Mesenchymal Stem Cells (MSC) Derived from Induced Pluripotent Stem Cells (iPSC) Equivalent to Adipose-Derived MSC in Promoting Intestinal Healing and Microbiome Normalization in Mouse Inflammatory Bowel Disease Model

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ABSTRACT

Cellular therapy with allogeneic or autologous mesenchymal stem cells (MSC) has emerged as a promising new therapeutic strategy for managing inflammatory bowel disease (IBD). However, MSC therapy ideally requires a convenient and relatively homogenous cell source (typically bone marrow or adipose tissues) and the ability to generate cells with stable phenotype and function. An alternative means of generating allogeneic MSC is to derive them from induced pluripotent stem cells (iPSC), which could in theory provide an indefinite supply of MSC with well-defined phenotype and function. Therefore, we compared the effectiveness of iPSC-derived MSC (iMSC) and adipose-derived MSC (adMSC) in a mouse model of IBD (dextran sodium sulfate-induced colitis), and investigated mechanisms of intestinal protection. We found that iMSC were equivalent to adMSC in terms of significantly improving clinical abnormalities in treated mice and reducing lesion scores and inflammation in the gut. Administration of iMSC also stimulated significant intestinal epithelial cell proliferation, increased in the numbers of Lgr5intestinal stem cells, and increased intestinal angiogenesis. In addition, the microbiome alterations present in mice with colitis were partially restored to resemble those of healthy mice following treatment with iMSC or adMSC. Thus, iMSC administration improved overall intestinal health and healing with equivalent potency to treatment with adMSC. This therefore is the first report of the effectiveness of iMSC in the treatment of IBD, along with a description of unique mechanisms of action with respect to intestinal healing and microbiome restoration.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn’s disease and ulcerative colitis, results in intestinal inflammation due to immune dysregulation in the gut [1–4]. These diseases are characterized by infiltration of inflammatory cells and variable degrees of intestinal injury [5]. While substantial progress has been made in treating IBD with new immune modulatory drugs, there still remains a need for new approaches to treatment and management of the disease, especially treatments with few side effects and long duration of action.

Cellular therapy with immune-modulatory mesenchymal stem cells (MSC) has emerged as a promising new therapeutic strategy for managing IBD. For one, MSC are strongly immune modulatory, and suppress a number of different inflammatory processes [6–10]. Importantly, it has now been demonstrated in several studies in rodent models of IBD that systemic administration of MSC by the i.v. or i.p. routes can significantly...
ameliorate intestinal inflammation [11–14]. Moreover, these same positive effects have also been observed in humans with Crohn’s disease and ulcerative colitis treated with cellular therapy, and in a canine spontaneous animal model of IBD [15–21]. Thus, there is strong evidence that cellular therapy with MSC may be an effective management option for IBD, even in individuals with drug-refractory disease.

Despite the compelling evidence of the efficacy of cellular therapy for management of IBD, our understanding of the mechanisms by which MSC improve clinical and immunological abnormalities in the disease remains incomplete. For example, animal models of IBD (principally the dextran sodium sulfate (DSS)-induced colitis model) have demonstrated that MSC administration can reduce inflammatory cell infiltrates in the gut [13, 22–24]. There are conflicting data on the degree to which MSC actually localize to the gut following i.v. delivery, although the consensus seems to be that overall recruitment to the gut is relatively inefficient [13, 25, 26]. To date, most studies of MSC cellular therapy for IBD have focused on immune modulation by MSC. However, MSC are also suggested to have a stimulatory effect on the gut epithelium itself, including stimulation of intestinal stem cell proliferation in radiation-induced GI injury models [27–29].

However, despite the promise of cellular therapies, challenges remain in the application of cellular therapy as a viable option for management of IBD. For example, the source of MSC (whether autologous or allogeneic) is a major variable impacting the efficacy of therapy [30–32] as well as donor age [32–34] and donor-to-donor intrinsic MSC variability [35, 36]. Thus, the routine use of MSC cellular therapy with consistent benefit would benefit from a uniform cell source, with stable phenotype and function.

