extent of edema, ulceration, crypt loss and infiltration of immune cells as previously described[36–38] and valuated in a blind manner. The histological score was determined as the sum of each score.

Cytokine measurements. To examine the concentration of several cytokines in colon tissues, the colons obtained from PBS-infused and mcMSCs-infused colitis mice at day 6 were rinsed with cold PBS and whole colon tissues were homogenized in cold PBS with protease inhibitor cocktails (Roche Molecular Biochemicals, Indianapolis, IN, 1 ml/100 mg tissue) using a Polytron-type homogenizer. The homogenized solution was centrifuged for 10 min at 3,000 rpm at 4°C twice and the supernatant was stored in aliquots at –80°C until analysis. Protein concentration of the supernatant was measured by a Bradford assay kit (Intron Biotechnology, Seoul, Korea) and spectrophotometer. To identify the concentrations of IL-1β (BD bioscience), IL-6, IL-10, IL-17A, IL-23, TNF-α, IFN-γ, and TGF-β (ebioscience, San Diego, CA), an ELISA assay of each cytokine was performed according to the manufacturer's instructions. The 250 μg homogenate obtained from the colon was used for the ELISA assays.

Immunohistochemistry. Immunohistochemistry (IHC) was performed using a standard protocol. In brief, methanol-fixed frozen section tissue (7 μm) slides obtained from mice at day 6 were incubated 3% H_2O_2 in 10 min at room temperature, and each slide was blocked with 0.3% Triton-X 100 and 5% normal goat serum in TBS (20 mM Tris pH 7.6, 137 mM NaCl) buffer. Blocking solution was removed and each slide was incubated with the following primary antibodies overnight at 4°C, respectively: anti-TNF-α (ab6671, Abcam, 1:200 dilution), anti-F4/80 (14-4801-85, ebioscience, 1:500 dilutin), anti-NF-κB (sc-8008, Santa Cruz, 1:100 dilution), and anti-NIMP (ab2577, Abcam, 1:100 dilution) antibodies. After washing three times in TBS buffer, each slide was incubated with secondary biotinylated anti-rat IgG (Vector laboratories, Inc., Burlingame, CA), which was diluted 1:100 in blocking solution, for 1 h at room temperature and followed by activin/biotin ABC reagent reaction (Vectastain ABC kit, Vector laboratories, Burlingame, CA). Each slide was counterstained with methyl green and hematoxylin.

TUNEL assay. Apoptosis was visualized with terminal deoxynucleotidyl transferase (TdT) *Frag*EL DNA fragmentation

detection kit (Promega, Madison, WI). After routine deparaffinization, rehydration, and washing in 1× PBS (pH 7.4), tissues were digested with proteinase K (20 μg/ml in 1× PBS) for 20 min at room temperature and washed. After then tissues were incubated in equilibration buffer for 10 min and were treated with terminal deoxynucleotidyltransferase (*TdT*) enzyme at 37°C for 1 h. To determine the apoptotic index (AI) in each group, we first scanned *TdT*-nick end labeling (TUNEL)-immunostained sections under low power magnification (×100) to locate the apoptotic hot spots. The AI at ×400 field was then scored by counting the number of TUNEL-positive cells. At least five hot spots in a section were selected and average count was determined. Data were expressed as a mean percentage of total cell numbers.

T cell analysis. Lymphocytes were isolated from mesenteric lymph nodes (MLNs), which were obtained from mice at day 6, and stained with the following antibodies. CD3-FITC, CD4-APC, IFN-γ-FITC, IL-4-PE, CD25-PE and Foxp3-FITC were purchased from ebioscience (San Diego, CA), and CD8-PE and IL-17A-PE were purchased from BD bioscience (San Jose, CA). The Cytofix/Cytoperm kit (BD bioscience) and Foxp3 staining buffer set (ebioscience) were used to measure intracellular IL-17A and Foxp3. PMA/Ionomycin (Sigma, St. Louis, MO) and BD Golgi stop (BD bioscience) were used for amplification of the IL-17A signal. Live cells were gated by Canto II (BD Bioscience) and analyzed.

Localization of injected mcMSCs. To confirm the localization of infused mcMSCs in the inflamed colon tissues and mesenteric lymph nodes (MLNs), we labeled mcMSCs with PKH26 dye that shows red fluorescence at the cell membrane for *in vivo* cell tracking. The MINIPKH26 kit (Sigma) was used to label mcMSCs. The labeled mcMSCs were injected into mice through the tail vein by the same protocol described above. The colon tissues and MLNs isolated at day 3 were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and horizontally cut into 5 μm slices. Sectioned colon tissue samples were incubated in distilled water (DW) for 20 min and stained with 4',6-diamidino-2 phenylindole (DAPI). The samples were subsequently washed with DW for 5 min three times and observed by confocal microscopy (Carl Zeiss, Göttingen, Germany).

Myeloperoxidase (MPO) activity. MPO in colon tissue was measured as a marker of neutrophil infiltration into the colon tissue. The accumulation of MPO levels was determined by an enzyme assay, as previously described with minor modifications.[39] The colon tissues obtained from mice at day 6 were homogenized in lysis buffer [200 mM NaCl, 5 mM EDTA, 10 mM Tris pH 7.4, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 μg/ml leupeptide and 28 μg/ml aprotonine] and the homogenates were centrifuged twice at 1,500 *g* for 15 min. Protein concentrations of the whole colon supernatants were measured by a Bradford assay kit and spectrophotometer. To determine the activity of MPO, 250 μg of homogenates were used with the MPO assay kit (Hycult Biotech, Uden, Netherlands).

Statistical analysis. All data are expressed as means ± SD. Statistical significance was calculated by using the GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA). The statistical analysis of survival rate was performed by a log-rank test. Significant differences were assessed by one-way ANOVA and Student's *t* test. Results were considered significant when p values are <0.05.

Results

Features of homogenous mouse clonal MSCs (mcMSC). As reported before,[28,29] we could establish homogenous mcMSCs from the BM of C57BL/6 mice by SCM. The established mcMSCs morphologically showed fibroblast-like shapes in culture plates, expressing positive MSC markers such as CD29, CD44, Sca-1 and Netsin. However, markers such as CD34, CD45, CD103, CD117 and MHC-II were robustly negative and CD73 marker was slightly increased (Fig. 1A). The interesting finding in mcMSCs was that current mcMSCs did not express CD90 and CD105 (Fig. 1A). The reason seems to be due to the difference of MSC markers between human and mouse MSCs. Although CD90 and CD105 have been suggested as positive markers in human MSCs,[40] some studies have reported that CD90 is not or little expressed on mouse MSCs.[41,42] A recent study showed that CD105-negative mouse MSCs have the stem cell properties and better differentiation potential and immunomodulatory capacity than CD105-positive MSCs,[43] suggesting that CD90 and CD105 might not be critical determinants in the identification of mouse MSCs. However, when we examined the differentiating capability of our mcMSCs, they differentiated into several cell types such as adipocytes, osteoblasts, and chondrocytes as shown in Fig. 1B. Since immune suppression of mcMSCs modulating immune cells is another known characteristic,[23] we additionally investigated whether mcMSCs are able to suppress T cell proliferation under two different situations, anti-CD3/CD28 stimulation and mixed lympocyte reaction (MLR). When mcMSCs were respectively co-cultured with splenocytes under anti-CD3/CD28 stimulation and MLR, T cell proliferation was significantly suppressed (Fig. 1C, $p<0.001$). All of these results strongly suggest that our BM-derived mcMSCs showed MSC properties as well as phenotypes and imposed immune suppression.

