Stromal cells in inflammatory bowel disease : perspectives of local mesenchymal stromal cell therapy

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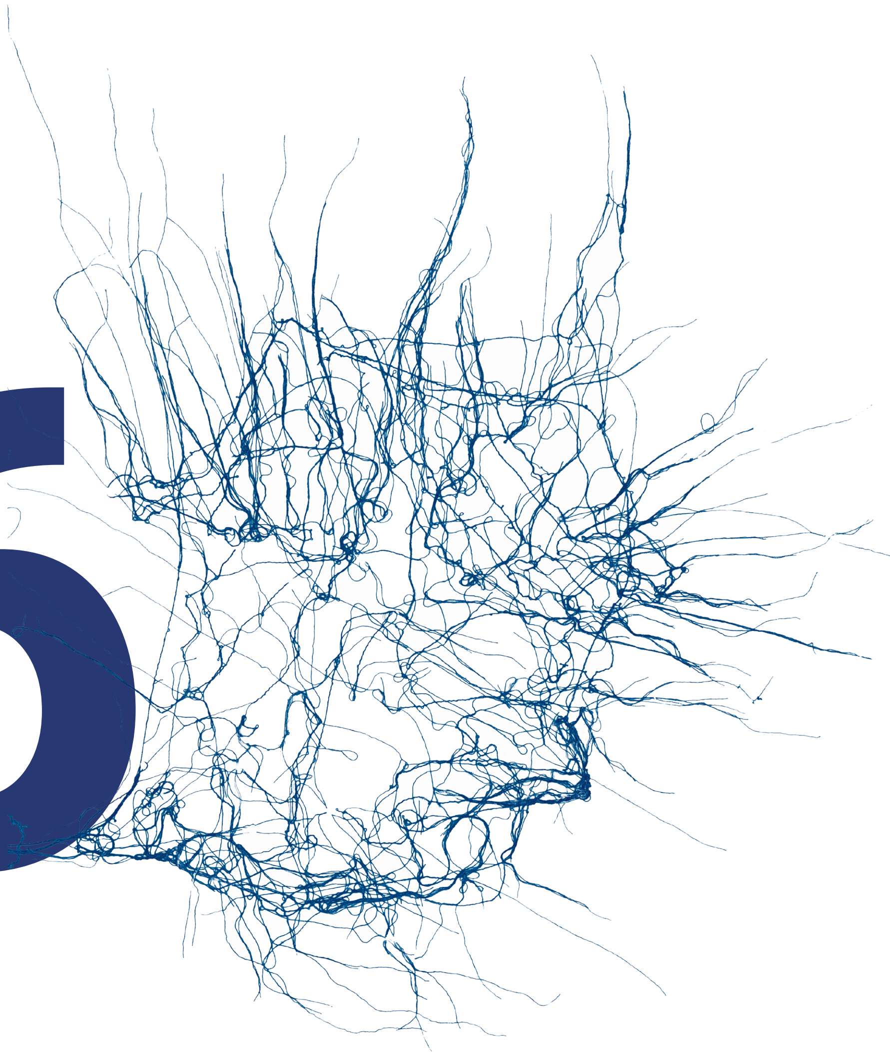

INTRALUMINAL INJECTION OF MESENCHYMAL STROMAL CELLS IN SPHEROIDS ATTENUATES EXPERIMENTAL COLITIS

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ABSTRACT

Background and Aim

In recent years mesenchymal stromal cells (MSCs) emerged as a promising therapeutic option for various diseases due to their immunomodulatory properties. We previously observed that intraperitoneally injected MSCs in experimental colitis form spherical shaped aggregates. Therefore, we aggregated MSCs *in vitro* into spheroids and injected them intraluminally in mice with established colitis to investigate whether these MSC-spheroids could alleviate the colitis.

Methods

We injected 0.5×10^6 MSCs in spheroids, 2.0×10^6 MSCs in spheroids, or phosphate-buffered saline (PBS) as a treatment control, via an enema in mice with established dextran sulphate sodium (DSS)-induced colitis. Body weight was measured daily and disease activity score was determined at sacrifice. Endoscopy was performed to evaluate mucosal healing. After sacrifice, both systemic and local inflammatory responses were evaluated.

Results

Intraluminally injected MSC-spheroids alleviated DSS-induced colitis, resulting in significantly less body weight loss and lower disease activity score at sacrifice when a high dose of MSC-spheroids was administered. However, the percentage of mucosal lesions in the distal colon and endoscopy scores were not significantly lower after treatment with 2.0×10^6 MSCs in spheroids compared with PBS-treated mice. The systemic inflammation marker serum amyloid A (SAA) was significantly reduced after treatment with 2.0×10^6 MSCs in spheroids. In addition, local cytokine levels of IFN-γ, TNF-α, IL-6 and IL-17A, as well as numbers of macrophages and neutrophils, showed a clear decrease - though not always significant - after intraluminal injection of the MSC-spheroids.

Conclusion

Intraluminally injected MSC-spheroids at least partially attenuate experimental colitis, with fewer phagocytes and proinflammatory cytokines, when a high dose of MSCs in spheroids was administered.

INTRODUCTION

Due to their immunomodulatory properties and their ability to actively participate in tissue repair mesenchymal stromal cells (MSCs) emerged as a potential therapeutic alternative in the treatment of several diseases[1]. Promising reports on the use of MSCs as a treatment for both experimental colitis as well as human inflammatory bowel disease (IBD) have been published in the last few years[2-6]. Currently, their exact mode of action is under investigation. Previously it became clear that MSC-therapy is not beneficial under all conditions of an ongoing immune response and that the timing of administration is important to induce the full immunosuppressive and tissue regenerative properties of MSCs[7]. In recent years, potentiating the therapeutic efficacy of MSCs by priming with proinflammatory cytokines such as interferon-gamma (IFN-γ) and/or tumor necrosis factor-alpha (TNF-α) to create an immunostimulatory milieu *in vitro* before use *in vivo* was also evaluated[8,9].

Although encouraging results have been obtained in different preclinical studies using these primed MSCs[10,11], caution should be taken as MSCs can participate in antigen presentation by upregulation of major histocompatibility class (MHC) class II molecules when not primed sufficiently. This could finally result in a potential risk of rejection and exaggeration of an ongoing immune response, ultimately worsening the disease[7, 12-15]. Therefore, new methods to increase the immunomodulatory effects of MSCs should be assessed.

