Endoscopic Administration of Mesenchymal Stromal Cells Reduces Inflammation in Experimental Colitis

Marieke Barnhoorn, MD, Eveline de Jonge-Muller, BSc, Ilse Molendijk, MD, PhD,* Mandy van Gulijk, BSc, Oscar Lebbink, BSc, Stef Janson, BSc, Mark Schoonderwoerd, MSc, Danny van der Helm, MSc, Andrea van der Meulen-de Jong, MD, PhD, Lukas Hawinkels, PhD,* and Hein Verspaget, PhD**

Background: Mesenchymal stromal cells (MSCs) are a potential therapeutic modality in inflammatory bowel diseases (IBDs) because of their immunomodulatory and regenerative properties. However, when injected systemically, only a small portion of the cells, if any, reach the inflamed colon. In this study, we assessed whether endoscopic injections of MSCs into the intestinal wall of the inflamed colon affect the course of experimental colitis. Furthermore, we investigated if injection of aggregated MSCs in spheroids could enhance their therapeutic ability.

Methods: Expression levels of in vivo MSC aggregates and in vitro MSC spheroids were compared with monolayer cultured MSCs for both anti-inflammatory and pro-regenerative factors. Subsequently, MSCs and MSC spheroids were injected endoscopically in mice with established dextran sulfate sodium (DSS)–induced colitis.

Results: Endoscopically injected MSCs and MSC spheroids both alleviated DSS-induced colitis. Furthermore, both in vivo and in vitro MSC spheroids showed increased expression of factors important for immunomodulation and tissue repair, compared with monolayer cultured MSCs. Despite differential expression of these factors, MSC spheroids showed similar clinical efficacy in vivo as single-cell suspension MSCs. Analysis of serum samples and colon homogenates showed that local MSC therapy resulted in increased levels of interferon-γ, indoleamine 2,3-dixoygenase, and interleukin-10.

Conclusions: Endoscopic injections of MSCs and MSC spheroids in the inflamed colon attenuate DSS-induced colitis. Our data show that endoscopic injection can be a feasible and effective novel application route for MSC therapy in patients with luminal IBD.

Key Words: mesenchymal stromal cells, experimental colitis, inflammatory bowel diseases

INTRODUCTION

Inflammatory bowel diseases (IBDs) affect approximately 4 million people in Europe and the United States.[1, 2] IBD can be subdivided into Crohn's disease and ulcerative colitis.[3, 4] Patients with IBD suffer from abdominal pain, diarrhea, and fatigue. The exact etiology of IBD is unknown, but defects in immune mechanisms play an important role. Patients with Crohn's disease have an imbalance between T-helper (Th) 17 and regulatory T (Treg) cells, whereas ulcerative colitis is associated with an atypical Th2 response.[3, 4] Current therapies consist of medical treatment with immunomodulators and biologicals and surgery to remove the severe inflamed parts of the intestine. Despite the introduction of biological therapy, there is still need for surgical intervention in 7.6%–24.2% of IBD patients 5 years after diagnosis.[5] Active disease negatively affects the quality of life of IBD patients,[6, 7] and therefore novel therapies are needed. One of these novel treatment options could be treatment with mesenchymal stromal cells (MSCs).

MSCs are pluripotent (stem) cells that are currently tested as a cellular therapy in several autoimmune diseases. Recent reports from clinical trials of our group and others[8, 9] have shown that MSCs have promising therapeutic effects in the local treatment of refractory Crohn's disease–associated perianal fistulas. Although clinical benefit of MSC therapy has been shown in these patients, the exact working mechanism of MSC therapy has not been elucidated yet. Currently, it is thought that the therapeutic potential of MSCs in IBD is associated with their multilineage differentiation, regenerative capacities, and especially their immunomodulatory effects. MSCs are able to act anti-inflammatory by suppressing the proliferation and activation of Th cells[10] and the stimulation of Treg cells.[11, 12] Furthermore, MSCs have been reported to suppress the proliferation of B cells[13] and promote the conversion of monocytes and pro-inflammatory macrophages into anti-inflammatory macrophages.[14] Taken together, these studies indicate that MSCs have immunoregulatory capacities, positioning them as an attractive potential therapy for IBD.

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From the Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands.

*Equal contribution.

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Address correspondence to: Hein Verspaget, PhD, Leiden University Medical Center, Department of Gastroenterology and Hepatology, PO Box 9600, C4-P, 2300 RC Leiden (h.w.verspaget@lumc.nl).

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Although the therapeutic efficacy of MSCs in the treatment of perianal fistulas has been shown, no clear therapeutic effect of systemic MSC therapy has been established yet in luminal IBD, despite promising studies in animal models. This could be due to the fact that only a small percentage of injected MSCs engraft in proximity to the inflamed bowel after systemic infusion.[15, 16] Therefore, the goal of our study was to investigate if the efficacy of MSC therapy could be improved by local administration, using endoscopic injections of MSCs in the bowel wall and thereby directly delivering the cells to the site of inflammation. In a clinical setting, this strategy would make it possible to treat only the affected bowel segments by endoscopic MSC injection. Secondly, we wanted to investigate if we could improve the therapeutic properties of MSCs by aggregation of MSCs in spheroids. In the present study, we show a favorable expression profile in MSCs after in vivo and in vitro aggregation and a clinical benefit of local MSC therapy for the treatment of experimental colitis.

METHODS

MSC Isolation, Culture, and Transfection

Green fluorescent protein (GFP)–expressing bone marrow MSCs were isolated from 8- to 12-week-old C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb (kindly provided by the Department of Anatomy, Leiden University Medical Center) and Tg(UBC-GFP)30Scha mice (The Jackson Laboratory, Bar Harbor, ME, USA), which both express GFP in all cells, as described previously.[17] In brief, mice were killed and femurs were removed. The bone marrow was flushed and filtered to remove debris. After centrifugation, bone marrow–derived cells were cultured in α-MEM medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS; Gibco, Gaithersburg, MD, USA), penicillin/streptomycin (Lonza, Verviers, Belgium), and 3 mM L-glutamine (Gibco). Nonadherent cells were removed after 24 hours, and the medium was refreshed every 3–4 days. For tracing experiments, some of the MSCs were transfected with a lentivirus encoding a codon-optimized luciferase construct (pGL4.51, Promega Corporation, Leiden, the Netherlands; kindly provided by Dr. M. Paauwe). The MSCs in the experiments were used between passages 4 and 9.

To confirm that the isolated cells are MSCs, they were stained with anti-CD44-APC, anti-CD29-PECy7, anti-SCA1-APC, anti-CD45-PE, anti-Ter119-V450, anti-CD105-PE, and anti-CD106-PE (all BD Biosciences, San Diego, CA, USA), and the fluorescent signal was measured using the LSR II flow cytometer with Diva Software (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). To test the multipotency of the isolated MSCs, cells were subjected to in vitro osteogenic and adipogenic differentiation assays, as described previously.[17] In brief, to induce osteoblast differentiation, MSCs were cultured with 10 mM β-glycerolphosphate, 50 µg/mL ascorbic acid, and 10 nM dexamethasone for 3 weeks (all Sigma-Aldrich, Zwijndrecht, the Netherlands). Adipogenic differentiation was induced by culturing MSCs in 0.5 mM 3-isobutyl-1-methylxantine, 100 µM indomethacin, 5 µM insulin, and 1 mM dexamethasone for 21 days (all from Sigma-Aldrich). To confirm differentiation into adipocytes, cells were stained with Oil-Red-O (Sigma-Aldrich). To confirm osteogenic differentiation, cells were stained with Alizarin Red for calcium deposition and with Fast Blue for alkaline phosphatase activity (both Sigma-Aldrich).