Therapeutic effects of autologous mcMSCs in animal model of DSS-induced colitis. We evaluated the therapeutic potential of the autologous mcMSCs in DSS-induced colitis model in C57BL/6 mice. DSS-induced colitis showed similar symptoms to human UC such as a shortening of the colon, loss of body weight, and bloody diarrhea, resulting in an increased DAI score (Fig. 2A and B). However, when mcMSCs were injected into DSS-induced colitis mice through tail vein on day 1, day 3, and day 5, the colon lengths were significantly restored compared with PBS-infused group (Fig. 2A, $p<0.001$) and the DAI scores including the changes of body weight, stool changes, blood changes, were all significantly reduced from day 4 to day 6, compared with the PBS-infused control group (Fig. 2B, $p<0.01$). All of these results clearly reflected the therapeutic effects of the mcMSCs in DSS-induced colitis. We next examined the histophatology of the distal colons, which were obtained from PBS-infused or mcMSCs-infused group with DSS-induced colitis at day 6, because the histological disruption of DSS-induced colitis was predominantly observed in the distal colon.[44] The mcMSCs-infused group ameliorated histological changes such as cryptic disruption, edema, ulceration, and infiltration of the inflammatory cells which were observed in the PBS-infused group of DSS-induced colitis mice (Fig. 2C). Histological scores, based on these pathological characteristics, were significantly improved, compared with the PBS-infused group (Fig. 2C, $p<0.0001$). Evaluating the influences of mcMSCs on survival in a DSS-induced colitis model, the administered mcMSCs were significantly rescued from DSS-induced mortality (Fig. 2D, $p<0.01$) from day 12 to day 15, with an average survival rate of 70% in mcMSC group, whereas PBS administered group showed 20% survival rate. These results strongly indicate that homogeneous mcMSCs used in this study have significant therapeutic potential against DSS-induced colitis.

Significant anti-inflammatory efficacy of mcMSCs in DSS-induced colitis. We investigated anti-inflammatory effects of mcMSCs regarding the inflammation widely observed in DSS-induced colitis. The H&E staining of colon sections obtained from PBS- or mcMSC-infused mice at day 6, obviously showed that mcMSCs significantly reduce the infiltration of the inflammatory cells into the distal colon and the inflammation scores (Fig. 3A, Table 1, $p<0.01$). These results prompted us to examine the infiltration of macrophages and neutrophils into the distal colons. The infiltrations of both macrophages and neutrophils were significantly decreased in the presence of mcMSCs (Fig. 3B and C,

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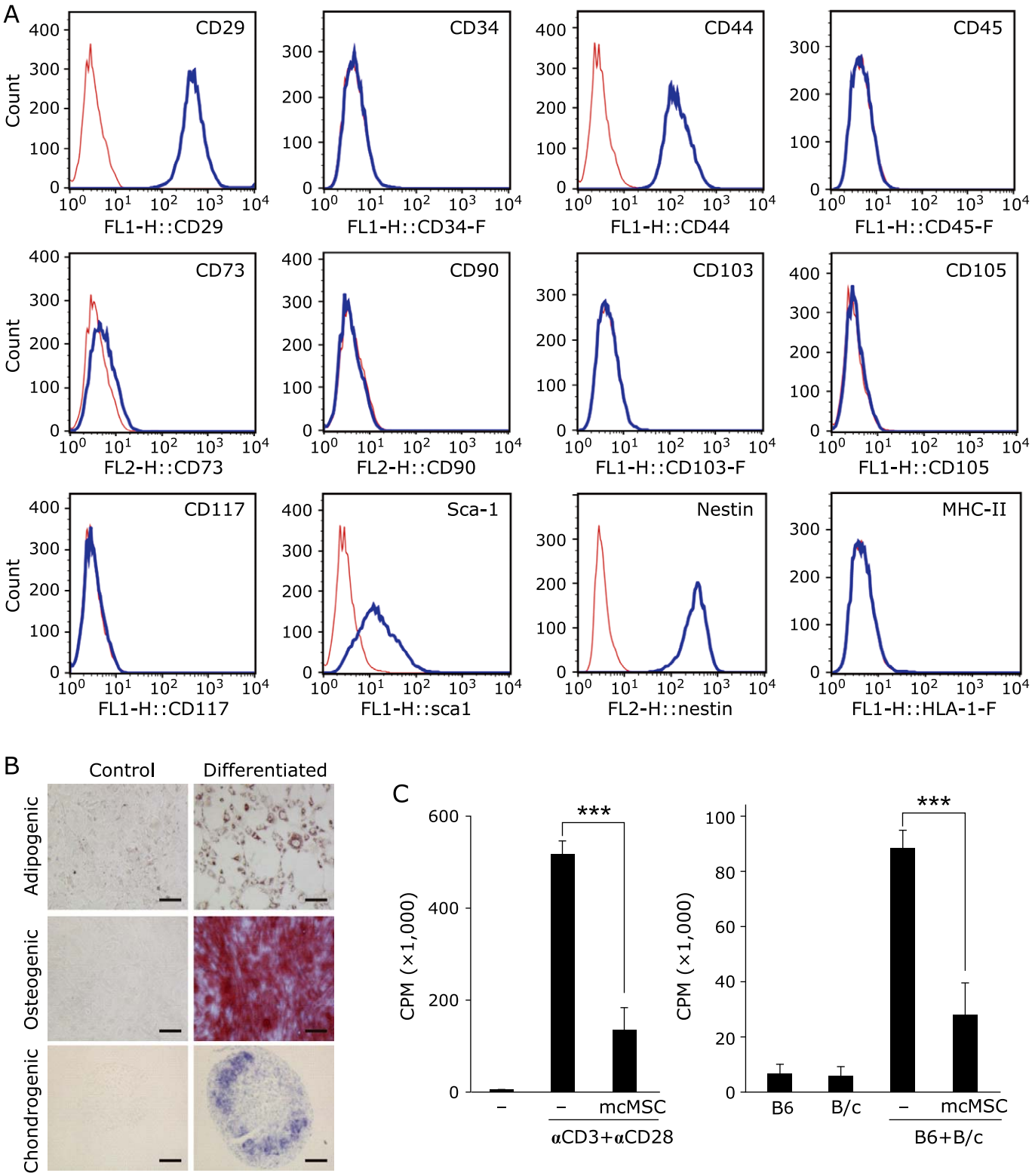