Recently, intraperitoneally injected MSCs were observed to cluster together to form aggregates which produced the anti-inflammatory protein tumor necrosis factor-inducible gene (TSG)-6 leading to attenuated dextran sulphate sodium (DSS)-induced colitis[16]. Remarkably, intravenously injected MSCs in experimental myocardial infarction entrapped within the lungs also formed clusters that produced TSG-6, thereby improving tissue damage[17]. In our previously performed experiments, we also observed that intraperitoneally injected MSCs form spherical shaped aggregates. To investigate whether MSCs aggregated into spherical clusters, i.e. spheroids, could alleviate experimental colitis, we created MSC-spheroids *in vitro* and injected them in mice with established DSS-induced colitis. Since the inflammation is in the mucosa of the distal colon, we injected the MSC-spheroids intraluminally via an enema, hypothesising that MSC-spheroids might integrate into the mucosa or release soluble factors which contribute to the attenuation of experimental colitis. We observed that intraluminal injection of *in vitro* generated MSC-spheroids alleviated DSS-induced colitis when given in a high dose, which was accompanied by a decreased invasion of distinct immune cells and a reduced mucosal production of some proinflammatory cytokines.

MATERIAL AND METHODS

MSC isolation

Murine bone marrow MSCs were isolated from 8 to 12-week-old C57BL/6Jico mice (Charles River Maastricht, The Netherlands). Mice were sacrificed by cervical dislocation and femurs and tibiae were removed, cleaned of tissue and flushed to retrieve bone marrow cells. Cells were plated in α-minimal essential medium (MEM) (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (Hyclone, Thermo Scientific, Landsmeer, The Netherlands), 3mM L-glutamine (Invitrogen, Bleiswijk, The Netherlands) and penicillin/streptomycin (Lonza, Verviers, Belgium) after a centrifugation step and expanded in a 37°C humidified incubator containing 5% CO_2. After 24 hours, non-adherent cells were removed and the medium was refreshed every 3-4 days. The protocol for the animal experiments was approved by the Committee on Animal Welfare of the Leiden University Medical Center.

MSC spheroid formation

Spheroids were formed in 96-wells culture plates (Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) of 2,500 MSCs per well. Thus, every spheroid consisted of 2,500 MSCs and 0.24% methyl cellulose (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) which was added to the medium in each well to enhance spheroid formation. MSCs from passages 6 to 8 were used to create spheroids for transplantation experiments. Spheroids were harvested after 2 days of culture. Before *in vivo* injection, MSC-spheroids were thoroughly washed with PBS. Either $0.5x10^6$ or $2.0x10^6$ MSCs in spheroids were injected locally, meaning 200 spheroids consisting of 2,500 MSCs per spheroid in case of $0.5x10^6$ MSCs in spheroids and 800 spheroids consisting of 2,500 MSCs per spheroid in case of $2.0x10^6$ MSCs in spheroids.

In indicated *in vitro* experiments 500 U/ml recombinant mouse IL-6 (Life Technologies, Bleiswijk, The Netherlands) or recombinant mouse TNF-α (R&D systems, Abingdon, UK) was added to each well at day -1. In these experiments, in total 12-15 96-wells culture plates per time point were used to generate spheroids resulting in 1152-1440 spheroids per time point.

MSC characterization

Immunophenotyping of MSCs was performed using the following primary antibodies: CD44, CD105, CD106 (BD Biosciences, San Diego, CA, USA), CD29, Sca-1 and CD45 (eBioscience, Vienna, Austria). Samples were analyzed using a FACSCanto II flow cytometer with Diva Software (BD Biosciences, San Diego, CA, USA) and the data were analyzed with FlowJo software (version 8.7.1., Tree Star Inc. Ashland, OR, USA). *In vitro* differentiation was performed in 24-wells culture plates with MSCs at 80% confluency. MSC-spheroids were transferred to 24-wells plates after 2 days of culture and 4 days before the start of differentiation. For osteogenic differentiation MSCs and spheroids were stimulated for 21 days in complete medium supplemented with 10^{-8} M dexamethason, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Alkaline phosphatase activity was shown with Fast Blue and calcium deposit with Alizarin Red (both Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). For adipogenic differentiation, MSCs and spheroids were stimulated for 21 days in adipogenic differentiation medium consisting of complete medium supplemented with dexamethasone (10^{-6} M), insulin (5 µM), indomethacin (100 µM) and 3-isobutyl- 1-methylxanthine (0.5 mM) (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Formation of lipid droplets was visualized with Oil-red O staining (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

Induction of colitis and intraluminal injection of MSCs

Animals were housed in individually ventilated cages and were given drinking water and food *ad libitum.* Colitis was induced in 8-week-old female C57BL/6Jico mice (Charles River Laboratories, The Netherlands) with 1.25% dextran sulphate sodium (DSS; MW 36,000-50,000 kDA; MP Biomedicals, Illkirch, France) supplied in the drinking water for 7 days. Five days after the introduction of DSS, mice received locally [with a 22 Gauge catheter (Abbocath, Hospira Benelux, Brussels, Belgium) approximately 3 cm from the anus] $0.5x10^6$ or $2.0x10^6$ MSCs in spheroids in 150 µl PBS (*n* = 7 and *n* = 14 respectively), or PBS alone (n = 7) as a treatment control. The healthy control group consisted of 3 mice that received $2.0x10^6$ MSCs in spheroids ('normal' group). In Supplementary Figure 1 the results of healthy control groups that received $0.5x10^6$ MSCs in spheroids (n = 3), PBS (n = 3) or without treatment (n = 3) are shown. Mice were anesthetized with isoflurane and kept upside down for 5 minutes after injection. Mice were sacrificed 10 days after the start of the experiment. The separate endoscopy study consisted of 10 mice with DSS-induced colitis treated with $2.0x10^6$ MSCs in spheroids and 9 mice treated with PBS. These mice were sacrificed 12 days after the start of the experiment to be able to evaluate mucosal healing.

Assessment of disease activity

Body weight was measured daily and disease progression and recovery were calculated as a percentage of weight loss from body weight at the start of the experiment. Furthermore, endoscopy was performed with the Coloview mini-endoscopic system of Karl Storz (Stöpler, Utrecht, The Netherlands)[18] at day 0, 5, 8, 10 and 12 to evaluate mucosal damage and subsequent healing. The murine endoscopic index of colitis severity (MEICS) was used to quantify the thickening of the colon, changes of the vascular pattern, fibrin deposit, granularity of the mucosal surface and stool consistency[18]. The MEICS was scored blindly and independently by two researchers. At sacrifice, blood was collected by cardiac puncture and centrifuged (10,000g for 10 minutes) to collect serum which was stored in -20°C. Colon length was measured, as an indicator of disease-related intestinal shortening, and colons were opened longitudinally to calculate the disease activity score consisting of the presence of loose stool, visible fecal blood and macroscopic inflammation using a scale of 0-3 per item, resulting in a maximum score of 9[19]. Colons were either stored in 4% neutral buffered formalin as a 'swiss roll'[20] and embedded in paraffin for (immuno) histological evaluation or the distal part of the colon was directly frozen in isopentane for protein extraction and cytokine measurements.