One solution to this problem of uniform cell sourcing is the use of MSC derived from induced pluripotent stem cells (iPSC). A number of studies have determined that iPSC-derived MSC (iMSC) closely resemble conventional bone marrow or adipose-tissue derived MSC in terms of both phenotype and function [37–40]. The ability of iMSC to suppress inflammation in vivo has also been demonstrated in mouse models, including allergic airway disease [38, 40, 41].

Therefore, in the present study, we investigated the effectiveness of iMSC for treatment of IBD, using a mouse model of DSS-induced colitis. Studies were done to investigate in greater detail the mechanisms by which MSC may ameliorate the intestinal abnormalities in IBD, including effects on intestinal healing and fecal microbiome populations, as well as inflammation. These studies revealed that iMSC were as effective as conventional, adipose-derived MSC (adMSC) for treatment of established and ongoing colitis in mice. In addition, these investigations also revealed important indirect stimulatory effects of MSC on intestinal healing and angiogenesis, as well as normalization of the intestinal microbiome. Taken together, these studies provide strong support for the use of iMSC as a more uniform and sustainable source of cells for IBD cellular therapy.

**Materials and Methods**

The animal experiments described here were conducted under protocols reviewed and approved Institutional Animal Care and Use Committee at Colorado State University (IACUC; protocol number 15-6194A).

**Animals**

Female CD-1 mice (age 8 weeks, weighing 25–30 g) were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed under a 12 hours-light/12 hours-dark cycle and a constant temperature at 25°C and were provided the standard diet and water ad libitum until starting the experiment.

**MSCs Isolation, Culture, and Tri-Lineage Differentiation**

The MSCs used for this experiment were isolated from abdominal and inguinal adipose tissues of 10 weeks old female CD-1 mice under sterile conditions. Approximately 0.5 g of isolated fat was washed with sterile Dulbecco’s phosphate-buffered saline (Sigma Aldrich, St. Louis, MO), then mechanically minced by scalpel blade and digested using collagenase (Sigma Aldrich; 1 mg/ml) for 30 minutes at 37°C. The stromal vascular fraction (SVF) was isolated by centrifugation at 380g for 5 minutes. After two washes with complete culture medium, the SVF was transferred into 75 cm² tissue culture flasks (Falcon, Thermo Fisher, Waltham, MA) and incubated at 37°C, 5% CO₂. After 72 hours, the flasks were washed to discard nonadherent cells, and refed with fresh complete medium. Proliferating colonies of adherent cells were allowed to reach 70% confluency, with medium changes every 48 hours. Thereafter, the cells were removed from flasks for passage by treating with trypsin (0.25% trypsin; EDTA, Gibco, Carlsbad, CA) and placed in 225 cm² flasks for further expansion. For studies reported here, adMSC were used at passage 3–4, and collected when approximately 80% confluent.

The iMSC line used in these studies was derived from a CD-1 mouse by the University of Colorado Denver Charles C. Gates Center for Regenerative Medicine and Stem Cell Biology iPS Core. Transgene integration-free iPS cells were generated from mouse skin fibroblasts using a CytoTune iPS Reprogramming kit (Life Technologies Corp. Grand Island, NY) according to manufacture instructions. The ability of adMSC and iMSC to undergo tri-lineage differentiation was assessed using a StemPro differentiation kit (Gibco). The phenotype of the differentiated cells was assessed by specific cytostaining (adipogenesis; Oil Red O, chondrogenesis; Alcian Blue, and osteogenesis; Alizarin Red).