MSC spheroids were generated by culturing MSCs in 96-well U bottom cell culture plates (Greiner Bio-One BV, Alphen a/d Rijn, the Netherlands) for 24 or 48 hours (1000 cells/well) in full medium containing 0.24% methylcellulose (Sigma-Aldrich). In indicated in vitro experiments, MSCs were stimulated with 500 U/mL recombinant mouse TNF-α (R&D systems, Minneapolis, MN, USA) and/or 40 U/mL recombinant mouse IFN-γ (Biolegend, San Diego, CA, USA) for 24 hours.

Intraperitoneal Aggregation and Endoscopic Treatment of Colitis With MSCs

To investigate in vivo aggregate formation, 2×10^6 MSCs were injected intraperitoneally in C57BL/6Jico mice (Charles River Laboratories, Saint-Germain-Nuelles, France). To trace MSCs in vivo, 100 mg/kg luciferin (Bachem, Bubendorf, Switzerland) was injected intraperitoneally, and mice were imaged on the IVIS Lumina-II (Caliper Life Sciences, Hopkinton, MA, USA). After 5 days, mice were killed, and MSC aggregates were collected in PAXgene RNA solution (Qiagen, Hilden, Germany) for RNA isolation or in 4% formalin for immunohistochemical analysis.

To induce colitis, female C57BL/6Jico mice were given 2.5% dextran sulphate sodium (DSS; MW 36000-50000kDA; MP Biomedicals, Brussels, Belgium) in their drinking water for 7 days. The DSS was refreshed every other day. Body weight was measured daily, and at day 5, mice were treated with 2×10^6 MSCs or 2000 MSCs in spheroids (equivalent to 2×10^6 cells) in 200 µL phosphate buffered saline (PBS) (n = 18–19 mice/group). In the control group, mice received 200 µL PBS. MSCs were injected in 4 spots (50 µL/spot), 1 in every quadrant of the distal bowel wall, with a 30-G needle during endoscopy using a high-resolution miniaturized colonoscope system (Karl Storz, Tuttlingen, Germany). Mice were killed 4 (n = 10–11/group) or 6 days after treatment (n = 8/group); then colons were opened longitudinally, and the macroscopic disease score[17, 18] was established for each mouse. This score was composed of the presence of loose stool (0–3), visible fecal blood (0–3), and macroscopic inflammation (0–3), resulting in a maximum score of 9. Bioluminescent imaging was performed in 4 mice to show the localization of injected MSCs.

Flow Cytometry

Lamina propria colonic cells were obtained by incubation of colonic tissue in 5 mM EDTA (Merck, Darmstadt,

Germany) and 1 mM dithiothreitol (Sigma-Aldrich) for 20 minutes, followed by matrix digestion in Liberase TL solution (Roche, Indianapolis, IN, USA) for 30 minutes at 37°C. Single-cell suspensions were measured using the LSR II flow cytometer, as described above.

RNA Isolation and Quantitative Polymerase Chain Reaction

RNA was isolated from cells or tissue using the NucleoSpin RNA Kit (Macherey Nagel, Düren, Germany) according to the manufacturer's protocol. RNA concentration and purity were determined using NanoDrop 3300 (ThermoFisher Scientific, Waltham, USA). cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to manufacturer's instructions. Quantitative polymerase chain reactions (qPCRs) were carried out using SYBR Green (Biorad, Hercules, CA, USA) and primers for GFP, CD45, stromal cell–derived factor (SDF)–1, C-X-C chemokine receptor type 4 (CXCR4), C-C motif ligand chemokine ligand (CCL)–2, CD200, vascular endothelial growth factor (VEGF)–A, VEGF receptor (VEGFR)–1, hepatic growth factor (HGF), interferon (IFN)-γ, indolamine 2,3-dioxygenase (IDO), interleukin-18-binding protein (IL-18bp), intracellular adhesion molecule (ICAM)–1, epidermal growth factor (EGF), transforming growth factor (TGF)–β1, TGF-β2, TGF-β3, cytokeratin-20 (CK20), leucine-rich repeat–containing G-protein coupled receptor 5 (LGR5) (all Invitrogen custom primers, Carlsbad, CA, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Qiagen; Supplementary Digital Content Video 1, primer sequences). cDNA samples were subjected to 40 cycles of real-time PCR analysis, as previously described.[19] All values were normalized for cDNA content by GAPDH expression.

Cytokine Measurement

Colonic tissue was homogenized in RIPA buffer [150 mM NaCL, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 2 mM EDTA, 1 mM NaVO4, 10 mM NaF, and 1 mM sodium orthovandate (BDH Laboratory, Poole Dorstet, UK)] using TissueLyser LT (Qiagen). Total protein content was determined using a BCA protein assay kit (ThermoFisher Scientific). Total levels of VEGF and TGF-β1 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) Duosets (R&D systems), as described before.[20] Briefly, wells were incubated overnight with capture antibody. After blocking, samples were incubated for 2 hours, followed by biotinylated antibodies and color development (Color reagent pack, R&D Systems). Absorption was measured at 450 nm. Additional cytokine levels in colon homogenates and blood were obtained using U-PLEX assay (Mesoscale, Rockville, MD, USA). All cytokine levels in colon homogenates were corrected for total protein amount. Serum amyloid A (SAA) levels were measured using a commercial ELISA kit according to the manufacturer's instructions (Tridelta Development Ltd, Maynooth, Ireland).

Immunoblot Analysis

Immunoblot analyses were performed as described previously.[19] Briefly, equal amounts of proteins were separated on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. Subsequently, separated proteins were transferred to polyvinylidine fluoride membranes (Whattman, Dassel, Germany). Nonspecific binding was blocked in 5% milk in tris-buffered saline with 0.05% Tween-20 (TBST). Blots were incubated overnight with primary antibodies: goat anti-CD200 (R&D Systems), rat anti-IDO (BioLegend), or rat anti-CXCR4 (BD Biosciences). Bands were visualized using horseradish peroxidase conjugated secondary antibodies and the enhanced chemiluminescence detection kit (Roche, Indianapolis, IN, USA). Blots were stripped and reprobed with mouse anti-GAPDH antibody (ThermoFisher Scientific) as a loading control. Densitometric analysis was performed with Image Lab Biorad software, version 5.2.

Immunohistochemical Analysis

Hematoxylin and eosin staining of the colons was used to visualize the microscopic signs of inflammation in the distal colon. The IBD histology score consisted of muscularis mucosa thickness (0–3), immune cell infiltrate (0–3), crypt architecture (0–3), and presence of goblet cells (0–1). Scoring was performed blinded to treatment by 2 individual observers.

Immunohistochemical stainings were performed as described before[17] using primary antibodies against GFP (goat; Rockland, Limerick, Ireland), Ki67 (rabbit; Merck), cleaved-caspase 3 (rabbit; Cell Signaling Technologies, Boston, MA, USA), CD3 (rabbit; Dako, Santa Clara, CA, USA), Foxp3 (rat; ThermoFisher Scientific), Ly6G (rat; Biolegend), F4/80 (rat; BD Biosciences), and MBP (rat; kindly provided by Dr. Lee, Mayo Clinic, Rochester, MN, USA). In brief, sections were deparaffinized, rehydrated, and incubated in 0.3% H_20_2/methanol for 20 minutes to block endogenous peroxidase. Antigen retrieval was performed using 0.01 M sodium citrate (pH 6.0), and primary antibodies were incubated overnight. The next day, slides were incubated for 60 minutes with biotinylated secondary antibody (Dako), followed by incubation with Vectastain (Vector Laboratories, Burlingame, CA, USA) and DAB (Dako) staining for 10 minutes. All slides were counterstained with hematoxylin. For Foxp3-staining, slides were blocked with Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCL, 0.25% gelatin, 0.05% Tween-20, pH 8). For MBP staining, slides were incubated with Pepsin Solution Digest-all (Invitrogen) for antigen retrieval. The amount of MBP-positive cells in the colon was evaluated by 2 independent researchers blinded to treatment groups using a score ranging from 0 to

4 for both the mucosal and submucosal layers. To evaluate Foxp3 staining, 3 microscopic images of the distal colon were made with 10× magnification, and positive cells were counted in the mucosal and submucosal layers using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between the 2 groups were calculated using the Student *t* test or Mann-Whitney test, whereas for more than 2 groups, one-way analysis of variance or Kruskal-Wallis tests were used, followed by Dunnett's multiple comparison test. Pearson's correlation test was used to analyze the correlation coefficient. All analyses were performed using GraphPad Prism software. *P* values ≤0.05 were considered statistically significant.