Histological evaluation of disease activity

The paraffin-embedded colons were cut into sections of 4 μm and stained with haematoxylin and eosin (HE) to evaluate the number of lesions in the distal 3 cm of the colon where DSS-induced colitis is most pronounced. A lesion was defined as a part of the colon without epithelium. Colon- and lesion length was measured with Olympus CellSens (version 1.7.1, Zoeterwoude, The Netherlands). All colons were measured twice and the researcher was blind for the treatment mice had received. The average percentage of mucosal lesions is shown in Figure 2F.

Immunohistochemistry

Apoptosis was shown in MSCs and MSC-spheroids embedded in paraffin with an anti-cleaved caspase-3 antibody (1:800, Cell Signaling, Leiden, The Netherlands). Macrophages were detected with an anti-F4/80 antibody (1:800, eBioscience, LTD, Hatfield, UK) and T regulatory cells (Tregs) with an anti-FoxP3 antibody (1:500, eBioscience, LTD, Hatfield, UK). In brief, slides were deparaffinised, hydrated and incubated in 0.3% H_2O_2/methanol for 20 minutes at room temperature. Slides were blocked with Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% (v/v) Tween-20, pH 8.0) for 30 minutes or proteinase K (50 μg/ml) for 10 minutes at 37°C, and subsequently incubated overnight at 4°C with primary antibody in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA) and followed by a peroxidase labelled polymer (EnVision+, Dako Netherlands BV, Heverlee, Belgium). For staining of macrophages and Tregs, slides were incubated for 1 hour (at room temperature) with a rabbit anti-rat HRP secondary antibody conjugated secondary antibody (1:200, Dako Netherlands BV, Heverlee, Belgium) diluted in PBS containing 0,01% Triton X-100 and 1% BSA instead of a peroxidase labelled polymer. Peroxidase activity was detected with 3,3'-diaminobenzidine tablets (DAB Fast Tablet, Sigma-Aldrich, St. Louis, MO). Sections were counterstained with hematoxylin, dehydrated, and mounted in Entellan (Merck KGaA, Darmstadt, Germany). Microscopic images of the distal 3 cm of the colon were made with a magnification of x20. The F4/80-positive cells in these slides were counted using ImageJ software (version 1.45s, National Institutes of Health, USA) to quantify their number in the mucosa of the distal 3 cm of the colon.

Serum amyloid A and cytokine measurements

Serum amyloid A (SAA) levels were measured in the serum collected upon sacrifice, using a solid phase sandwich ELISA kit (Life Technologies, Bleiswijk, The Netherlands).

Homogenates were prepared from distal colon with a Potter-Elvehjem glass homogenizer at 4°C in Greenberger lysis buffer (150 mM NaCl, 15 mM Tris, pH 7.4, 1 mM MgCl2, and 1% Triton X-100). Samples were centrifuged for 15 minutes (11,000g at 4°C) and stored at -80°C. The BCA Protein Assay Kit (Thermo Scientific Pierce, Etten-Leur, The Netherlands) was used to determine the total concentration of protein in the colons and cytokine levels of IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A and TNF-α were measured using the Cytometric Bead Array System (BD Biosciences, San Diego, CA, USA) following the manufacturer's instructions. Data was analysed with FlowJo software (version 8.7.1., Tree Star Inc. Ashland, OR, USA). Cytokine levels measured were corrected for the amount of total protein.

MPO determination

Myeloperoxidase (MPO) activity in the homogenates of distal colon was measured as an index for neutrophil infiltration. In briefly, homogenates were sonicated and 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in 50 mM in phosphate buffer (pH 5.5) was added to the sonicated homogenates and incubated for 30 minutes at room temperature. Supernatants were mixed with 150 µl of phosphate buffer (pH 5.5; 37°C) containing 0.26 mg/ml o-dianisidine dihydrochloride (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.52 mM 30% H_2O_2. Colorimetric reaction was measured at 450 nm for 30 minutes using a spectrophotometer. One µmol H_2O_2 (= 1 unit of MPO) split, gives a change in absorbance of 1.13×10^{-2}/min. The number of MPO units was determined as $\Delta A_{450}/(\Delta time \times 1.13 \times 10^{-2})$ and corrected for the total amount of protein per sample. Samples were analysed in duplicate.

RNA isolation and quantitative PCR

RNA from distal colons after sacrifice and from MSC-spheroids 1 to 5 days after the start of culture (*n* = 1152-1440 spheroids per time point) was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using RevertAid reverse transcriptase (Fermentas, St. Leon-Rot, Germany) and random primers (Promega, Leiden, The Netherlands). Quantitative PCR using SYBR green (QuantiFast SYBR Green PCR Kit, Qiagen) with a forward and reverse primer mix for COX-2 (NM_011198) (QuantiTect Primer Assay, Qiagen) was performed. The household gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, QiantiTect Primer Assay, Qiagen) was used to normalize the data. Samples were analysed in triplicate.

PGE2 ELISA

Prostaglandin E2 (PGE2) was measured in the homogenates of the distal colons at sacrifice and in the supernatants of MSC-spheroids 1 to 5 days after the start of culture using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) following the manufacturer's instructions. Samples were analysed in duplicate.

Statistical Analysis

To compare two groups, parametric or nonparametric analyses were performed using an unpaired Student's t-test or Mann-Whitney U-test, respectively. Numerical values were expressed as means ± standard error of the mean (SEM). All analyses were performed using GraphPad Prism software (GraphPad Software, version 5.01, San Diego, CA). *P*-values ≤0.05 were considered statistically significant.

RESULTS

Intraluminally injected MSC-spheroids alleviate DSS-induced colitis

The *in vitro* generated MSC-spheroids had an average size of 148.9±3.8 μm (n = 6), showed low expression of the apoptotic marker cleaved caspase-3 (Figure 1A right panel) and every spheroid was constructed from 2,500 by flow cytometry characterized single cell MSCs (Figure 1B). Similarly to single cell MSCs, MSC-spheroids were able to differentiate into adipocytes and osteoblasts (Figure 1C).

Subsequently, we examined whether our *in vitro* generated MSC-spheroids could attenuate DSS-induced colitis. Mice received either $0.5x10^6$ or $2.0x10^6$ MSCs in spheroids intraluminally via an enema at day 5 when colitis was established. Treatment with $2.0x10^6$ MSCs in spheroids resulted in significantly reduced body weight loss at sacrifice compared with treatment with $0.5x10^6$ MSCs in spheroids or PBS (9.2% vs 16.4% and 15.9% respectively; both $p = 0.02$; figure 2A). Moreover, disease-related shortening of the colon was significantly reduced after treatment with $2.0x10^6$ MSCs in spheroids compared with PBS ($p < 0.05$; Figure 2B and 2C) which was also reflected in the macroscopic disease activity score consisting of the presence of loose stool, visible fecal blood and macroscopic inflammation at sacrifice ($p = 0.01$ compared with PBS; Figure 2D). In addition, histological evaluation of the distal colon revealed a clear but non-significant trend towards less mucosal lesions by injection with $2.0x10^6$ MSCs in spheroids compared with PBS (Figure 2E and 2F).