**Flow Cytometry**

The surface phenotype of the adMSC and iMSC was determined as described previously [6]. Briefly, single cell suspensions were prepared at a concentration of 1 × 10⁶ cells per ml in FACS buffer, and 1 × 10⁵ cells were immunostained in single wells of round bottom 96-well plates (Corning Inc. Corning, NY). The primary antibodies were used as following: Sca-1-APC (clone eBR2a), CD11b-FITC (clone M1/70), CD29-biotin (clone HMb1-1), CD31-FITC (clone 390), CD44-FITC (clone IM7), CD45-PE (clone 30-F11), CD73-PE (clone eBioTv/11.8), CD90.2-eFlour 450 (clone 53-2.1), CD106-biotin (clone 429). All antibodies were obtained from eBioscience (San Diego, CA). For evaluation of leukocyte populations, the following primary antibodies were used: CD4-FITC (clone GK1.5), CD8-APC-e780 (clone 53-6.7), CD11c-FITC (clone N418), CD45-eFlour450 (clone 30-F11), FOXP3-PE (clone JFK-16s), B220-APC (RA3-682), Ly6G-APC-e780 (clone RB6-8C5), Ly6C-PE (clone HK1.4), F4/80-APC (clone BMB). In addition, intracellular staining was done using fixation permeabilization buffer for anti-FOX3 staining (Thermo Fisher), after cell surface staining was done. Cells were evaluated using a Beckman Coulter Gallios flow cytometer (Brea, CA) and data were analyzed using FlowJo Software (Ashland, OR). An example of gating scheme is shown in Supporting Information Fig. S1.
Intestinal Healing By iPSC-Derived MSC

DSS-Induced Colitis Model and Clinical Scoring of Disease Severity

Colitis was induced in mice using DSS administered at a concentration of 2.5% wt/vol in drinking water, as described previously [42]. Briefly, (DSS; Mr ≈ 40,000, Sigma-Aldrich, St. Louis, MO) by administering in drinking water daily throughout the study period. For each study, mice (n = 5 per group) were randomly assigned to the following groups: (a) untreated control group; (b) DSS treatment only; (c) DSS + adMSC administration; and (d) DSS + iMSC administration. Mice were monitored daily for body weight, clinical signs, stool consistency and color daily. On days 10, 13, and 16 of the study (with DSS administration initiated on day 0), MSC were administered by tail vein injection at a dose of 1 × 10^6 cells per mouse in 200 μl phosphate-buffered saline (PBS). The control and DSS only groups of mice were administered 200 μl of PBS by tail vein injection.

Clinical scoring was done using a modified scoring matrix, as described previously [13, 43]. Briefly, the mice were weighed, and fresh stool collected daily, and monitored for clinical signs every day. The percent weight loss was calculated daily and scored as 0 (no loss), −1 (1%–5%), −2 (5%–10%), −3 (10%–20%) and −4 for above 20%. Fecal occult blood was measured using a test kit (Fisher Healthcare, Houston, TX). The color reference guide was modified to quantitate the level of positive occult blood in a range of 2 (loose stool), −3 (watery). The final clinical score was the sum of the scoring from weight, fecal color, and fecal consistency, and the possible maximum score was −12.

Sample Collection for Histology and Immunofluorescent Staining

Mice were euthanized on day 19 of the study (3 days after the last MSC injection) and the colon was removed and weighed and the length determined. A 1 cm section of colon was cut and frozen at −80°C. Half of the remaining colonic tissue was fixed in 10% neutral buffered formalin for standard histological processing, while the other half was proceeded for immunofluorescent staining by fixation in a 1% paraformaldehyde-lysine-periodate (PLP) solution (1% paraformaldehyde in 0.2M lysine-HCl, 0.1M anhydrous dibasic sodium phosphate, with 0.21% sodium periodate) (Fisher Scientific, Hampton, NH) for 24 hours at 4°C. Following PLP fixation, colonic tissues were placed in a 30% wt/vol sucrose solution for 24 hours at 4°C, prior to embedding and freezing in O.C.T. compound (Tissue Tek, Tokyo).