Fig. 1. Characterization of mcMSCs isolated from female C57BL/6 mice. (A) Expressions of stem cell markers were analyzed by flow cytometry. Positive and negative markers for MSCs were analyzed to confirm the stem cell characteristics of mcMSCs. MHC-II: MHC Class II. (B) Multi-lineage differentiation potential of mcMSCs was evaluated by cell type-specific cytostaining. Adipogenic, osteogenic, and chondrogenic differentiations were verified by oil red O, alizarin red S, and toluidine blue staining, respectively. Magnification: ×200. Scale bar, 100 μm. (C) *In vitro* immunosuppressive activity of mcMSCs was determined by [^{3}H]-thymidine incorporation. A total of 1×10^5 splenocytes isolated from C57BL/6 mice were stimulated with 1 g/ml of anti-CD3/CD28 (left panel) in the presence of mcMSCs (5×10^3 cells). The cell numbers of splenocytes were twenty times higher than those of mcMSCs. A total of 1×10^5 splenocytes from C57BL/6 and Balb/c mice were mixed and cultured for a mixed lymphocyte reaction (MLR) (right panel). mcMSCs (5×10^3 cells) were co-cultured. Incorporation of [^{3}H]-thymidine (1 μCi/well) was done for the last 12–16 h in the culture dish. Radioactivity was measured in a -counter. Values are means ± SD (***p<0.0001, vs negatvie control without mcMSC). (–): negative control, B6: C57BL/6 splenocytes, B/c: Balb/c splenocytes.

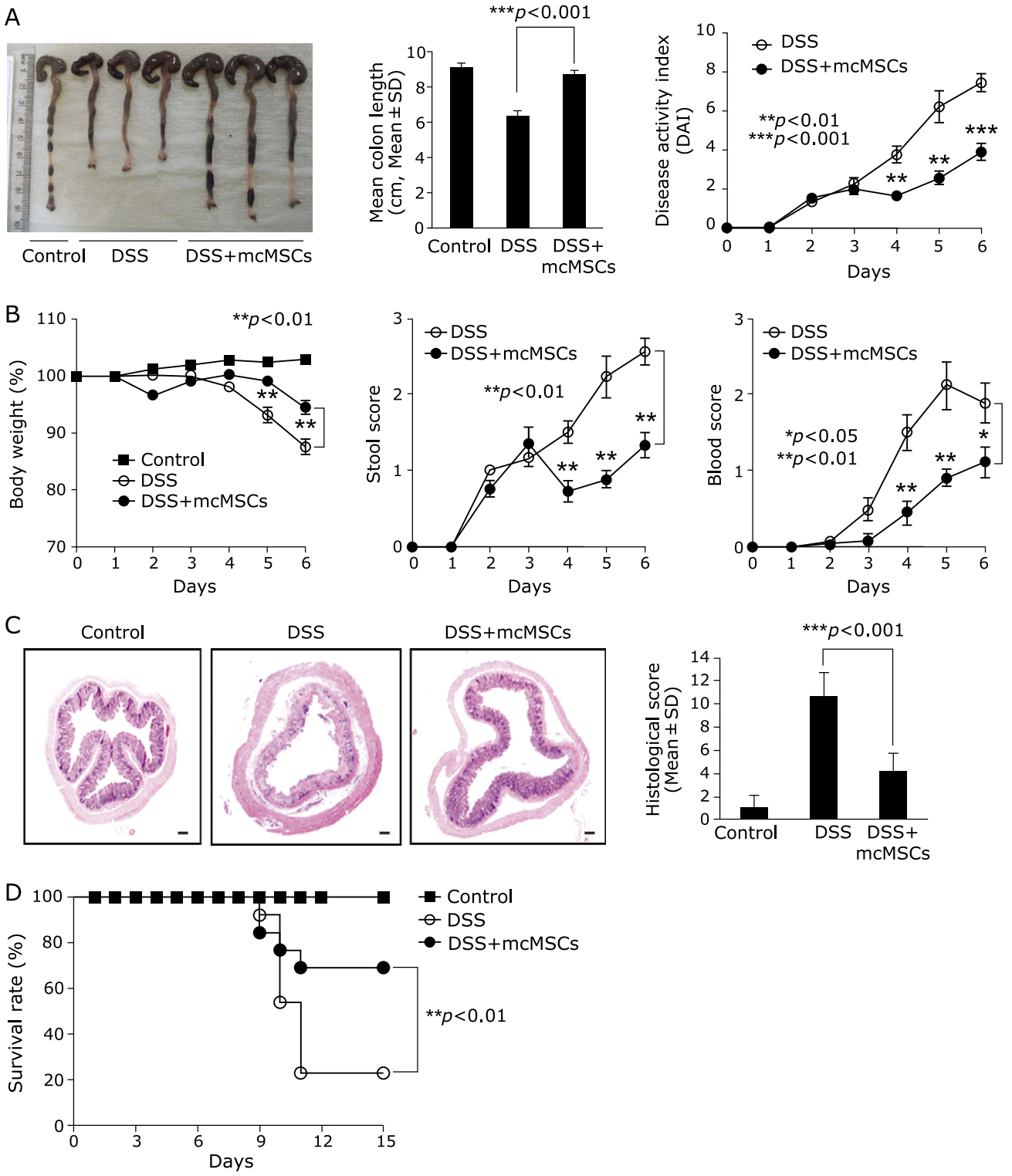

Fig. 2. Therapeutic effects of mcMSCs in DSS-induced colitis mice. (A) DSS-induced colitis was established by administration of drinking water with 2.5% DSS for 6 days. Colon lengths were described upon IV injections of mcMSCs and quantified in a bar graph. Values are the means ± SD [***p<0.0001 vs PBS-infused colitis group (DSS), t test, n = 9/group]. The scores of disease activity index (DAI), were examined in PBS-infused (DSS) and mcMSCs-infused (DSS + mcMSCs) colitis mice. DAI scores were summarized from day 0 to day 6. Values at day 4, 5 and 6 are means ± SD [**p<0.01, ***p<0.0001, vs PBS-infused colitis group (DSS)]. The control means mice which drink water without 2.5% DSS. (B) Loss of body weight, stool condition, and fecal occult blood, which are components of DAI, were examined from day 0 to day 6. Values are means ± SD [*p<0.05, **p<0.01, vs PBS-infused colitis group (DSS)]. (C) H&E staining of the distal colons of PBS-infused and mcMSC-infused colitis mice were described. The distal colons were isolated on day 6. The data are representative of at least three independent experiments. The histological scores were described in bar graph. Values shown on histological scores are the means ± SD [***p<0.0001 vs PBS-infused colitis group (DSS)]. Magnification: ×40. Scale bar, 200 μm. (D) Survival rates of the PBS-infused (DSS) and mcMSCs-infused (DSS + mcMSCs) colitis mice were analyzed. Values are the means ± SD [**p<0.01 vs PBS-infused colitis group]. Control: wild-type mice, DSS: PBS-infused colitis group, DSS + mcMSCs: mcMSCs-infused colitis group.