Ethical Considerations

All animal experiments were approved by the animal ethical committee of the Leiden University Medical Center. Mice were housed in individually ventilated cages and had access to drinking water and food ad libitum.

RESULTS

MSC Isolation and Characterization

MSCs were isolated from the bone marrow of GFP-expressing mice, and expression of mesenchymal stem cell precursor markers on the cells was confirmed using flow cytometry. MSCs showed expression of GFP, CD29, CD44, SCA-1, CD105, and CD106, whereas hematopoietic marker CD45 and erythrocyte marker TER-119 were absent (Supplementary Fig. 1A). Next, to confirm their multipotency, cells were subjected to differentiation assays, showing their ability to differentiate into adipocytes and osteoblasts (Supplementary Fig. 1B).

Intraperitoneally Injected MSCs Aggregate Into Spheroids and Show Upregulation of Immune-Regulating and Tissue Repair Genes

To analyze how intraperitoneally injected MSCs behave, the expression profiles of the in vivo formed aggregates were compared with MSCs from in vitro monolayer cultures. Five days postinjection of MSCs in single-cell suspension, abdominal MSC aggregates could be detected, which appeared as small clusters with a white-yellowish color (Fig. 1A). Bioluminescent imaging confirmed the presence of luciferase-expressing MSCs in these spots (Supplementary Fig. 2A). These aggregates could be isolated and subsequently cultured in vitro. Spindle-shaped GFP-positive cells were present in culture after 5 days (Fig. 1B). In mice with DSS- and trinitrobenzene sulfonic acid solution (TNBS)–induced colitis, similar MSC aggregates were formed after intraperitoneal injection of MSCs (Supplementary Fig. 2B). MSC aggregates directly isolated from healthy mice, without culturing, were used for immunohistochemistry and qPCR. GFP immunohistochemistry and qPCR confirmed their origin (Fig. 1C and Supplementary Fig. 2C). Low levels of CD45 RNA could also be detected in the MSC aggregates by qPCR (Supplementary Fig. 2D). To investigate the expression pattern of in vivo aggregated MSCs, RNA from the isolated MSC spots was evaluated by qPCR for genes involved in immunomodulation and tissue repair and compared with the identical MSCs, which were injected. Significantly higher RNA expression levels of the anti-inflammatory cytokine TGF-β1 and CD200, a transmembrane glycoprotein that stimulates the differentiation of T cells to Treg cells,[21] were found in the in vivo aggregates compared with the in vitro MSCs. Also, RNA expression of factors involved in tissue repair, like VEGF and HGF, were upregulated in the in vivo MSC aggregates. CXCR4 gene expression, important for the homing of MSCs toward damaged tissue,[22] was also significantly upregulated in the isolated aggregates. SDF-1, a ligand for CXCR4 and CCL-2 that is important for the recruitment of T cells, and VEGFR-1 expression were similar between in vitro MSCs and in vivo MSC aggregates (Fig. 1D). No correlation was found between the expression of these genes and the amount of CD45 RNA in these spots, indicating that the expression is not due to the low but detectable amount of contaminating CD45+ cells (data not shown). These results indicate that MSCs after in vivo aggregation show a favorable RNA expression profile (anti-inflammatory and pro-regenerative) for the treatment of colitis.

MSCs Aggregated in Spheroids In Vitro Mimic Their In Vivo Counterparts

To confirm the altered expression pattern of the aggregated MSCs in vitro, GFP-expressing MSCs were cultured in 3-dimensional spheroids (1000 MSCs/spheroid). After 48 hours, MSCs aggregated into single spheroids in every well (Supplementary Fig. 3A). These MSC spheroids showed high expression of the proliferation marker Ki-67 and low levels of the apoptotic marker cleaved caspase–3 (Fig. 2A), indicating that cells were viable and proliferating. Next, MSCs cultured in monolayer were compared with MSCs cultured in spheroids for 24 and 48 hours on RNA expression levels for 12 genes involved in immunomodulation, tissue repair, and cell trafficking. Expression levels of anti-inflammatory TGF-β1 and TGF-β2 were significantly higher in the spheroids compared with MSCs cultured in monolayer, whereas TGF-β3 expression did not differ (Fig. 2B; Supplementary Fig. 3B). In addition, CD200 showed an upregulation in MSC spheroids, corresponding to what was observed in the in vivo aggregates. CCL-2 showed a downregulation in spheroids compared with monolayer cultured MSCs. In contrast, genes involved in tissue repair, such as HGF, VEGF, and VEGFR-1, showed an upregulation when MSCs were cultured in spheroids (Fig. 2C). CXCR4 RNA