Histopathology

Formalin-fixed paraffin-embedded colon tissues were processed for histological evaluation by routine H&E staining. Histopathological scoring of colonic tissues was completed in a blinded fashion by a board-certified veterinary pathologist (DR). A previously published scoring system for colonic inflammation (score 1–6; two categories) [44] was modified to include a category for mucosal inflammation and damage as follows (score 1–3); 1 (leukocytic infiltration), 2 (mucosal erosion/ulceration +/− colonic gland ectasia and necrosis), 3 (collapse of mucosal architecture +/− replacement granulation tissue). The final score was the summation of all parts of the scoring index, with a maximum possible score of 9.

Immunofluorescence Staining

PLP-fixed, OCT-embedded frozen tissues were cryosectioned at 5 μm thickness and mounted on Superfrost slides Fisher Scientific for immunofluorescent staining. The following primary antibodies were used for immunolabeling: CD11b (clone M1/70), F4/80 (clone BM8), CD3 (clone 145-2C11), CD4 (clone 45M95), CD103 (clone 2E7; all from ebioScience), cytokeratin-20 (Abcam, Cambridge, MA), CD31 (clone MEC 13.3; BD Pharmingen); FOXP3 (clone JFK-16s), Ki-67 (clone SolA15; all from ebioScience), Lgr5 (Abcam); using 0.1% Triton X and 0.25% saponin (Sigma-Aldrich) as permeabilizing agent, with overnight incubation. Secondary antibodies were conjugated with Cy3 or Alexa Flour488 (1:200 in PBS, Jackson ImmunoResearch Laboratories). The tissue sections were washed and counter stained with DAPI (Molecular Probe, Eugene, OR). The slices were subsequently mounted with Prolong Diamond Antifade medium (Thermo Fisher, Waltham, MA) and placed under a coverslip. Appropriate isotype control mAbs were used for all studies. The tissues were visualized using an Olympus IX83 confocal microscope and Hamamatsu digital camera.

For quantitation of images by confocal microscopy, 10 fields (× 20 objective magnification) of colonic mucosa were randomly sampled using the DAPI channel, and images were acquired. The positive fluorescent cells expressing FOXP3, CD11b, F4/80, CD3, CD4+ T cell (CD3+, CD4+), CD103, Ki-67, Lgr5, or CD31 were quantified per mm² of tissue. For calculation of microvessel density, the percentage of CD31 fluorescence area was divided by the total pixels of mucosal area of each image tile, using ImageJ software [45] (Supporting Information Fig. S2).

For MSC tracking studies, MSC were labeled with fluorescent DiR or with DiD dye (Invitrogen, Eugene, OR) and mice were imaged using an IVIS instrument (PerkinElmer, Waltham, MA).

Microbiome 16S rRNA Sequencing

Fecal pellet DNA extraction was performed using a Mobio PowerSoil DNA Isolation kit (Qiagen, Valencia, CA) according to manufactures instructions. Extracted DNA was submitted to Novogene Corporation (Chula Vista, CA) for 16S rRNA sequencing. According the company report of analysis, DNA concentration and purity was monitored on 1% agarose gels. 16S rRNA genes of V4 region were amplified using V4: 515F-806R. All PCR reactions were carried out with Phusion High-Fidelity PCR Master Mix (New England Biolabs, MA). PCR products were purified with Qiagen Gel Extraction Kit. Sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA) following manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Then quality filtering on the raw tags were performed according to the QIIME version 1.7.0. Chimeric sequences were removed using UCHIME Algorithm. Operational Taxonomic Units (OTUs) were conducted at 97% sequence similarity using QIIME for taxonomically classified. For genus level, naive Bayesian classifier retrained on the GreenGenes 16S rRNA gene database was used at 0.8 confidence threshold for taxonomic assignment. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. Alpha diversity

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was calculated using Shannon diversity index. Beta diversity on both weighted and unweighted unifrac were calculated by QIIME software (Version 1.7.0).