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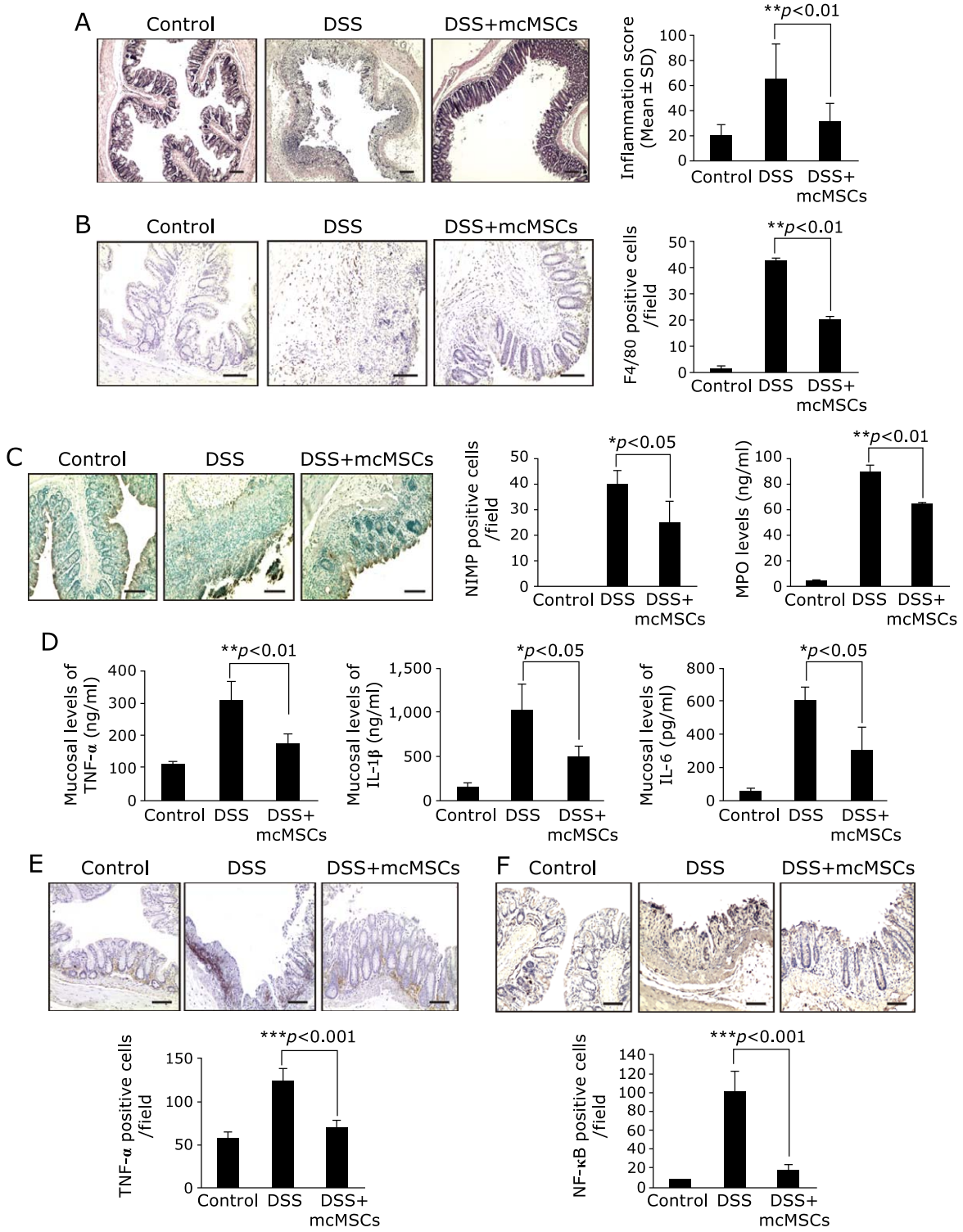

Fig. 3. mcMSCs reduce the inflammation induced by DSS. (A) H&E staining of the distal colons was described. Each colon was obtained from mice treated without DSS (control), PBS-infused colitis mice (DSS), and mcMSCs-infused colitis mice (DSS + mcMSCs). Inflammation scores were evaluated in bar graph. Values are the means ± SD [**p<0.01 vs PBS-infused colitis group (DSS)]. Magnification: ×100. Scale bar, 100 μm. (B) IHC analysis for the macrophage-specific marker F4/80 was performed and subsequently positive cells were evaluated. Values are the means ± SD [**p<0.01 vs PBS-infused colitis group (DSS)]. Magnification: ×200. Scale bar, 100 μm. (C) IHC analysis for the neutrophil-specific marker NIMP was performed and subsequently positive cells were evaluated. Myeloperoxidase activities for neutrophils were analyzed using colon homogenates obtained from each mouse. Values are the means ± SD [*p<0.05, **p<0.01 vs PBS-infused colitis group (DSS)]. Magnification: ×200. Scale bar, 100 μm. (D) Colons from PBS-infused (DSS) and mcMSCs-infused (DSS + mcMSCs) colitis mice were homogenized and the concentrations of the TNF-α, IL-1β, and IL-6 cytokines in the homogenates were measured by the respective ELISA kits. The data are means ± SD (*p<0.05, **p<0.01 vs PBS-infused colitis group). (E) IHC analysis for TNF-α expression in colons was performed and subsequently positive cells were evaluated. Values are the means ± SD [***p<0.0001 vs PBS-infused colitis group (DSS)]. Magnification: ×200. Scale bar, 100 μm. (F) IHC analysis for NF-κB expression in colons was performed and subsequently positive cells were evaluated. Values are the means ± SD [***p<0.0001 vs PBS-infused colitis group (DSS)]. Magnification: ×200. Scale bar, 100 μm. control: wild type mice, DSS: PBS-infused colitis group, DSS + mcMSCs: mcMSCs-infused colitis group.