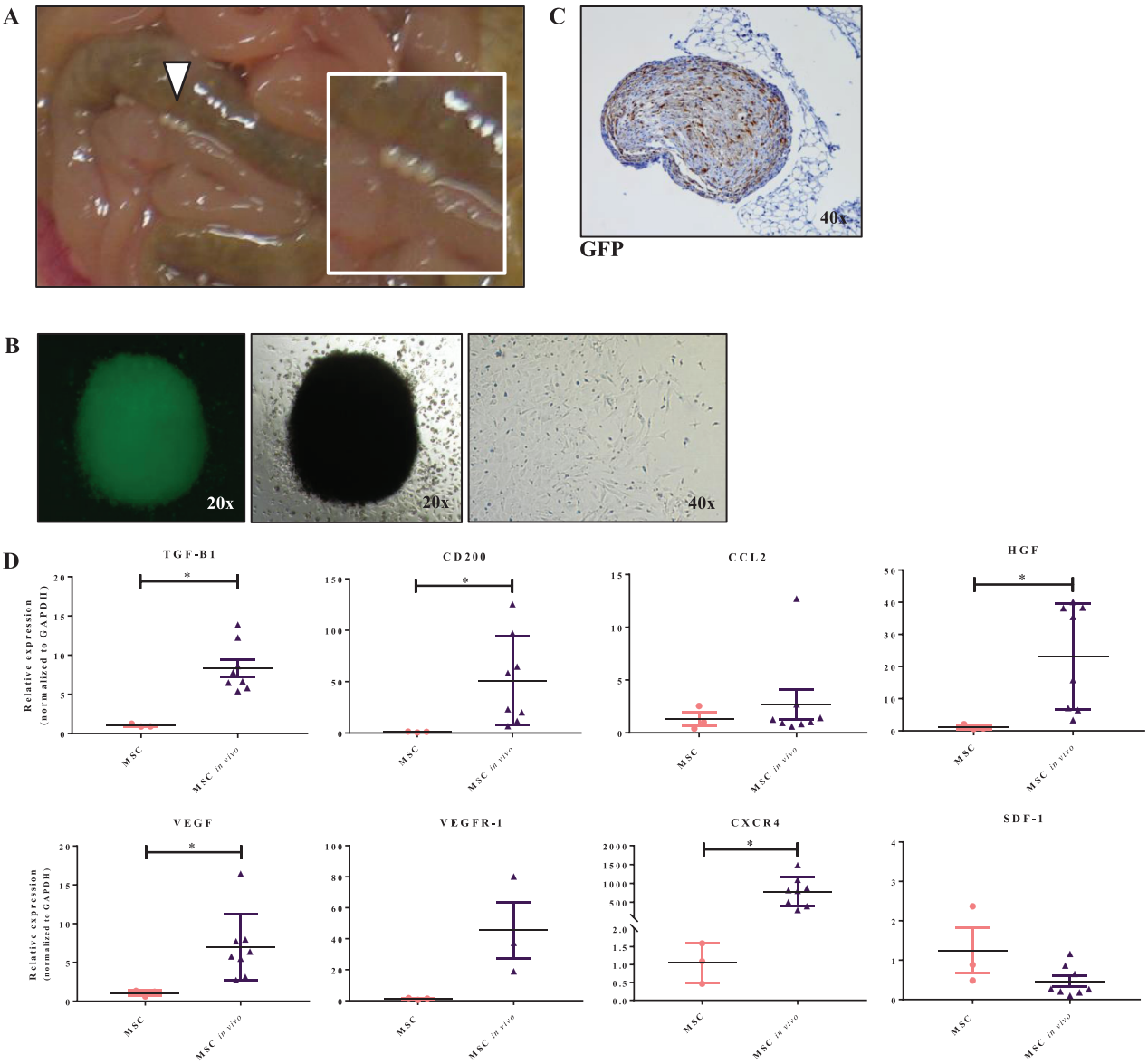

FIGURE 1. MSC aggregates, formed after intraperitoneal injection, show upregulation of immune regulatory and tissue repair genes. A, MSC aggregates in the murine abdomen 5 days after injection. B, Immunofluorescent and bright field images of MSC aggregates in culture, confirming the presence of GFP-positive MSCs. Spindle-shaped cells grew from the aggregate after 5 days of culture (right picture). C, GFP immunohistochemistry confirming the presence of MSCs in the aggregates. D, qPCR comparing isolated RNA from in vitro MSCs (n = 3) with abdominal in vivo MSC aggregates (n = 3–8). RNA expression levels were determined in triplicate and normalized to GAPDH. Relative expression of MSCs in vivo was calculated compared with in vitro MSCs (set to 1). Data are expressed as mean ± SEM. $^{*}P \leq 0.05$.

expression was also strongly increased in the spheroids, whereas SDF-1 was slightly downregulated (Fig. 2D).

To confirm that increased RNA expression results in increased protein expression, CD200, CXCR4, TGF-β1, and VEGF protein levels were evaluated using immunoblot or ELISA. These data indeed confirmed the increased expression in spheroids (Fig. 2E and F). Overall, these results indicate that in vitro aggregated MSCs in spheroids have a more favorable expression pattern, similar to in vivo aggregated MSCs, for the alleviation of colitis.

Endoscopically Injected MSCs and MSC Spheroids Alleviate DSS-Induced Colitis

The therapeutic effects of in vitro cultured MSCs in spheroids and in monolayer were investigated in a DSS mouse model for colitis. To increase their potential effectiveness, 2×10^6 GFP-expressing MSCs cultured in single-cell suspension or in spheroids (1000 MSCs/spheroid) were injected during endoscopy in 4 spots in the inflamed distal colon at day 5 of DSS administration. Successful injections during endoscopy were confirmed

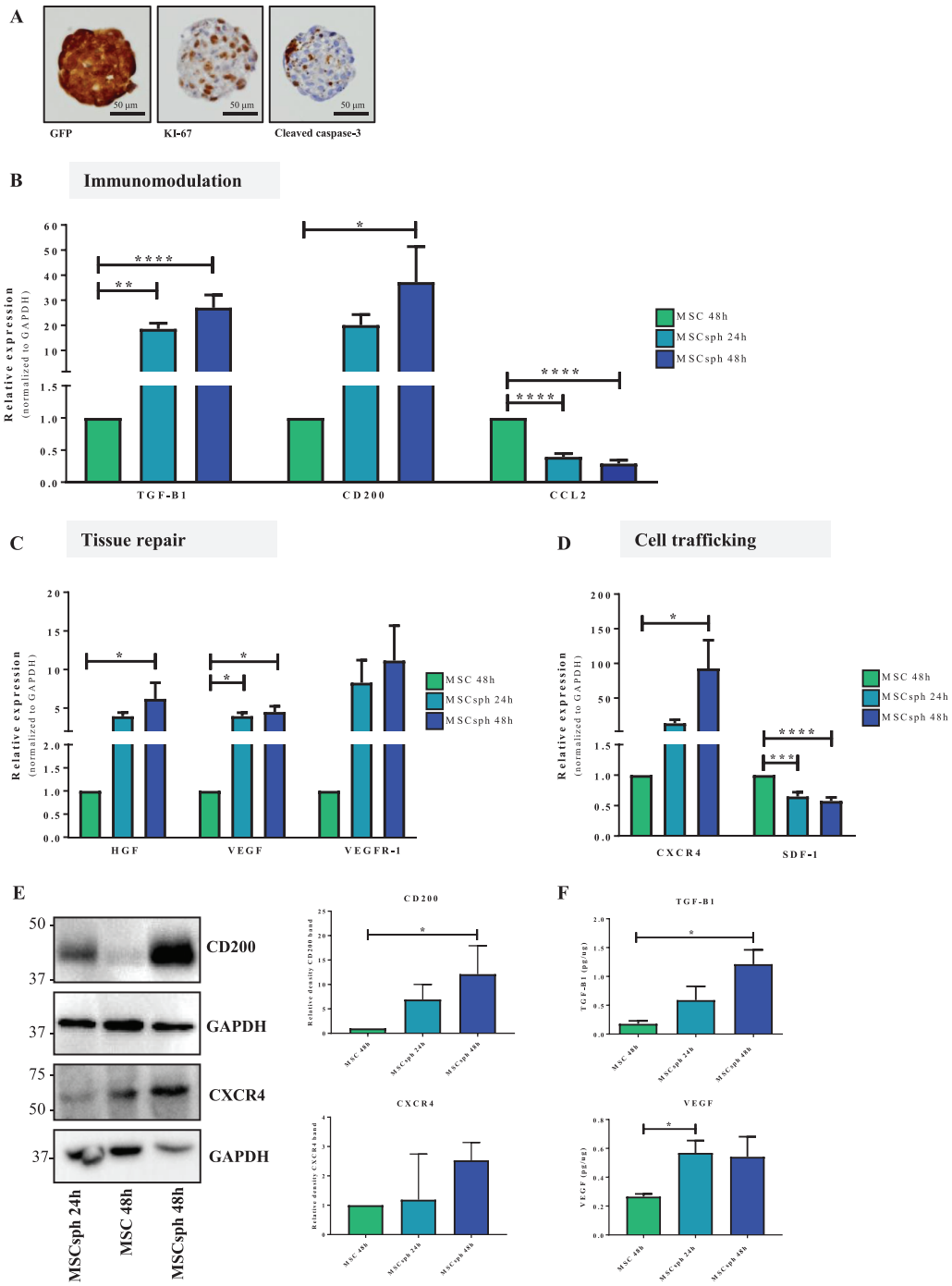

FIGURE 2. In vitro aggregation of MSCs in spheroids shows differential expression of genes involved in immunomodulation, tissue repair, and cell trafficking. A, Immunohistochemistry on embedded in vitro MSC spheroids for GFP, KI-67, and cleaved caspase-3. B–D, qPCR comparing expression levels of MSCs cultured in monolayer for 48 hours (MSC 48h), in spheroids for 24 (MSCsph 24h) and 48 hours (MSCsph 48h) for genes involved in immunomodulation (B), tissue repair (C), and cell trafficking (D). The data are the result of 6 independent experiments in triplicate. Expression levels were normalized to GAPDH and compared with MSC 48h. E, Immunoblot analysis for CD200 and CXCR4. GAPDH was used as a loading control. A representative immunoblot from 3 independent experiments is shown. Quantifications are corrected for GAPDH. F, TGF-β1 and VEGF levels in cell lysates of MSC 48h, MSCsph 24h, and MSCsph 48h measured by ELISA. Experiments were performed in triplicate. All data are expressed as mean ± SEM. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

by the observation of a blister in the bowel wall (Fig. 3A; Supplementary Digital Content Video 1, which demonstrates endoscopic injection in the bowel wall). Bioluminescence imaging using luciferase-expressing MSCs was performed. Both MSCs and MSC spheroids were detectable up to 6 days after endoscopic injection in the distal colon (Fig. 3B), indicating successful injection and survival of the cells.