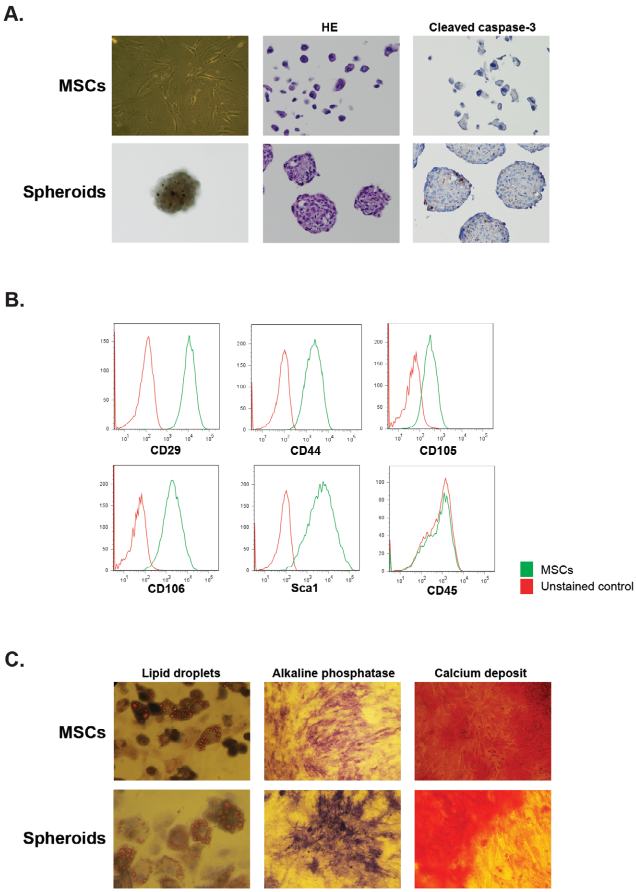

FIGURE 1. Immunophenotypical characterisation of single cell mesenchymal stromal cells (MSCs) and MSC-spheroids. Spheroids were created in 96-wells plates with 2,500 MSCs per well. A. Macroscopic picture (left panel), hematoxylin and eosin (HE) staining (central panel) and cleaved caspase-3 staining (right panel) of spindle shaped MSCs and MSC-spheroids. Magnification x20. B. MSC surface markers CD29, CD44, CD105, CD106, Sca1 (positive) and CD45 (negative) as analysed by flow cytometry. C. Differentiation into adipocytes (lipid droplets) and osteoblasts (alkaline phosphatase activity and calcium deposit) of MSC-spheroids (lower panel) was similar to single cell MSCs (upper panel). Magnification x32.

To quantify mucosal damage during the colitis, a second experiment was performed. Mice were treated with 2.0×10^6 MSCs in spheroids or PBS at day 5 when colitis was established and endoscopy was performed at day 0, 5, 8, 10 and 12 (Figure 3A). The MEICS score was calculated to quantify the thickening of the colon, changes of the vascular pattern, fibrin deposit, granularity of the mucosal surface and stool consistency. The endoscope could be inserted in the colon up to approximately 3-4 cm from the anus. At day 0, endoscopy showed a smooth and translucent mucosal surface with a normal blood vessel architecture and solid stools as DSS was not yet introduced in the drinking water resulting in a MEICS score of 0 (Figure 3B). Five days after the start of DSS, the colon transparency was reduced and the mucosa was vulnerable, which in some mice resulted in bleedings. At day 8, signs of severe inflammation were present. Mice presented with diarrhoea and non-transparent mucosa with moderate granularity and some fibrin deposit. The highest MEICS score was reached at day 10. At day 12, treatment with 2.0×10^6 MSCs in spheroids seemed to partially reduce the MEICS score compared with PBS-treated mice (Figure 3B). Body weight, disease activity and colonic TNF-α, IFN-γ, IL-6 and IL-17A at day 12 are shown in Supplementary Figure 2.

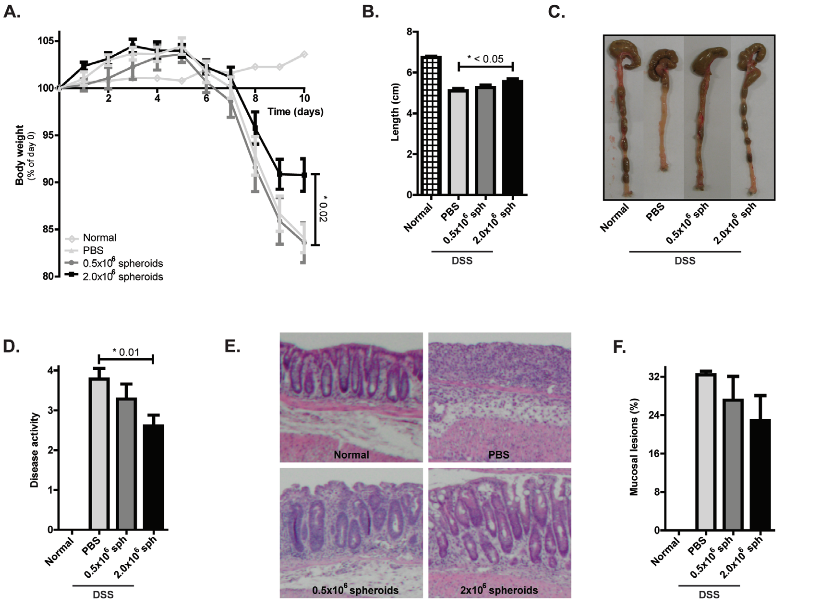

FIGURE 2. Intraluminally injected mesenchymal stromal cells (MSC)-spheroids alleviate dextran sulphate sodium (DSS)-induced colitis. DSS was introduced in the drinking water for 7 days. Mice received 0.5×10^6 or 2.0×10^6 MSCs in spheroids or phosphate-buffered saline (PBS) via enema at day 5. Mice were sacrificed at day 10. Data are expressed as mean ± SEM. n = 7-14 per group from two separate experiments. The 'normal' group consisted of three healthy mice that received 2.0×10^6 MSCs in spheroids. A. Body weights were measured daily and expressed as the percentage of body weight at day 0. *, p = 0.02: 2.0×10^6 MSCs in spheroids vs both PBS and 0.5×10^6 MSCs in spheroids. B. Disease-related shortening of the colon. C. Macroscopic images of representative colons at sacrifice (day 10). D. Disease activity score based on the presence of loose stool, fecal blood and macroscopic inflammation determined at sacrifice (day 10). E. Representative histological sections of mouse colons stained with HE. Magnification x20. F. Quantification of mucosal lesions defined as parts in the colon without mucosa expressed as a percentage of total colon. n = 3-6 per DSS-group and n = 1 in healthy control group.