Table 1. Assessment of inflammation score

Inflammation	Score
<30%	1
30–50%	2
50–70%	3
70%<	4

$p<0.05$). To further confirm the alleviating action of neutrophils by mcMSCs, we performed a MPO assay in colon homogenates prepared from each group at day 6 because transmigration of a number of neutrophils across epithelial tight junctions and subsequent accumulation in the crypt of the colon have been reported to be an important cause of crypt abscesses which are a representative feature of IBD.[18] The increased MPO activity was significantly reduced in the mcMSCs-infused colitis group, indicating that mcMSCs decrease neutrophil infiltration into the inflamed colon (Fig. 3C, $p<0.01$). Next, we measured inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which have been known to be linked to colitis,[16,17,35,45,46] in colon homogenates prepared from each group at day 6, respectively. The control PBS-infused group showed an increased level of pro-inflammatory cytokines, whereas these pro-inflammatory cytokines were significantly decreased in mcMSCs-infused colitis mice (Fig. 3D, $p<0.05$ or $p<0.01$). To support the decreased expression of pro-inflamamtory cytokines by mcMSCs, we performed the immunohistochemical staining using TNF-α antibody. The immunohistochemical stainings for TNF-α clearly showed that TNF-α expression is significantly decreased with mcMSCs (Fig. 3E, $p<0.001$). Because expressions of these inflammatory cytokines are mainly regulated by transcription factor NF-κB, expression of the NF-κB was evaluated by immunohistochemical staining with NF-κB p50 antibody. Expression of the NF-κB transcription factor was also significantly decreased in mcMSC treated group (Fig. 3F, $p<0.001$). All of these results strongly indicated that mcMSCs have significant anti-inflamamatory effects in DSS-induced colitis.

Significant regeneration spurting with mcMSC in DSS-induced colitis. Since the mucosal ulceration shown in DSS-induced colitis has been known as one of the characteristic findings of IBD,[47] we analyzed the contribution of mcMSCs on ameliorating colonic ulceration in DSS-induced colitis. DSS administration induced severe epithelial ulcerations of the mucosal tissues and inflammatory infiltrates, whereas mcMSCs significantly decreased colonic ulceration in DSS-induced colitis (Fig. 4A,

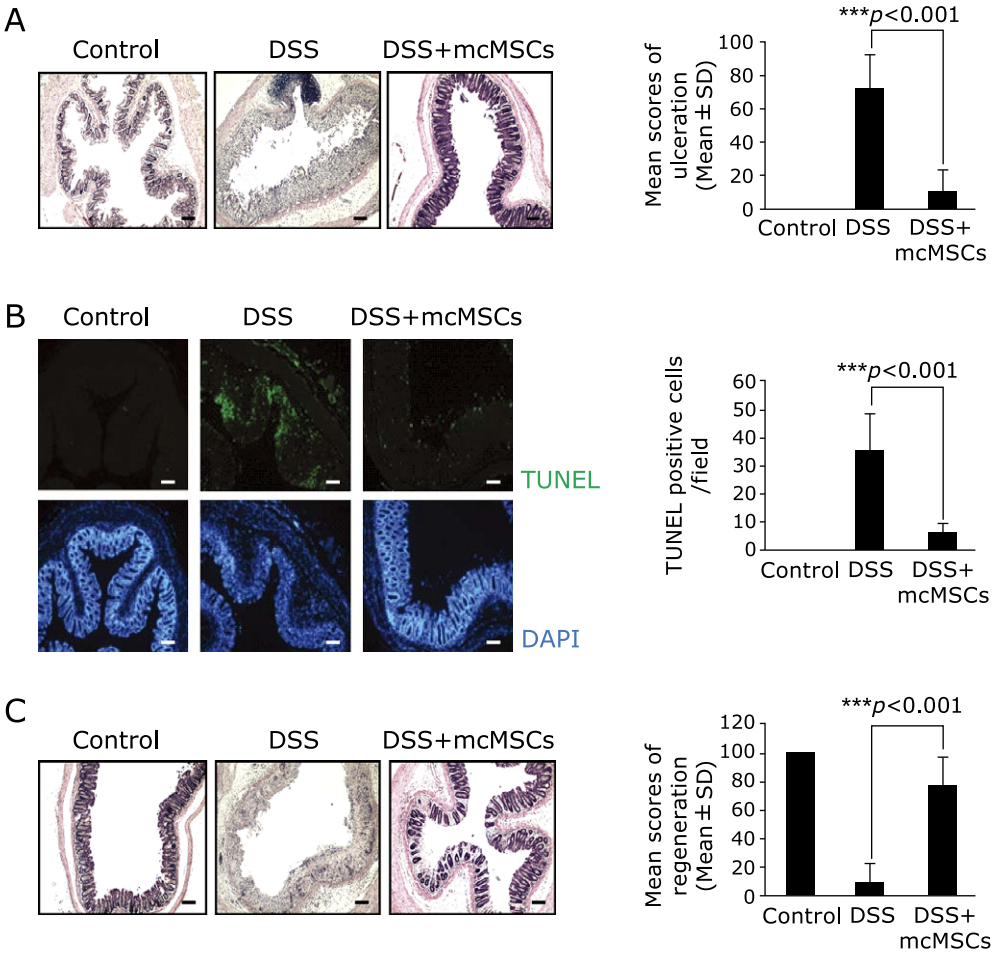

Fig. 4. mcMSCs are involved in regeneration of the damaged colons. (A) Ulcerations caused by DSS treatment and the infusion of mcMSCs were evaluated and described in bar graph. Values are the means ± SD [***$p<0.001$ vs PBS-infused colitis group (DSS)]. Magnification: ×100. Scale bar, 100 μm. (B) Cell death was analyzed by TUNEL assay in the respective group. Values are the means ± SD [***$p<0.001$ vs PBS-infused colitis group (DSS)]. Magnification: ×100. Scale bar, 100 μm. (C) Regenerations caused by the infusion of mcMSCs in DSS-induced colitis were evaluated and described in bar graph. Values are the means ± SD [***$p<0.001$ vs PBS-infused colitis group (DSS)]. Magnification: ×100. Scale bar, 100 μm. The data are representative of at least three independent experiments. control: wild type mice, DSS: PBS-infused colitis group, DSS + mcMSCs: mcMSCs-infused colitis group.

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Table 2. Assessment of ulceration score

Ulceration	Score
1/3 portion of mucosa	1
2/3 portion of mucosa	2
3/3 portion of mucosa	3
Perforated ulcers	4

Table 3. Assessment of regeneration score

Regeneration	Score
<30%	1
30–50%	2
50–70%	3
70%<	4

Table 2, $p<0.001$). Since ulceration is ultimate outcome of accumulated apoptosis, we have performed TUNEL in each group. As seen in Fig. 4B, DSS administration led to significant increases in apoptosis, but mcMSC significantly decreased DSS-induced apoptosis ($p<0.001$). Apart from apoptosis changes, prominent pathological findings with mcMSC administration were noted in degree of regeneration. Hardly, regenerative glands were seen in DSS-induced control group, but mcMSC provoked remarkable emergence of regenerative colon glands as seen in Fig. 4C (Table 3, $p<0.001$). In summary, the core rescuing actions of mcMSC in DSS-induced colitis might be anti-inflammatory, anti-apoptotic, and strong regenerative mechanisms.