Mice treated with MSCs or MSC spheroids showed a higher relative body weight at days 9, 10, and 11 compared with the PBS-treated group (Fig. 3C). Four or 6 days after start of treatment, the mice were killed and the colons were evaluated. In agreement with the significantly higher body weight after MSC therapy, the macroscopic disease score was lower in mice treated with local MSCs and MSC spheroids (Fig. 3D) at day 6. For tracing MSCs, part of the lamina propria cells of the colon was isolated for flow cytometric analysis of GFP. Sixteen out of 21 (76%) mice injected with MSCs or MSC spheroids had GFP-positive cells in the lamina propria 4 days after treatment, whereas no GFP-positive cells were observed in the control mice (Fig. 3E).

Histological analysis revealed a lower IBD histology score in MSC-treated mice compared with the control mice (Fig. 4A). To confirm the presence of MSCs in the bowel wall, sections were stained for GFP. These data show the presence and engraftment of MSCs until the end of the experiment, confirming the bioluminescent imaging and flow cytometry

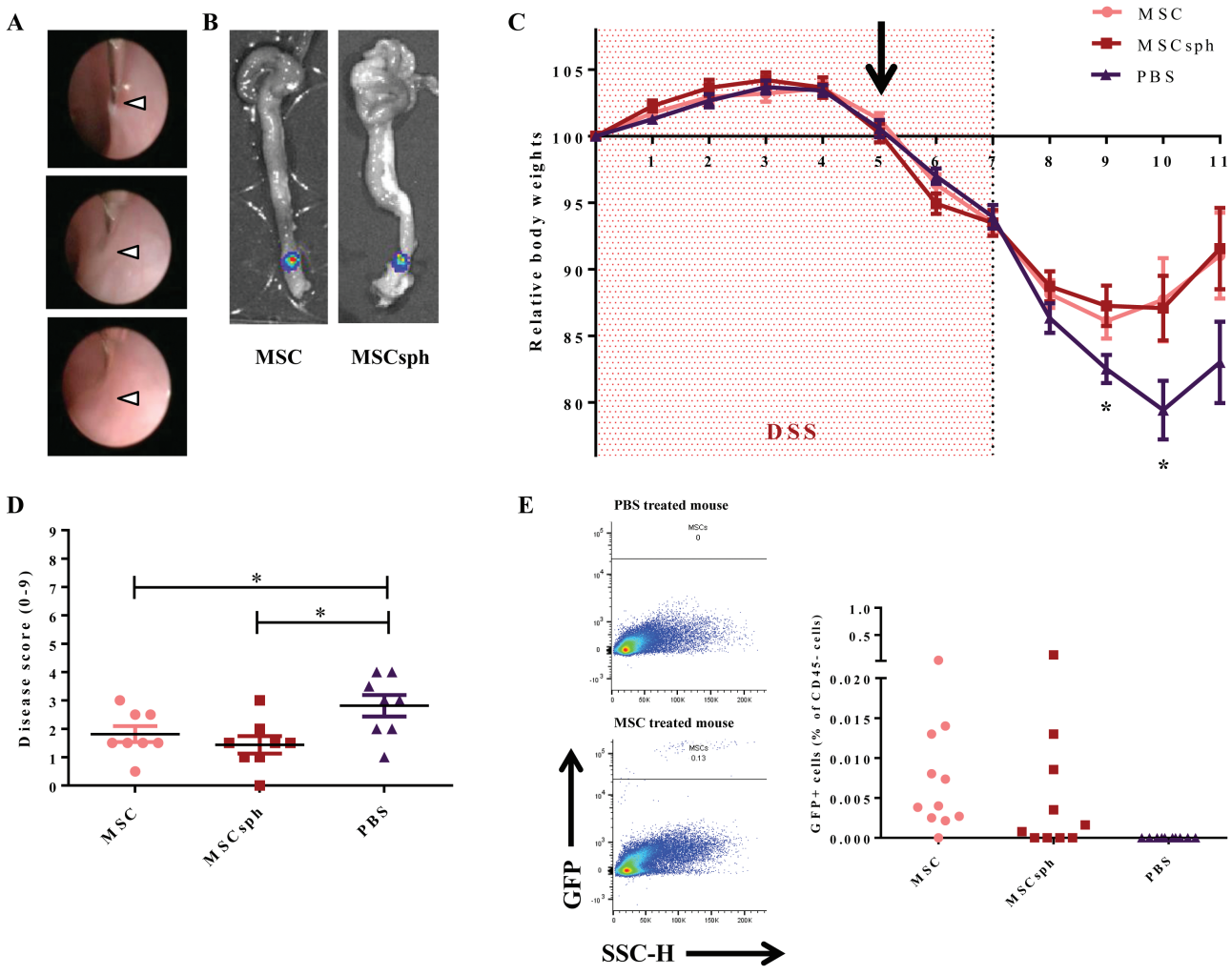

FIGURE 3. Endoscopic injections of MSCs alleviate DSS-induced colitis. A, Sequential pictures of an endoscopic injection of MSCs in the bowel wall, showing the appearance of a submucosal blister. B, Bioluminescent images of colons 6 days after endoscopic injections with luciferase-expressing MSCs or MSC spheroids (MSCsph), showing the presence of MSCs in the distal colon. C, Relative body weights of the mice during the experiment (n = 18–19 mice/group). Mice were endoscopically treated at day 5 and killed at day 9 (n = 10–11/group) or day 11 (n = 8/group). A significantly higher body weight was observed in both MSC treatment groups at days 9 and 10 compared with the PBS-treated group. D, A higher macroscopic IBD disease score, based on the presence of loose stool, fecal blood, and macroscopic signs of inflammation, was observed in PBS-treated mice. E, Flow cytometry analysis of lamina propria cells isolated from the colon confirmed the presence of GFP-positive MSCs in MSC-treated mice. Data are expressed as mean ± SEM. $^{*}P \leq 0.05$.