Statistical Analysis
Data were analyzed using Prism 7 software (GraphPad, San Diego, CA) and results were shown as mean ± SD (unless otherwise stated) and statistical significant was set at *p* < .05. The normality of data was analyzed using Shapiro-Wilk normality test. The clinical score and histopathological inflammatory score data were transformed to normal distribution. Statistical differences between groups were evaluated using one-way ANOVA with multiple means comparisons using Tukey's adjustment or otherwise stated.

RESULTS
Phenotypic Characterization of iMSC and adMSC
Both iMSC and adMSC were assessed for MSC characteristics and identification criteria, as established by the International Society for Cellular Therapy [46]. Flow cytometric analysis revealed high expression of MSC markers, including Sca-1, CD29, CD44, CD73, and CD106. In contrast, there was no expression of leukocyte markers, including CD45, CD11b, and CD31 (Fig. 1A). We found that whereas adMSC highly expressed CD90, the iMSC were negative for CD90 expression. Both cell types were positive for expression of CD106, which was expressed exclusively on MSC and not on skin fibroblasts (not shown). Tri-lineage differentiation studies also revealed that both adMSC and iMSC could be differentiated into adipocytes, osteoblasts, and chondrocytes (Fig. 1B).

Effects of MSC Administration on Clinical Signs and Colonic Lesions in Mice with DSS-Induced Colitis
Mice treated with DSS in their drinking water exhibited clinical signs consistent with colitis, including sustained weight loss, bloody diarrhea and abnormal fecal consistency resulting in decreased clinical scores over time (Fig. 2). Following the onset of signs of colitis, mice (*n* = 5 per treatment group) were treated with MSC (1 × 10⁶ MSC per mouse per injection, i.v.) on days 10, 13, and 16. In mice treated with either iMSC or adMSC, the clinical illness scores were significantly reduced compared to mice treated with DSS only (for iMSC-treated mice, *p* = .003 and for adMSC treated mice, *p* = .001 respectively) (Fig. 2A). The improvement in clinical scores became apparent within 1 day of the first MSC injection and was maintained during the period of MSC injections, whereas clinical scores continued to worsen in DSS-only treated animals.

Gross lesion scores and histopathology were evaluated in distal colon tissues collected in mice euthanized at 72 hours after the third MSC injection (day 19 of DSS treatment). In mice treated with DSS only (compared to untreated control animals), the colon appeared shortened and hyperemic (data not shown). Histologically, colonic tissues from DSS-treated mice exhibited severe infiltration of inflammatory cells, variable degrees of colonic gland ectasia and necrosis, extensive mucosal erosion to ulceration, and occasional complete loss and collapse of mucosal architecture (Fig. 2B) consistent with previous studies [42, 44, 47]. In contrast, colonic tissues from mice treated with either iMSC or adMSC exhibited an overall reduction in transmural inflammation, with significantly less infiltration of inflammatory cells in the lamina propria, diminished mucosal ulceration, and decreased mucosal collapse and granulation tissue formation. The overall histological
inflammatory scores were significantly improved in mice treated with either iMSC or adMSC, compared to the PBS-treated group (iMSC, \( p = 0.002 \); adMSC, \( p = 0.003 \)) (Fig. 2C). These results indicated therefore that iMSC were equivalent to adMSC in their ability to ameliorate the signs of intestinal inflammation induced by DSS treatment in this model of IBD.

**Trafficking of MSC to Intestinal and Extraintestinal Tissues**

To investigate the distribution of MSCs to sites of colonic inflammation, MSC were labeled using DiR dye immediately prior to injection. Cell distribution was monitored using IVIS live animal imaging. These studies revealed that labeled MSCs were primarily distributed to lungs initially, and at later time points labeled MSC could also be localized in the liver and spleen (Fig. 3A). However, labeled cells could not be detected by IVIS imaging in intestinal tissues.