Direct immune suppression of mcMSCs in colon, not via mesenteric LN. Since mcMSCs reduced the inflammation of DSS-induced inflamed colons and helped to mitigate DSS-induced colitis, we moved to the next experiment how the homogenous mcMSCs contributed to the alleviation of inflammatory responses induced by DSS treatment, seeing whether the infused mcMSCs affect the populations of T cells besides of neutrophil and macrophages infiltration (Fig. 2 and 5). First, we examined the production of important cytokines, which are involved in T cell differentiation, using the colon homogenates obtained from PBS-infused and mcMSC-infused mice in DSS-induced colitis of day 6. IFN-γ and IL-17A, which are secreted from Th1 and Th17, respectively, were significantly increased in DSS-induced colitis mice, but these cytokines were significantly decreased in the presence of mcMSCs (Fig. 5A, $p<0.01$ or $p<0.05$). In contrast, other cytokines such as IL-4, IL-10, IL-22, IL-23 and TGF-β1 were not changed between two groups (Fig. 5A). Second, we further investigated the subpopulations of T cells in mesenteric lymph nodes (MLNs) obtained from PBS-infused or mcMSCs-infused DSS-induced colitis mice at day 6. Normal control mice without DSS treatment were used as a negative control. The populations of $CD4^+$ and $CD8^+$ T cells were not changed in the presence of mcMSCs (Fig. 5B). The subpopulations of $CD4^+$ T cells, including Th1, Th2, regulatory T cells (T_{reg}), and Th17 cells, also did not show significant statistical difference among three groups. Though the mcMSCs significantly reduced the level of IL-17A and IFN-γ in the colon tissues (Fig. 5A), but we did not find significant changes of Th17 populations in MLN. All of these results suggest that mcMSCs might show anti-inflammatory effects through direct influence on the inflammatory cells *in vivo*, not through immune regulation onto T cells existing in LN.

Direct migration of mcMSCs into the inflamed regions of the colon. The findings that mcMSCs help to restore the structural alteration of colons induced by DSS and mitigate inflammation prompted us to investigate whether the mcMSCs actually migrate to the inflamed regions upon IV injection in the colitis model and orchestrate the above direct beneficiary mechanisms. Since mcMSCs were labeled with PKH26 dye, we performed confocal imaging analysis in resected colon tissue as well as mesenteric lymph node (MLN). Larger amounts of mcMSCs stained with PKH26 were only detected in either colon or regional mesenteric LN in the DSS-induced colitis group, mostly observed in the distal part of colon, scant in the proximal part (Fig. 6). Based on these findings, we suggest that mcMSCs migrated in both inflamed colon and mesenteric MLNs, thereby exerting anti-inflammatory and regenerative actions. However, we do not exclude the possibility that mcMSCs contribute to indirect immune modulation to quench propagation of colon inflammation. Taken all together, mcMSC cell therapy can be applied to two clinical conditions, highlighting regenerative activities and anti-inflammatory actions, which can be usefully applied to prevent relapse of IBD and to mitigate inflammatory activities, leading to quality of ulcer healing in IBD treatment (Fig. 7).

Discussion

A valuable merit of mcMSCs obtained through SCM was that they consist of homogenous cells compared with MSCs obtained by the conventional gradient centrifugation method,[28,29] by which they can produce consistent therapeutic outcomes. We hypothesized that though mcMSCs that have the same characteristics observed in other adult MSCs such as differentiation potential and stemness,[26,30–32] therapeutic potential of the homogenous cMSCs might be much better than those of heterogenous MSCs as already evidenced with our previous trial of mcMSC against acute necrotizing pancreatitis.[26] In this study, we could add potential therapeutic effects of mcMSCs in a DSS-induced colitis model supported with evidences of significantly attenuated infiltration of the inflammatory cells, imposing higher regenerative activities, and higher achievement of functional restoration.

Emerging data indicated that IBD might be caused by uncontrolled immune responses, intolerance to gut flora, and environmental factors,[2–4,8] by which conventional treatment for IBD includes corticosteroids, 5-aminosalicylates (5-ASA), 6-mercaptopurine (6-MP), sulfasalazine, and antimicrobial therapy, but addition of immunosuppressive agents, and biologics in refractory to aforementioned bottom-up treatment.[48] However, complicated case, unresponsive patients, and cases suffering from adverse effects requires further top-down therapeutics or surgery in some cases, although they are prerequisite for maintaining remission.[49] Therefore, it is worth considering MSC administration as another therapeutic option in IBD. In practice, several groups have reported that MSCs imposed significant therapeutic effects on IBD in clinical trials and animal models[50–55] and cMSCs have been successfully applied to several disease models, including models for acute pancreatitis, salivary gland damage, vocal cord damage, and graft-versus-host disease,[26,30–32] suggesting that MSC or cMSCs possess the immune modulatory activity against inflammatory diseases as well as tissue repair activity in damaged organs, emphasizing their therapeutic potential as a source of cell-based therapy. However, although cMSCs are a promising tool for clinical application in terms of their homogeneity and easy purification, the therapeutic effects of cMSC on IBD was unknown before our study. Current study might be the first proof showing clonal MSC as cell therapeutics for IBD.

Remarkable achievement was seen in strong regenerative capability of damaged colon. Striking finding from the current study was that tissue repair activity of mcMSCs in DSS-induced colitis was excellent, much better than the previous finding that adipose tissue-derived stem cells (ADSCs) facilitated colonic mucosal repair in TNBS-induced colitis.[56] Considering our results and recent findings about the effects of cMSCs in other disease model of pancreatitis,[31,57] mcMSCs have imposed higher