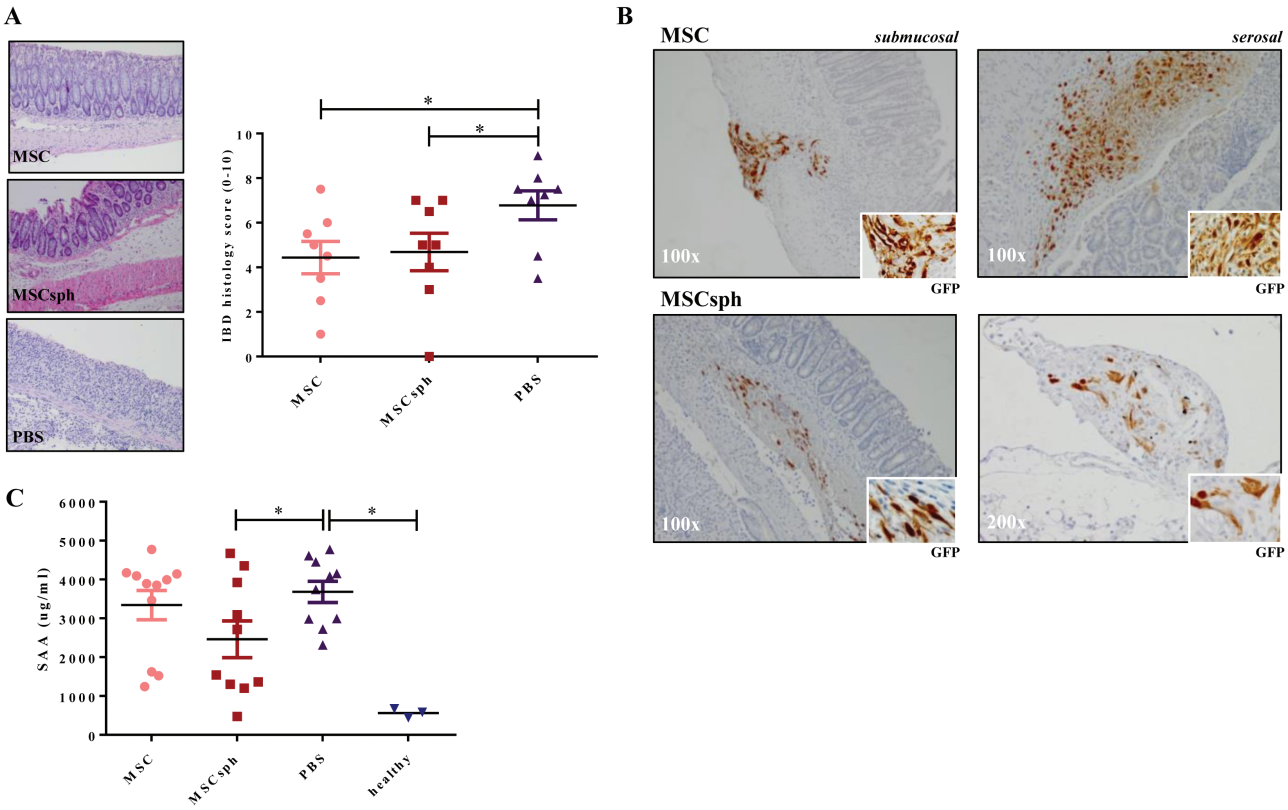

FIGURE 4. MSCs in the submucosal and serosal layer of the bowel lower microscopic signs of inflammation. A, Lower IBD histology scores (muscularis mucosa thickness, immune cell infiltrate, crypt architecture, and presence of goblet cells) were observed in mice treated with MSCs. B, MSCs were detected in both the submucosal and serosal layers of the colon up to 6 days after endoscopic injection using GFP staining. Magnification ×100 or ×200, inserts higher magnification. C, The systemic inflammation marker SAA was strongly elevated in mice with DSS-induced colitis. Treatment with MSC spheroids significantly lowered the SAA levels in the blood. Samples were measured in duplicate. Data are expressed as mean ± SEM. $^{*}P \leq 0.05$.

data. Clusters of GFP-positive cells were found both in the submucosal area and at the serosal side of the colon in mice injected with MSCs and MSC spheroids 6 days after injection (Fig. 4B). Circulating SAA levels, a general inflammation marker, were determined as well. Our data show high upregulation of SAA in mice with DSS-induced colitis compared with healthy mice. Lower serum SAA levels were detected in mice treated with MSC spheroid therapy compared with the control group (Fig. 4C). Taken together, these data show that locally applied MSCs and MSC spheroids are both able to ameliorate DSS-induced colitis and show similar clinical effects, including improvement in the macro- and microscopic IBD score.

T Cells and Macrophages Surround MSCs Injected Into the Bowel Wall

As MSCs and MSC spheroids can modulate immune cells, colonic tissue sections were analyzed for various subtypes of immune cells. CD3, Foxp3, Ly6G, and F4/80 stainings were performed to identify, respectively, T cells, Treg cells, neutrophils, and macrophages near injected MSCs (Fig. 5A). A substantial number of CD3-positive cells were found in proximity of MSC clusters. In contrast, only a few Foxp3-positive Treg cells were seen close to the MSCs. No differences were found between the number of Treg cells in the distal colon of the different treatment groups (Fig. 5B). Also, the number of eosinophils was not affected by MSC therapy (Fig. 5C). A few Ly6G-positive neutrophils could also be detected in close proximity to the MSCs. F4/80+ macrophages, on the other hand, were well detectable near the MSCs. No differences between the amount and type of immune cells surrounding MSCs injected in monolayer or as spheroids was observed. These data indicate that in DSS-induced colitis, T cells and macrophages are the predominant cell types surrounding MSCs in the bowel wall. Ki67 staining showed normal crypt proliferation in the 3 treatment groups (Supplementary Fig. 4A), whereas no ectopic crypt formation was detected. No differences between the RNA expression of the stem cell marker LGR5 were found between MSC- and PBS-treated colon samples. In contrast, higher CK20 expression, a marker for differentiated epithelial cells, could be detected in the colons of mice treated with MSC spheroids, suggesting more epithelial repair in these mice (Supplementary Fig. 4B).