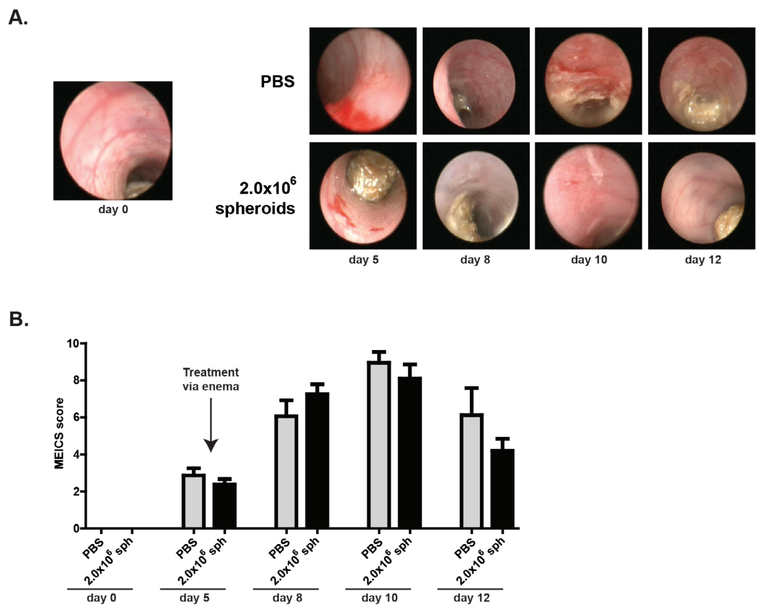

FIGURE 3. Endoscopic evaluation of colonic inflammation in mice with dextran sulphate sodium (DSS)-induced colitis. DSS was introduced in the drinking water for 7 days. Mice received 2.0×10^6 mesenchymal stromal cells (MSCs) in spheroids or PBS via enema at day 5. Endoscopy was performed at day 0 before DSS introduction and at day 5, 8, 10 and 12. Mice were sacrificed after the endoscopy at day 12. Data are expressed as mean ± SEM. *n* = 9-10 per group. A. Representative endoscopic images of mice with colitis treated with either 2.0×10^6 MSCs in spheroids or PBS via enema. B. Endoscopic evaluation of the colonic inflammation using the murine endoscopic index of colitis severity (MEICS) based on the thickening of the colon, changes of the vascular pattern, fibrin deposit, granularity of the mucosal surface and stool consistency[18].

Local treatment with MSC-spheroids results in reduction of some inflammatory mediators

The systemic marker of inflammation SAA was drastically upregulated when DSS was induced indicating a severe colitis (Figure 4A). Intraluminal treatment with 2.0×10^6 MSCs in spheroids resulted in a significant reduction of SAA in the serum at sacrifice compared with PBS-treated mice ($p = 0.04$; Figure 4A). Locally, the number of neutrophils, as reflected by MPO activity, was significantly lower in the distal colons of mice treated with 2.0×10^6 MSCs in spheroids compared with PBS ($p = 0.03$; Figure 4B). In addition, lower numbers of macrophages were observed in the distal colon after MSC-spheroids treatment ($p = 0.06$ for 0.5×10^6 MSCs in spheroids vs PBS and $p = 0.08$ for 2.0×10^6 MSCs in spheroids vs PBS; Figure 4C and 4D).

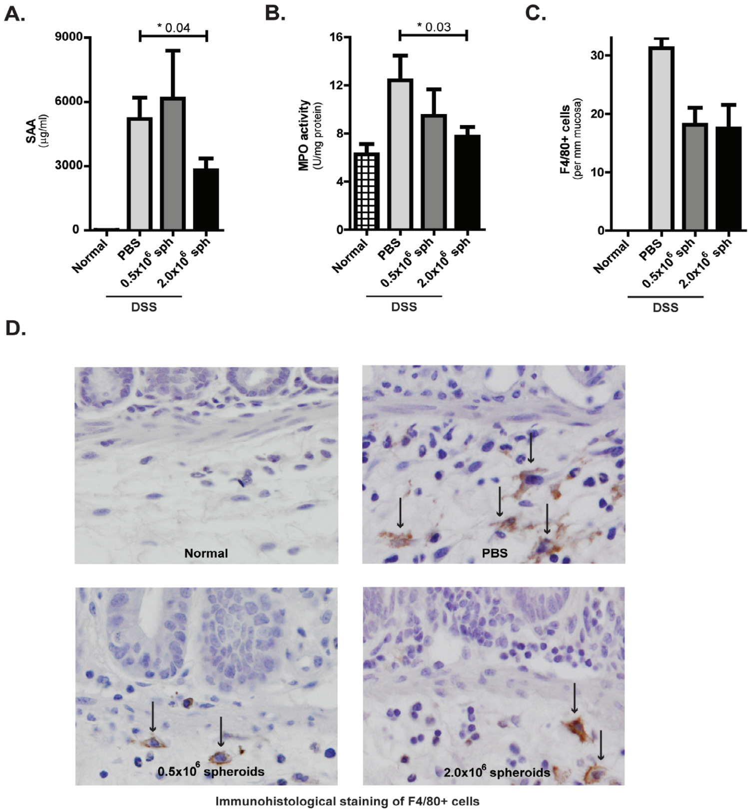

FIGURE 4. Attenuated colitis is accompanied by reduced systemic and local inflammatory responses. Dextran sodium sulphate (DSS) was introduced in the drinking water for 7 days. Mice received 0.5x10^6 or 2.0x10^6 MSCs in spheroids or phosphate-buffered saline (PBS) via enema at day 5. Mice were sacrificed at day 10. Data are expressed as mean ± SEM. *n* = 4-8 per DSS-group and *n* = 2 in healthy control group that received 2.0x10^6 MSCs in spheroids from 2 separate experiments. A. Measurement of systemic inflammation marker serum amyloid A (SAA). B. Myeloperoxidase (MPO) activity was measured in homogenates of the distal colons to evaluate the amount of neutrophils. C. Number of macrophages per mm in the distal colon. *n* = 2-3 per DSS-group, *n* = 1 in healthy control group. D. Representative immunohistological sections of mouse colons stained with a F4/80-antibody to reveal macrophages (arrows). Magnification x20.