To increase the sensitivity of cell detection, additional studies were done with mice injected with DiD-labeled MSC (Fig. 3B), followed by immunohistochemical examination of tissues from injected mice (Fig. 3C–3G). These studies revealed that labeled MSC could be detected only very rarely in the colonic mucosa and submucosa (Fig. 3D, 3E), in Peyer’s patches (Fig. 3F, 3G) and mesenteric lymph nodes (Fig. 3C) of treated animals. In contrast, labeled MSC were much more numerous in the spleen (Fig. 3C) and lung tissues (data not shown). The relatively scarcity of MSC in colonic tissues following i.v. injection in DSS models is consistent with several previous studies [13, 22, 48, 49], but differs markedly from findings in other published studies in which high numbers of labeled MSC were found in intestinal tissues [23, 50,
Our findings, with very low numbers of MSC in colonic tissues, suggest that the therapeutic benefits of injected MSC were more likely to have been mediated by paracrine secreted factors than by direct cell-to-cell effects between MSC and colonic epithelial cells.

**Effects of MSC Administration on Epithelial Regeneration**

The histologic appearance of colonic tissue from mice treated with MSC demonstrated remarkable recovery of intestinal epithelial integrity (see Fig. 2B). These findings suggested therefore that...
MSC treatment may have affected directly the reconstitution of epithelial integrity in the colon. Stimulation of epithelial regeneration could be mediated by several factors, including enhanced neovascularization, increased intestinal stem cell regeneration, and greater crypt cell proliferation and differentiation [52, 53]. Therefore, immunohistochemistry was used to investigate these processes in colonic tissues of DSS-treated control mice and mice treated with iMSC and adMSC (Fig. 4).

First, we observed a significant increase in the numbers of Ki-67\(^+\) intestinal epithelial cells in the colon of mice treated with iMSC and with adMSC, compared to the numbers of Ki-67\(^+\) epithelial cells in DSS-only treated animals and in untreated control animals (Fig. 4A, 4B). In addition, we found that the numbers of Lgr5\(^+\) intestinal stem cells were significantly greater in the colonic mucosa of animals treated with MSC, compared to DSS-only treated or control animals (Fig. 4C, 4D). Finally, MSC-treated animals demonstrated increased angiogenesis as determined by significantly increased numbers of CD31\(^+\) endothelial cells, as well as increased mean vessel density and area compared to control animals (Fig. 4E, 4F).

Taken together, these findings indicate that systemic administration of iMSC or adMSC exerted an important trophic effect on intestinal epithelial cells, by stimulating proliferation and recruitment of intestinal stem cells, and on the overall intestinal blood supply, by stimulating local angiogenesis. Thus, these findings suggest secretion of multiple trophic factors by i.v. delivered MSC. The stem cell tracking data, and the paucity of MSC detected in colonic tissues, strongly suggests that these trophic factors were likely to have been produced at sites distant from the GI tract.

Administration of MSC Reverses Microbiome Dysbiosis

Previous studies have found that DSS-induced colitis causes alterations in the gut microbiome [54]. In our studies, we used 16S sequencing to investigate the composition of the gut microbiome of DSS-treated animals, and we also found marked alterations in the populations of several important gut phyla (Fig. 5). For example, we observed a significant relative increase in *Proteobacteria* (Fig. 5A), along with increased *Bacteroides*, and decreased *Firmicutes* in mice treated with DSS, compared to untreated control animals (Fig. 5D). Overall, the DSS alone group had the least...
microbial community diversity measured within a sample as shown in an alpha diversity graph (using Simpson index) (Fig. 5B). Also, as shown in the Venn diagram (Fig 5E); the iMSC and adMSC treated groups shared more OTUs (operational taxonomic units) with the healthy group compared to the DSS only group.

Notably, in mice treated with either iMSC or adMSC, after 10 days of stem cell treatment, and despite the continued administration of DSS, the composition of the microbiome in these animals had returned to a population that much more closely resembled that of the microbiome of healthy untreated mice (Fig. 5C). For example, the iMSC-treated group of animals had the most similar taxa distribution relative to heathy control animals, compared to DSS only animals or DSS animals treated with adMSC (Fig. 5D). These results suggest that treatment with MSC helped restore the normal colonic flora, although the exact mechanism of the effect remained undetermined.