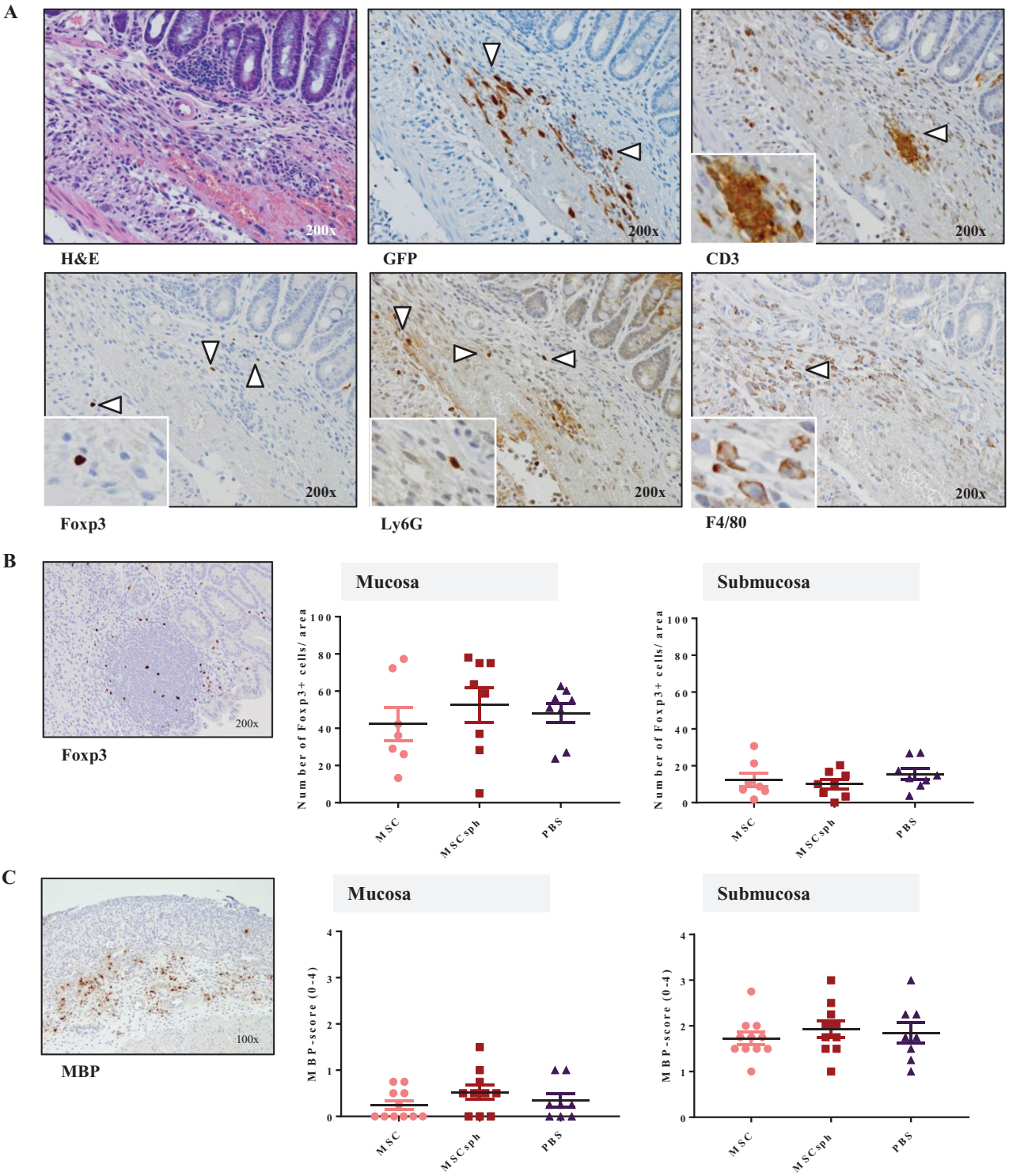

FIGURE 5. CD3- and F4/80-positive cells surround MSCs injected into the bowel wall. A, Immunohistochemical stainings for GFP reveal the localization of MSCs after endoscopic injection. CD3, Foxp3, Ly6G, and F4/80 stainings show the presence of, respectively, T cells, Treg cells, neutrophils, and macrophages in proximity to the MSCs. White arrowheads indicate positive cells. Magnification ×200, inserts higher magnification. B/C, Immunohistochemical staining with anti-Foxp3 (B) to detect Treg cells and anti-MBP (C) to detect eosinophils. Foxp3-positive nuclei were quantified using ImageJ. The percentage of MBP-positive cells was determined by 2 observers blinded to treatment. No differences in the number or percentage of positive cells in the mucosa (left panel) or submucosa (right panel) were detected between the treatment groups. Data are expressed as mean ± SEM.

Local MSC Therapy Induces IFN-γ, IDO, and IL-10 Upregulation

To assess the immunomodulatory effects of local MSC therapy on DSS-induced colitis, cytokines in serum and colon homogenates were measured. The levels of the pro-inflammatory cytokines IL-17, IL-6, IL-8, and TNF-α were elevated in the colons of mice receiving DSS in their drinking water. MSC therapy showed a consistent tendency of reduced levels of these cytokines in the colon 4 days after treatment (Fig. 6A). No differences in VEGF or TGF-β levels were found in the colons of mice injected with MSCs or MSC spheroids (data not shown). Six days after treatment, colon homogenates showed comparable IL-17, IL-6, IL-8, and TNF-α levels between the 3 treatment groups (data not shown). Higher levels of the anti-inflammatory cytokine IL-10 were found in the serum of mice treated with MSCs, particularly in the MSC spheroid group (Fig. 6B). Serum IFN-γ levels were below the detection limit in all PBS-treated mice, but in 7 out of 10 MSC-treated mice, detectable levels were observed (Fig. 6C). Moreover, in colon homogenates, higher IFN-γ levels were observed in mice that received MSC therapy (Fig. 6C), although this did not reach statistical significance. IFN-γ is known to upregulate a series of anti-inflammatory IFN-γ-inducible genes in immune cells, DSS-damaged epithelial cells, and MSCs.[23] These genes include the anti-inflammatory proteins IDO and IL-18bp. Our data indeed show upregulation of RNA levels of IDO and IL-18bp in the colons of MSC-treated mice (Fig. 6D). The upregulation of IDO in the colons of MSC-treated mice was confirmed by immunoblot analysis (Fig. 6D). Interestingly, the expression levels of both IDO ($R = 0.859$) and IL-18bp ($R = 0.819$) were directly correlated with the level of IFN-γ in the colon homogenates 6 days after treatment (Fig. 6E). The same trend was observed 4 days after treatment, with a higher IFN-γ level corresponding with a higher IDO expression (Supplementary Fig. 5A and B). To investigate if the injected MSCs are indeed capable of inducing IDO production after stimulation with cytokines, we performed additional in vitro experiments. MSCs stimulated with IFN-γ showed a remarkable upregulation of IDO expression, which could even be further enhanced by combining IFN-γ with TNF-α stimulation (Fig. 6F). Taken together, these results suggest that local MSC therapy can induce an anti-inflammatory response via activation of the IFN-γ, IDO, and IL-10 pathways.

DISCUSSION

In this study, we have shown that local endoscopic administration of MSCs is feasible and effective in mice with experimental colitis. Furthermore, we have demonstrated that MSCs in aggregates (in vivo or in vitro) express increased levels of immunosuppressive and tissue-regenerative factors, like TGF-β1, CD200, HGF, and VEGF. Our data show that generating in vitro spheroids is not required to obtain similar clinical effects.

MSC therapy is a promising novel approach for the treatment of perianal fistulas in Crohn's disease, based on recent clinical data.[8, 9] Besides highlighting the feasibility and safety of local MSC therapy in IBD, these trials also show a response rate of up to 80% in patients with perianal fistulas refractory to standard treatment. Potentially, MSC therapy could also be beneficial for the treatment of luminal IBD, but several improvements with regard to homing and immune-regulatory properties should be made to enhance their efficacy. Our results demonstrate that aggregation of MSCs in spheroids enhances the expression of at least TGF-β1, CD200, HGF, VEGF, and CXCR4. Upregulation of CXCR4, in addition to the upregulation of anti-inflammatory tumor necrosis factor–inducible–6 (TSG-6), stanninocalcin-1, and leukemia inhibitory factor, was also observed by others in human MSCs cultured in spheroids.[24] These spheroids were also found to be more effective in suppressing inflammation in a mouse model of zymosan-induced peritonitis, when compared with monolayer cultured MSCs.[24] Surprisingly, our results show that in experimental colitis, administration of MSCs aggregated in spheroids in vitro did not result in better clinical responses than single-cell MSCs. Although in vitro expression profiles show an upregulation of VEGF and TGF-β1 in MSC spheroids, similar levels were found in the bowel wall of mice after endoscopic injection with MSCs or MSCs aggregated in spheroids. These data together suggest that in an acute disease model, high cytokine levels are sufficient to activate the anti-inflammatory and pro-regenerative features of MSCs and polarize MSCs in a favorable phenotype for treating autoimmune diseases.[25] However, this might imply that in chronic conditions it could still be critical to pre-activate the MSCs in vitro by culturing in spheroids or prestimulating them with pro-inflammatory cytokines. Another explanation for the comparable results between MSCs and MSC spheroids in vivo is the spontaneous aggregation of MSCs that was observed after injection in the bowel wall.

Detection of MSCs after administration has been proven difficult, and most studies show that only a small number of MSCs, if any, will reach the inflamed tissue.[15, 17, 26] Here we could show that MSCs injected as single cells or in spheroids are detectable until the end of the experiment, 6 days after injection. Moreover, we could show that the MSCs are localized in the submucosal and serosal areas and colocalize with T cells and macrophages. In a previous study, MSCs injected intraperitoneally in mice with experimental colitis were also found to be surrounded by macrophages and T cells, beside some B cells and Ly6G-positive cells.[26] The close proximity of endoscopically injected MSCs to colonic immune cells gives them the opportunity to modify these cells directly through cell-to-cell contact and indirectly through the production of short-living cytokines, like TGF-β1 and CD200.

Several cytokines have been proposed to mediate the regenerative and anti-inflammatory features of MSCs. Manieri et al.[27] recently showed that endoscopically applied MSCs could promote intestinal repair and angiogenesis in intestinal wounds, mainly by short-living factors, like VEGF, one of the factors that was upregulated by aggregation into spheroids.