Next, we measured the proinflammatory cytokines locally in the distal colon. In our hands, the Th1 and Th17-skewing cytokines TNF-α, IFN-γ, IL-6 and IL-17A were upregulated in the experimental colitis compared with normal controls. IFN-γ and, to a lesser extent, TNF-α were reduced in the distal colons in colitis after treatment with 2.0x10^6 MSCs in spheroids (IFN-γ, $p < 0.05$; Figure 5A and 5B). In addition, the level of local IL-6 was significantly reduced after treatment with MSC-spheroids ($p = 0.03$ for both 0.5x10^6 and

2.0x10⁶ MSCs in spheroids vs PBS; Figure 5C). IL-17A was, although not significant, also decreased after MSC-spheroid treatment compared with PBS (Figure 5D). IL-2 and IL-4 were not upregulated compared with healthy control; however, treatment with 2.0x10⁶ MSCs in spheroid significantly reduced both cytokines compared with PBS (Figure 5E and 5F). Although MSC-spheroid treatment reduced inflammatory responses and thereby alleviated colitis, levels of the regulatory cytokine IL-10 were not elevated compared with PBS or healthy controls (Figure 5G). In addition, FoxP3 staining of the distal colons did not reveal major differences between Tregs in the MSC-spheroid treated groups and the PBS treated mice (Figure 5H).

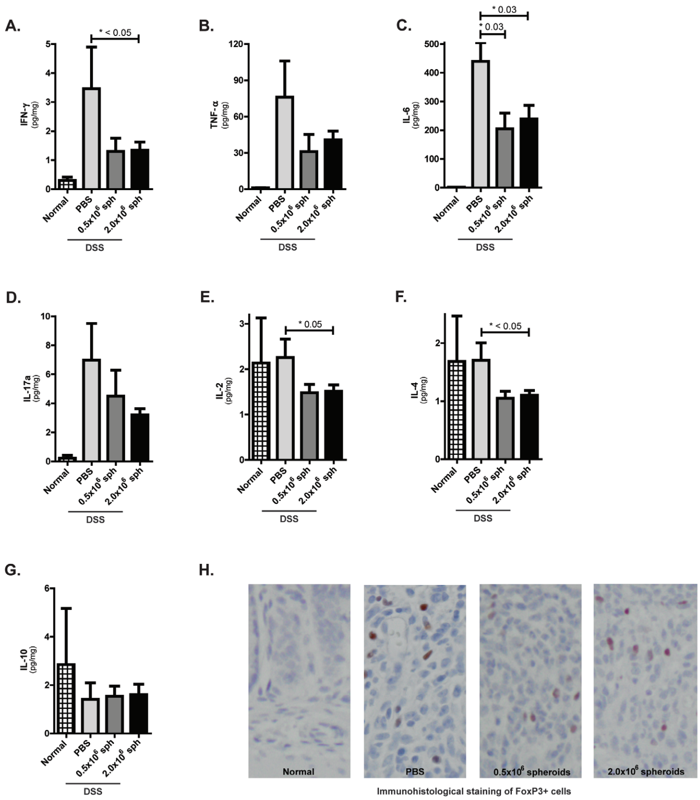

FIGURE 5. Local treatment with mesenchymal stromal cell (MSC) spheroids alters colonic cytokine production. Dextran sodium sulphate (DSS) was introduced in the drinking water for 7 days. Mice received 0.5x10⁶ or 2.0x10⁶ MSCs in spheroids or phosphate-buffered saline (PBS) via enema at day 5. Mice were sacrificed at day 10. Data are expressed as mean ± SEM. *n* = 4-8 per DSS-group and *n* = 2 in healthy control group that received 2.0x10⁶ MSCs in spheroids from two separate experiments. Cytokine levels of (A) IFN-γ, (B) TNF-α, (C) IL-6 and (D) IL-17A were upregulated after colitis induction with DSS. No differences between healthy controls and DSS-colitis mice were observed in the cytokine levels of (E) IL-2, (F) IL-4 and (G) IL-10. H. Representative immunohistological sections of mouse colons stained with a FoxP3-antibody to reveal Tregs (arrows). Magnification x20.

6

As a measure of mucosal healing the PGE2 concentration and the amount of COX-2 RNA were determined in the homogenates of the distal colons. Both PGE2 and COX-2 levels were higher in mice with colitis compared with healthy controls at time of sacrifice (Figure 6A and 6B). Remarkably, the PGE2/COX-2 ratio in the mucosa of the colons of the MSC spheroid-treated group showed a clear trend to increase towards that of healthy control mice, and related to a decrease in disease activity of the intestinal mucosa (Figure 6C).

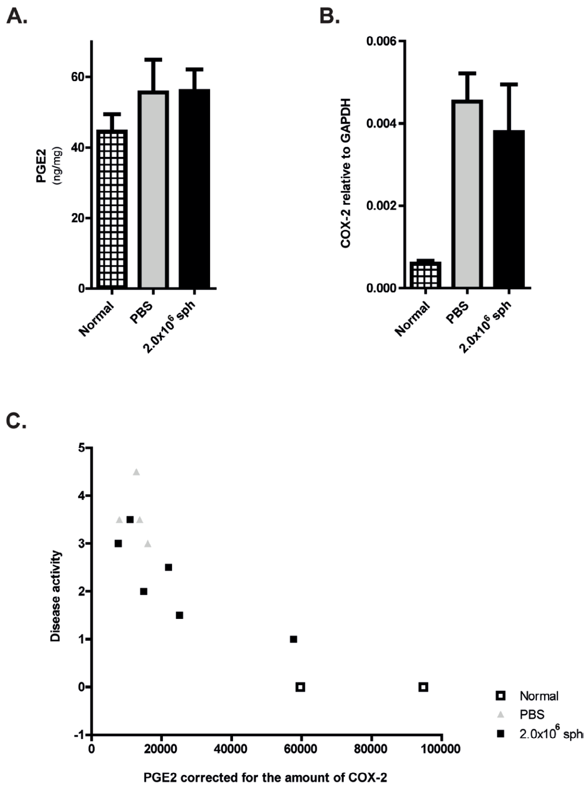

FIGURE 6. PGE2 and COX-2 RNA in the homogenates of the distal colon. Dextran sodium sulphate (DSS) was introduced in the drinking water for 7 days. Mice received $2.0x10^6$ mesenchymal stromal cells (MSCs) in spheroids or phosphate-buffered saline (PBS) via enema at day 5. Mice were sacrificed at day 10. Homogenates were prepared from distal colons. RNA was isolated from the homogenates and cDNA was prepared. COX-2 RNA expression was measured in triplicate and normalized to GAPDH and PGE2 levels in duplicate and corrected for the amount of protein. Disease activity score was based on the presence of loose stool, fecal blood and macroscopic inflammation and determined at sacrifice. Data are expressed as mean ± SEM. *n* = 4-6 per DSS-group and *n* = 2 in healthy control group that received $2.0x10^6$ MSCs in spheroids from two separate experiments. A. Mean concentrations of PGE2 (ng/mg) and B. mean amount of COX-2 RNA relative to GAPDH in the homogenates of the distal colons. C. Disease score vs PGE2 concentration corrected for the amount of COX-2 RNA present in the homogenates of the distal colons.