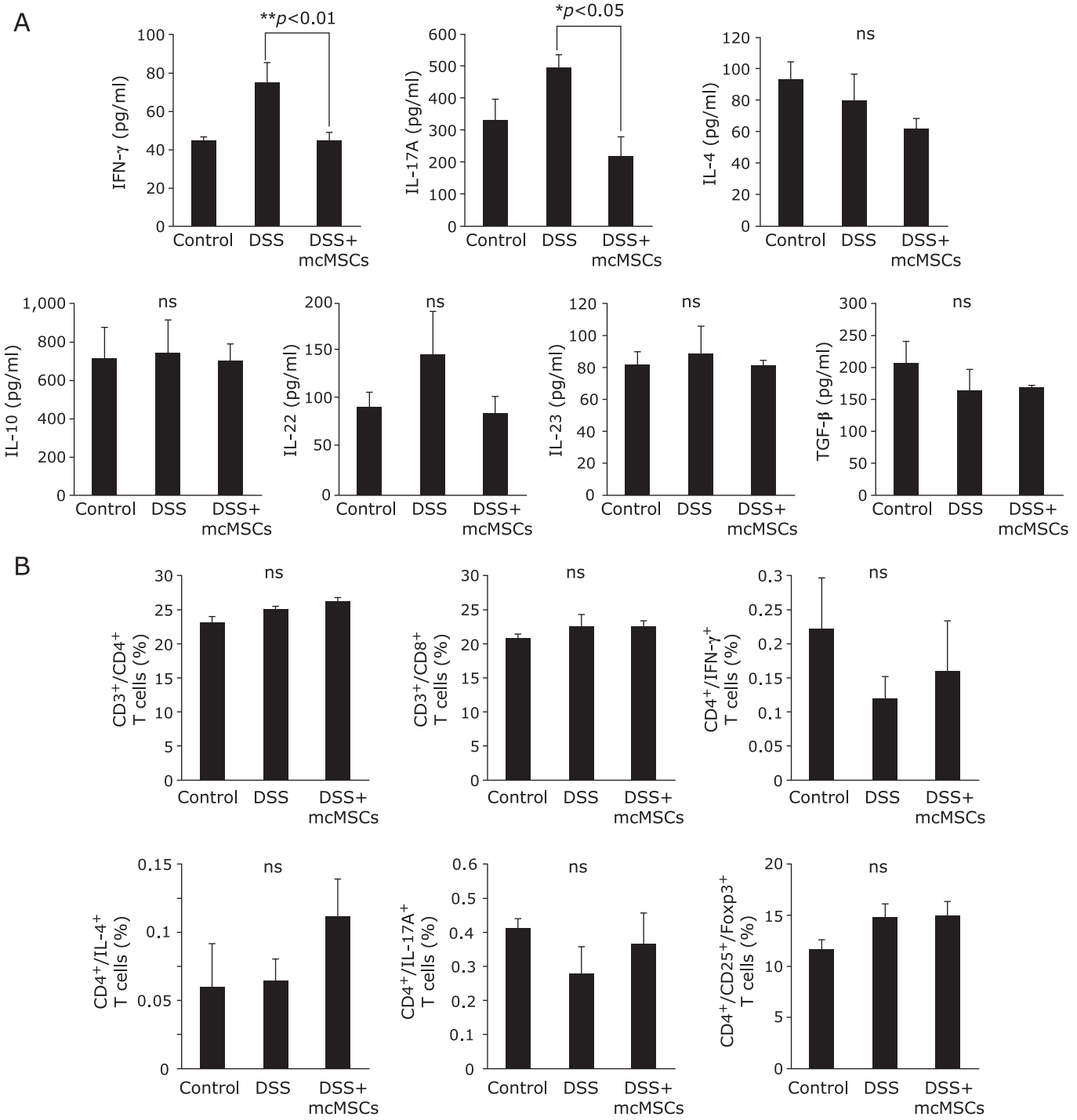

Fig. 5. mcMSCs do not affect the distribution of T cell populations in MLNs. (A) Colons from PBS-infused (DSS) and mcMSCs-infused (DSS + mcMSCs) colitis mice were homogenized and the concentrations of the IFN-γ, IL-17A, IL-4, IL-10, IL-22, IL-23, and TGF-β cytokines in the homogenates were measured by the respective ELISA kits. The data are means ± SD (*p<0.05, **p<0.01 vs PBS-infused colitis group). (B) Lymphocytes were isolated from MLNs at day 6 from normal mice (control) group, PBS-infused (DSS) colitis group, and mcMSCs-infused (DSS + mcMSCs) colitis group, respectively. The populations of $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+/IFN\text{-}\gamma^+$ Th1 cells, $CD4^+/IL\text{-}4^+$ Th2 cells, $CD4^+/IL\text{-}17A^+$ Th17 cells, and $CD4^+/CD25^+/Foxp3^+$ T_{reg} cells were analyzed by FACS analysis. All data represent the mean values ± SD of three independent experiments.

regeneration activities to the injured colitis. Although we still do not completely reveal the exact mechanism of regeneration process imposed by mcMSCs in colitis model, we speculate that mcMSCs may stimulate the proliferation of colonic epithelial cells as well as the inhibition of the cell deaths essential in tissue injury, endow restitution activities of damaged colonocytes and the possibility of transdifferentiating into subepithelial myofibroblast. Another differentiating and interesting finding was that mcMSCs did not affect the distribution of T cell populations in the MLN, including Th17 cells, although mcMSCs migrated to the MLNs as well as the inflamed colons (Fig. 6) and significantly inhibited the expression of IL-17 in colon (Fig. 5A). Although it is

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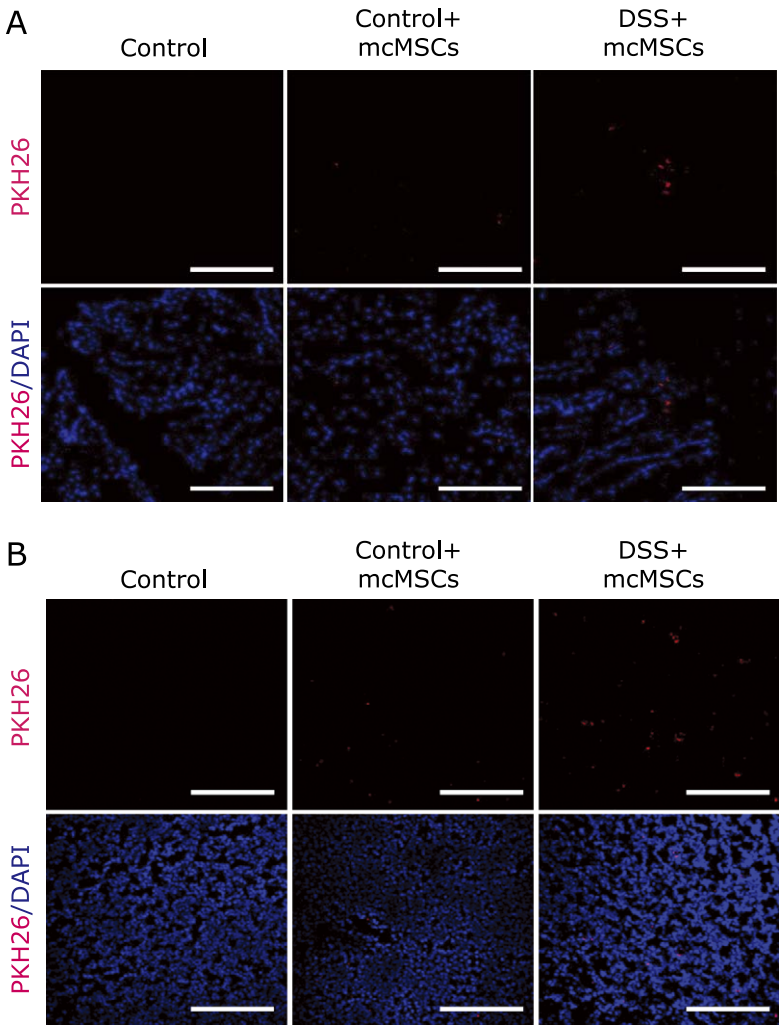