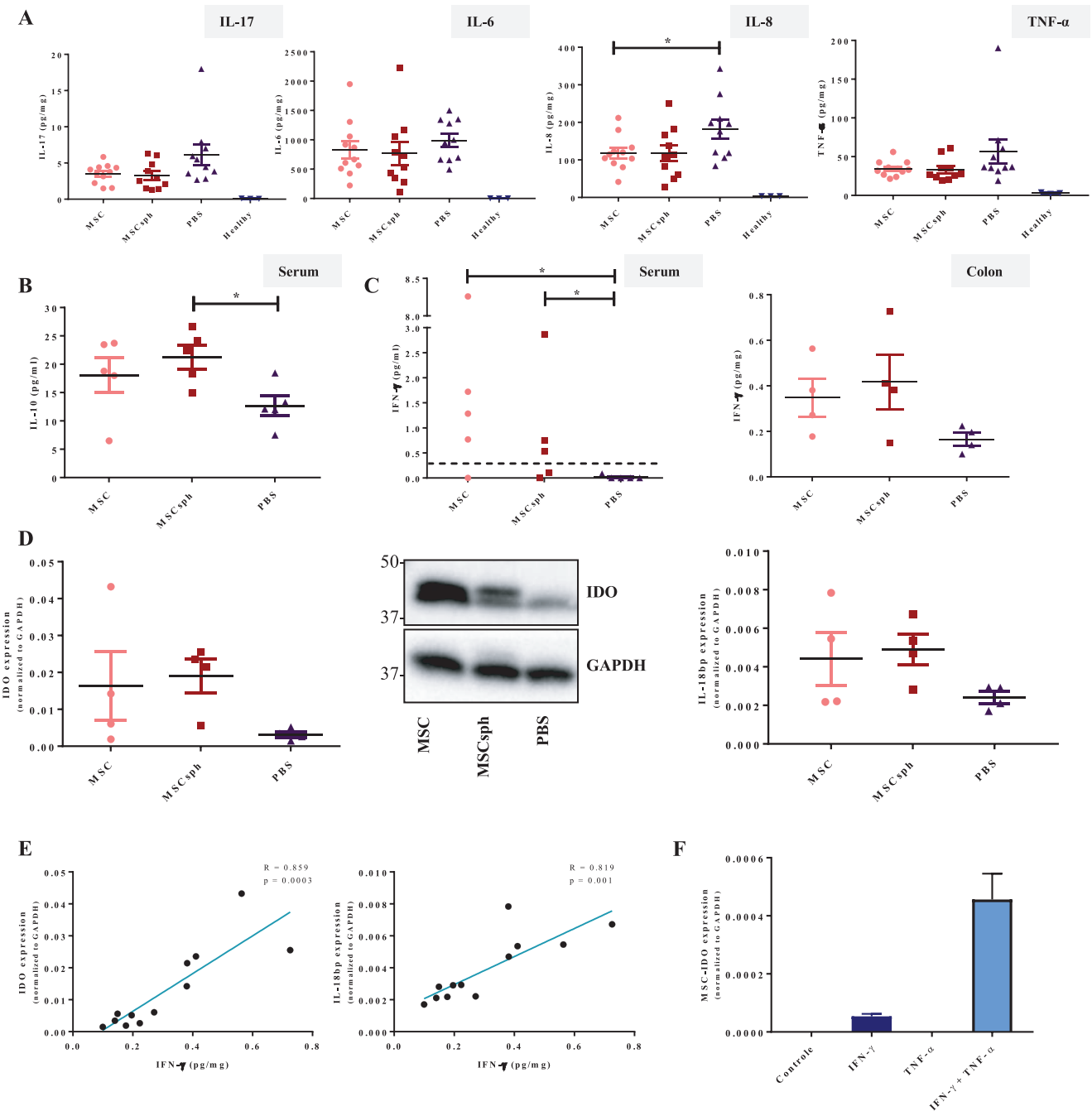

FIGURE 6. Endoscopic injections of MSCs alter cytokine and enzyme production. A, Cytokine levels of IL-17, IL-6, IL-8, and TNF-α in the colon were upregulated after DSS-induced colitis. Local MSC therapy significantly reduced IL-8 levels. B, IL-10 levels in the serum were significantly higher in mice treated with MSC spheroids compared with PBS. C, IFN-γ was only detectable in the serum of mice treated with MSCs or MSC spheroids (left panel). IFN-γ levels were also higher in colon homogenates after MSC therapy (right panel). D, RNA expression levels of IDO (left panel) and IL18bp (right panel) were elevated in MSC-treated mice 6 days after MSC therapy. Higher RNA expression levels for IDO after MSC therapy were confirmed at the protein level by immunoblot (middle panel). E, IFN-γ levels in the colon were significantly correlated with IDO and IL-18bp expression levels ($R = 0.859$ and 0.819, respectively; $P \leq 0.001$). F, MSCs were stimulated in vitro for 24 hours with IFN-γ, TNF-α, or a combination of both, and IDO expression was determined. IFN-γ stimulation induced IDO expression, which was further enhanced by the addition of TNF-α. Data are expressed as mean ± SEM. $^{*}P \leq 0.05$.

Sala et al.[26] reported TSG-6 to be responsible for the clinical effect of intraperitoneally injected MSCs in experimental colitis. Our data indicate that not a single pathway, but the interplay between several pathways is most likely responsible for the anti-inflammatory effects of MSCs. This will probably also depend on the disease model, host, and donor. Our study shows that the pathways of IFN-γ, IDO, and IL-10 are all involved in the anti-inflammatory mechanisms of local MSC therapy in acute colitis. No significant differences in the levels of IL-17, IL-6, and TNF-α in the colon between MSC-treated mice and controls were observed, which could imply that these cytokines correlate with resolution of inflammation rather than being directly related to MSC therapy. However, higher levels of IFN-γ were observed in MSC-treated mice. The role of IFN-γ in experimental colitis and MSC therapy is complex. IFN-γ is known for both its pro-inflammatory and anti-inflammatory effects, but, especially at early stages of inflammation, IFN-γ has homeostatic functions by stimulating Paneth cells to release antimicrobial peptides[28] and modulating anti-inflammatory molecules like IDO and IL-18bp.[23] Important with regard to IBD is that IFN-γ is capable of suppressing Th17 cells and thereby restoring the balance between Th17 and Th1 cells.[29] In line with our observations, in a mouse model of rheumatoid arthritis, enhanced levels of IFN-γ were measured in the spleens of mice treated with combined MSC and type I regulatory T-cell (Tr1) therapy compared with mice treated with Tr1 therapy alone.[30] IFN-γ levels strongly influence the suppressive effect of MSCs by inducing IDO production. Recently, the importance of IDO in the working mechanism of MSCs in IBD was revealed by showing that MSCs dampen the inflammatory response of mucosal T cells in Crohn's disease through the upregulation of IDO activity.[31] MSCs are known to stimulate certain cell types, like T cells and macrophages, to produce IL-10, through the production of IDO or prostaglandin E2.[32] Previously, it was found that human MSCs are more capable than murine MSCs in upregulating IDO.[33] However, in the current study, we show that murine MSCs are also able to increase their IDO expression after IFN-γ stimulation, and even further after the addition of other cytokines next to IFN-γ. Taken together, these data indicate that local MSC therapy alleviates experimental colitis, possibly through the elevation of IFN-γ, leading to increased IDO production by MSCs and eventually IL-10 production by immune cells.

In conclusion, our data show the potency of local MSC therapy for the treatment of luminal IBD. Endoscopically applied MSCs can be used as a novel application route for luminal IBD with substantial advantages compared with systemic application. The next step will be to determine the clinical efficacy of local MSC therapy in patients with active proctitis.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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