TNF-α-stimulation of MSC-spheroids enhances COX-2 dependent PGE2 secretion *in vitro*

To evaluate if MSC-spheroids produce COX-2 dependent PGE2, we determined COX-2 RNA in MSC-spheroids and PGE2 levels in the supernatant, as a proof of principle. In addition, MSC-spheroids were stimulated with IL-6 or TNF-α at the start of culture since these cytokines were significantly elevated in DSS-induced colitis. Adding IL-6 to MSCs at the start of spheroid induction did not affect the expression of COX-2 RNA (Figure 7A). In concordance with this result, PGE2 levels in the supernatants were comparable to those in non-stimulated MSC-spheroids (Figure 7B). When TNF-α was added at the start of spheroid induction, already 1 day later COX-2 RNA expression was increased compared with non- or IL-6 stimulated MSC-spheroids (Figure 7A). Moreover, PGE2 levels in the supernatants of TNF-α-stimulated MSC-spheroid culture were elevated compared with non- or IL-6-stimulated MSC-spheroids (Figure 7B).

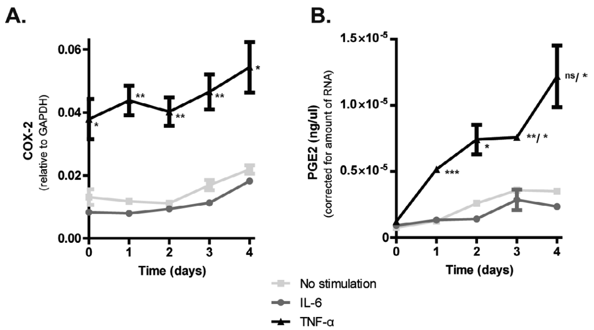

FIGURE 7. TNF-α-stimulation of mesenchymal stromal cell (MSC)-spheroids enhances COX-2 dependent PGE2 secretion *in vitro*. Spheroids were created in 12-15 96-wells plates with 2,500 MSCs per well resulting in 1152-1440 MSC-spheroids per time point. Culture medium was centrifuged to get rid of debris and stored. RNA was isolated from the MSC-spheroids and cDNA was prepared. COX-2 RNA expression was measured in triplicate and normalized to GAPDH and PGE2 levels in duplicate. Data are expressed as mean ± SEM. A. MSC-spheroids were created without stimulation, with IL-6-stimulation (500 U/ml) or TNF-α-stimulation (500 U/ml) at day -1. Every day MSC-spheroids were harvested to evaluate COX-2 RNA expression in the MSC-spheroids (day 0: *, p = 0.02 no stimulation vs TNF-α; *, p = 0.01 IL-6 vs TNF-α; day 1: **, p = 0.002 both no stimulation and IL-6 vs TNF-α; day 2: **, p = 0.003 no stimulation vs TNF-α; **, p = 0.002 IL-6 vs TNF-α; day 3: **, p = 0.007 no stimulation vs TNF-α; **, p = 0.003 IL-6 vs TNF-α; day 4: *, p = 0.02 no stimulation vs TNF-α; *, p = 0.01 IL-6 vs TNF-α). B. PGE2-levels in culture medium of MSC-spheroids stimulated with IL-6, TNF-α or without stimulation (day 0: ns; day 1: ***, p = 0.0001 both no stimulation and IL-6 vs TNF-α; day 2: *, p = 0.05 no stimulation vs TNF-α; *, p = 0.03 IL-6 vs TNF-α; day 3: **, p = 0.002 no stimulation vs TNF-α; *, p = 0.03 IL-6 vs TNF-α; day 4: ns no stimulation vs TNF-α; *, p = 0.05 IL-6 vs TNF-α).

DISCUSSION

Recently, MSCs were observed to form aggregates in the peritoneum, which produced TSG-6 and thereby attenuated DSS-induced colitis[16]. In addition, in an experimental myocardial infarction model, intravenously injected MSCs were entrapped within the lungs where they also formed aggregates that produced TSG-6, resulting in less myocardial damage[17]. Not only in experimental disease models, but also in healthy state, MSCs tend to spontaneously form spheroids[16,23]. Therefore, we hypothesised that the formation of spheroids is important for MSCs to gain their immunosuppressive effects. In this present study, we observed that intraluminal treatment with *in vitro* generated MSC-spheroids alleviated moderately-severe DSS-induced colitis but only when a high dose of 2.0×10^6 MSCs in spheroids was given. Body weight loss and disease activity score at sacrifice were significantly reduced after treatment with 2.0×10^6 MSCs in spheroids. The percentage of mucosal lesions in the distal colon and endoscopy scores were not significantly reduced after MSC-spheroid treatment which resembles human IBD: clinical remission does not per definition imply histological and/or endoscopic remission[24,25]. In addition, a retrospective study showed that in patients with clinical remission, the presence of mucosal inflammation was not associated with more complications or exacerbations during a median follow-up of 6.8 years[26].

Biochemically, the systemic marker of inflammation SAA was elevated after DSS-induced mucosal damage, possibly as a result of bacterial invasion. Similarly to other acute phase proteins, SAA is mainly produced in the liver. In addition, SAA is also secreted into the lumen by colonic epithelial cells, especially in case of inflammation and tissue destruction[27,28]. Locally, SAA can promote IL-6 and TNF-α production by macrophages[29,30]. Indeed, both IL-6 and TNF-α were drastically upregulated after DSS administration. Although not significantly, histological evaluation demonstrated that the number of macrophages in the mucosa of the distal colon was decreased after local MSC spheroid treatment compared with PBS. Moreover, SAA-, IL-6- and TNF-α-levels were reduced. We cannot ensure that IL-6- and TNF-α were only produced by macrophages, as dendritic cells and T cells are also able to produce these cytokines; however, macrophages are identified as of crucial importance in the innate intestinal immunity. Mice deficient for macrophages or depleted for local gut macrophages have less susceptibility to experimental colitis than wild type mice[31,32]. Moreover, DSS administration to lymphocyte-deficient mice leads to the development of colitis, indicating that macrophages are the key players in establishing DSS-induced colitis and that lymphocytes are of less importance herein[33]. In our experiments, IL-17A was elevated in DSS-induced colitis and dose-dependently reduced after intraluminal treatment with MSC-spheroids. Since secreted IL-17A is reported to regulate migration of neutrophils to the place of inflammation[34,35], we examined the amount of neutrophils in the distal colons. The amount of neutrophils was significantly lower after intraluminal treatment with 2.0×10^6 MSCs in spheroids.