Effects of MSC Administration on Intestinal Inflammation

The effects of MSC injection on inflammatory responses in the colon and regional lymphoid tissues were examined next. In colonic tissues of DSS-treated animals, increased infiltrates of F4/80+ and CD11b+ macrophages, CD3+, CD4+ T cells, and CD103+ inflammatory monocytes were observed, compared to colonic tissues of healthy untreated animals.

In animals treated with MSC, the numbers of macrophages and monocytes were significantly reduced compared to DSS only treated animals treated (Fig. 6A). FOXP3+ regulatory T cells were significantly more numerous in colonic tissues of mice treated with either iMSC or adMSC, compared to control animals or DSS-only animals (Fig. 6A). These results are consistent with the results of previous studies [13, 22] and suggest that MSC treatment ameliorated colonic inflammation, as reflected by increased numbers of regulatory T cells and reduced macrophage and neutrophil infiltration into colonic submucosa. However, MSC treatment in our model did not alter the numbers of infiltrating T cells in colonic tissues (Fig. 6A).

Notably, iMSC and adMSC were comparable in their effects on reducing the severity of colonic inflammation. However, there were no consistent changes in T cell or B cell or myeloid cell populations in the spleen or mesenteric lymph node tissues of animals treated with MSC, compared to animals with DSS-induced colitis (Fig. 6B). Thus, overall administration of MSC reduced local colonic inflammation, in addition to stimulating epithelial cell proliferation and angiogenesis, but had little effect on immune cell populations in extraintestinal tissues.

DISCUSSION

The use of MSCs to treat IBD has shown considerable promise in preclinical studies in rodent models, and in small clinical trials in...
humans [13, 14, 17, 23, 32, 50, 55, 56]. However, the use of autologous or allogeneic bone marrow derived MSC or adMSC for larger scale clinical cellular therapy studies is subject to several important drawbacks, including the limited proliferative capacity of older MSC, donor-to-donor MSC variability, the costs associated with donor screening, and the time and expense associated with expanding MSC in primary culture [33, 36, 57]. Therefore, alternative cell sources for standardized and renewable, functional populations of MSC for cellular therapy are desirable. In the present study, we have investigated the potential for use of iMSC as an alternative source of cells for treatment of IBD. The potential advantages of iMSC for cellular therapy have been described previously [37, 38, 41, 58].

In our study, we found that iMSC were equivalent or in some cases superior to conventional adMSC for treatment of IBD, in terms of ameliorating clinical signs of colitis and stimulating intestinal healing. For example, the overall clinical score in DSS-treated mice was reduced by 26% at day 19 in mice treated with iMSC, compared to untreated mice (see Fig. 2A), while the reduction in clinical score was 19% in mice treated with adMSC. In addition, intestinal inflammation was reduced equivalently in mice treated with iMSC versus mice treated with adMSC (see Fig. 2C). Also, iMSC administration elicited significant stimulation of intestinal epithelial cell proliferation, increased in the numbers of Lgr5+ intestinal stem cells, and increased in intestinal angiogenic responses (see Fig. 4). Notably, in our study the long-term therapeutic effect was maintained by repeated MSC injections, suggesting a relatively short duration of action following a single MSC administration [59, 60].