Fig. 6. mcMSCs were localized in the inflamed colons and MLN. (A) Control group was not injected PKH-stained mcMSCs as negative control. PKH26-stained mcMSCs were IV injected into normal wild type mice (control + mcMSCs) and mcMSCs-infused colitis mice (DSS + mcMSCs). The distal colons were isolated from each group and observed by confocal microscope. Magnification: ×400. Scale bar, 100 μm. (B) The mesenteric lymph nodes (MLNs) were isolated from the colon of each group and observed by confocal microscope. Magnification: ×400. Scale bar, 100 μm. All data are representative of at least three independent experiments. Red fluorescence indicates the PKH26-labedled mcMSCs.

widely accepted that Th17 cells, which mainly secrete IL-17A, play an important role in the pathogenesis of IBD,[9] it is possible that these results might be due to the unaffected levels of TGF-β and IL-23 because both cytokines are required for the differentiation of $CD4^+$ T cells into Th17 cells. Further experiments are likely to be needed to demonstrate the exact reason that mcMSCs do not affect the distribution of T cell populations.

We deduced that differences in the cellular context of mcMSCs in colitis model might lead to better outcome than other MSCs. Although MSCs have been recognized as a new cell therapy source, several groups have recently reported the presence of multiple subsets of human primary MSCs by fluorescence activated cell sorting (FACS)-based purification method,[58–60] limiting in producing consistent and homogenous benefits. Since whole population of MSCs might be mixed with a variety of subpopulation of MSCs, which may distinguish from their surface markers, differentiation potential and immune modulating activity, our current findings definitely emphasize an importance of the purity of therapeutic homogenous MSC products in clinical and academic studies. In terms of its homogenous properties and easy production of cMSCs purified by SCM, homogenous cMSCs, originated from BM and isolated after picking up a single colony among a number of colonies,[28,29] may be a promising tool as a cell therapy source in the treatment of intractable diseases like frequently relapsed IBD. That is, SCM procedure for the isolation of cMSCs indicates that the homogenously purified cMSCs are eventually isolated from a single cell with stemness in BM. Therefore, if we clarify therapeutic potential of cMSCs according to their distinct markers, it will overcome the heterogeneity of MSCs and be very useful in clinical applications of cMSCs.

Although we revealed therapeutic effects of mcMSCs on IBD in this study, further detailed comparison studies between cMSCs and conventional MSCs are prerequisite. However, we believed that mcMSCs have profound benefit to control the quality of MSCs for clinical application. Featured with our present experiments, we hope that cMSC cell therapy can be applied to two clinical conditions of IBD after further investigations, evident regenerative activities of cMSCs might prevent relapse of IBD and potent anti-inflammatory actions of cMSCs might induce higher remission induction in mild to moderate degree of IBD (Fig. 7).

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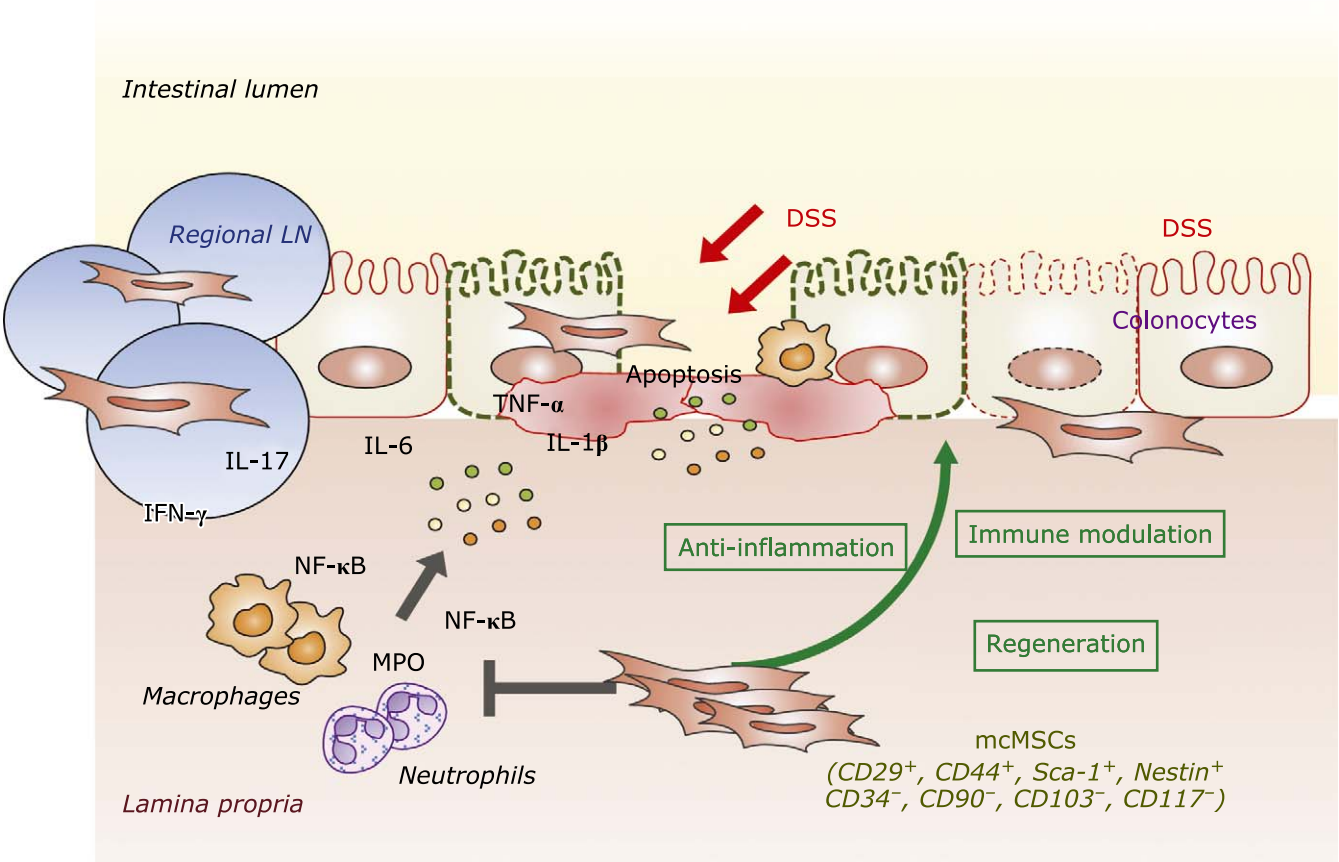

Fig. 7. Schematic summary regarding therapeutic effects of mcMSC in DSS-induced colitis, through stimulating regeneration, anti-inflammation, and immune regulation. All of these orchestrated beneficiary actions might be based on homogenous characteristics of mcMSCs compared to other reported MSC.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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