The anti-inflammatory properties of MSC have been described previously, and these effects certainly may account for a portion of the overall beneficial effect of MSC on colitis. For example, studies have shown that MSC suppresses Th17 responses, increase the Treg: Th17 ratio, and lead to loss of effector T cells [61–64]. In addition, our study suggests alternative mechanisms by which MSC may also exert an important effect on resolution of lesions in the gut associated with IBD. For example, stimulation of epithelial regeneration may be as important to healing as suppression of inflammation. The significant increase in the number of Lgr5+ cells observed in the epithelium of mice treated with iMSC versus control DSS mice (see Fig. 4C, 4D), is indicative of stimulation of local stem cell proliferation and/or recruitment. Our results are in agreement with previous studies of radiation-induced GI injury which suggest that MSC may secrete molecules leading to activation of the Wnt/β-catenin signaling pathway required for Lgr5+ cell proliferation [27]. Also reduced inflammatory cytokines may improve self-renewal or return normal differentiation or apoptotic rate of Lgr5+ cells [65, 66]. Therefore, suppression of inflammation and stimulation of local stem cell proliferation by MSC may work together...
synergistically to stimulate intestinal healing. Furthermore, we observed significantly greater proliferation of mature epithelial cells in the colonic mucosa, consistent with a stimulatory effect of MSC secreted factors. Given the rather sparse distribution of MSC found in the colonic mucosa or submucosa (see Fig. 3D, 3E), this MSC effect is most likely mediated by trophic factors secreted by MSC residing in extraintestinal tissues, including the lung and spleen. Finally, we also observed that angiogenic responses were significantly enhanced locally in colonic tissues in animals treated with MSC, consistent with the release of proangiogenic cytokines such as VEGF, as has been reported previously [67].

A second potential mechanism of intestinal healing by MSC involves microbiome alterations. The gut microbiome is known to exert an important stimulatory effect on intestinal integrity and local immune responses [68]. For example, intestinal healing has been shown to be significantly impaired in mice in which the microbiome has been disrupted, and in mice unable to sense intestinal bacteria due to disrupted TLR signaling [69, 70]. In the present study, we observed that the microbiome alterations induced by DSS injury were to a large degree reversed by the administration of MSC, including administration of either iMSC or adMSC (see Fig. 5). For example, the diversity of the gut microbiome phyla was significantly increased in MSC-treated animals, compared to DSS only treated animals (Fig. 5B). These findings suggest therefore that by generating a more diverse microbiome more closely resembling that of healthy control animals, MSC administration improved overall intestinal health and healing.

However, it should also be acknowledged that is not clear whether the microbiome changes were a consequence of improved intestinal healing, or whether intestinal healing resulted from the improved microbiome. More likely, both mechanisms were likely to have been operative, with MSC-induced changes in the colonic epithelium altering the microbiome, and the microbiome changes further stimulating intestinal epithelial regeneration and angiogenesis.

In summary, our studies reveal that iMSC are equally effective as adMSC when administered systemically for resolving IBD in a rodent model, without inducing detectable adverse effects, even after repeated i.v. administration. Moreover, the effects of MSC on IBD appear to be multifactorial, involving both improved intestinal epithelial regeneration, beneficial microbiome alterations, and suppression of intestinal inflammation. To evaluate the safety and efficacy of iMSC for treatment of IBD in a more translationally relevant setting, we have initiated studies of iMSC treatment in a spontaneous IBD model in pet dogs, based on early evidence of efficacy of MSC efficacy in a similar model [16]. Moreover, we have recently conducted safety studies for systemically administered canine iMSC in dogs and found no evidence of teratoma or tumor formation (L. Chow et al., manuscript submitted for publication). Thus, the use of systemically administered iMSC as a new cellular therapy is an option for management of refractory IBD, provided regulatory and potential safety issues can be adequately addressed.

**CONCLUSION**

These studies are the first to demonstrate the equivalence of iMSC and adMSC for treatment of IBD, in a widely used rodent DSS colitis model. In all aspects evaluated, iMSC were equally potent in terms of suppressing intestinal inflammation and stimulating intestinal healing. These studies also revealed a significant stimulatory effect of iMSC cellular therapy on epithelial proliferation and angiogenesis, and restoration of normal microbiome populations.

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**AUTHOR CONTRIBUTIONS**

S.S., L.C., and S.D.: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; V.J., J.C., W.W., and D.R.: collection and assembly of data; S.D.: conception and design, financial support, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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