The exact mechanism of MSC-spheroid-mediated attenuation of DSS-induced colitis remains partially unclear, as no MSC-spheroids were found in the colon of treated mice at sacrifice. Both MSCs as well as the supernatants from MSCs are reported to be able to reduce the levels of IL-6 and TNF-α secreted by activated macrophages and to increase the production of IL-10 resulting in the polarisation towards regulatory M2 macrophages[36-38]. We observed that IL-6 and TNF-α levels, and the number of macrophages in the mucosa of the distal colons were reduced, suggesting that in this model at time of sacrifice no regulatory macrophages were induced but rather that the number of macrophages was decreased as a sign of reduced inflammation after MSC spheroid treatment. In line with these results, an increased recruitment of macrophages to the place of acute myocardial infarction was found to facilitate cardiac muscle repair by MSCs as no attenuation of disease was observed when macrophage recruitment was diminished or MSCs were removed after local macrophage infiltration[39]. This indicates that the interaction between MSCs and macrophages might be an important factor in restoring cardiac function suggesting that the number of local macrophages will be decreased as a sign of attenuated disease when cardiac function is restored.

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In our hands, IL-10 levels in homogenates of the distal colons at sacrifice were similar between MSC-spheroids-treated mice and mice that received PBS and not significantly elevated compared with healthy controls. Although IL-10 has been described to be of major importance in the homeostasis of the colonic milieu[40,41], clinical trials using IL-10 as a treatment for Crohn's disease have failed to show superiority of IL-10 therapy[42]. In addition, MSCs cocultured *in vitro* with activated NK cells resulted in reduced levels of IL-10[43]. On the other hand, when MSCs were cocultured *in vitro* with dendritic cells, IL-10 production was elevated[44]. In DSS-induced colitis, an abundance of distinct immune cells is present, likely resulting in more complex interactions between those cells and MSCs compared with the 'simple' interaction of MSCs with only one type of immune cell in *in vitro* models. Another possible explanation for the lack of IL-10 elevation in MSC spheroid treated mice is the time of sacrifice. It is possible that IL-10 produced by either MSCs or Tregs is one of the main factors involved in the immunosuppressive process mediated by MSCs directly after injection. However, we have not sacrificed animals in the first 24-48 hours after MSC spheroid injection to evaluate this hypothesis. In line with the observed IL-10 levels in the homogenates of the distal colons at sacrifice, no apparent differences in the number of Tregs were found in the distal colons of mice treated with MSC-spheroids compared with PBS-treated mice.

MSCs are reported to be able to inhibit Th17 cell differentiation and subsequent IL-17A production, which was restored when COX-2 was inhibited or PGE2 secretion by MSCs was blocked, suggesting that COX-2 dependent PGE2 is at least one of the paracrine factors MSCs produce to gain their immunosuppressive effects[45-47]. Whether or not direct cell-to-cell contact between MSCs and CD4-positive T cells is needed for the suppression of Th17 cell differentiation is doubtful, as previous published papers are not consistent. In our present study, however, we do not assume that direct contact between IL-17A-producing cells and intraluminally injected MSC-spheroids is the explanation of the alleviated colitis, since even with scrutinous histological evaluation, we have never observed the intraluminally injected MSC-spheroids in the damaged or healed mucosa. In addition, we generated MSC-spheroids with GFP-positive MSCs and injected them intraluminally in mice with DSS-induced colitis and these MSCs were not found in the mucosa (Supplementary Figure 3). In addition, for confirmation, we performed spheroid tracing experiments with luciferase-transduced MSCs (luc-MSCs) and subsequent bioluminescence imaging (Supplementary Data). Four hours after the intraluminal injection of these luc-MSC-spheroids, hardly any bioluminescence signal was observed when luciferin was given intraperitoneally, indicating that the spheroids had not engrafted into the colonic mucosa (Supplementary Figure 4A and 4B). However, when luciferin was given as an enema 4 hours after intraluminal injection of luc-MSC-spheroids, a clear signal was observed distally in the colon (Supplementary Figure 4C and 4D), indicative of luminally present luc-MSC-spheroids. Further, 16 hours after administration of luc-MSC-spheroids via enema, no bioluminescent signal in the colons was present anymore when luciferin was given either intraperitoneally and/or intraluminally. Thus, engraftment of the luminally administered MSC-spheroids into the mucosa does not seem to occur.

COX-2 expression and subsequent production of PGE2 in inflamed colons has been identified as an important factor in the wound healing process in experimental colitis[21,22]. We observed that the PGE2/COX-2 ratio was increased in the homogenates of the distal colons of colitic mice treated with MSC-spheroids towards healthy control mice, in concert with the decrease in disease score at sacrifice. We hypothesise that this is a reflection of the healing process initiated by the MSC-spheroids but it is certainly not a result of direct MSC activity within the intestinal mucosa simply because these spheroids were not present in the mucosa at sacrifice.

Interestingly, supernatants from co-cultures between Th17 cells and MSCs have been reported to contain elevated PGE2 levels compared with culture of only CD4-positive T cells or MSCs suggesting that this soluble factor is at least one of the key player in the suppressed differentiation[47]. In addition, spheroid formation was reported to induce increased expression of COX-2-dependent PGE2 both *in vitro* as *in vivo*[23]. In line with those results, we observed that MSC-spheroids constantly produced considerable but relatively low levels of COX-2 and PGE2 *in vitro* which increased over time especially when stimulated with TNF-α. However, in our *in vitro* model as proof of principle, we started with the priming of MSCs at the initiation of the aggregation into spheroids, whereas *in vivo,* if at all, stimulation would take place after the formation of spheroids. Interestingly, TNF-α induces the expression of COX-2 in colonic epithelial cells of patients with IBD and mice with DSS-colitis thereby promoting epithelial wound healing[48-51]. Wound healing was even impaired in COX-$2^{-/-}$ mice with DSS-induced colitis as a result of an inability to increase colonic PGE2[22]. Moreover, intraperitoneal administration of PGE2 restored DSS-induced decrease of proliferating epithelial cells indicating a key role for PGE2 in mucosal repair[52]. We hypothesise that the observed elevated levels of TNF-α in the inflamed colons possibly resulted in priming of intraluminally injected MSC-spheroids to produce high amounts of PGE2 subsequently promoting colonic epithelial wound healing without engraftment of the spheroids within the damaged mucosa. Supporting our hypothesis, rectal administration of basic fibroblast growth factor ameliorated DSS-induced colitis by activating COX-2 RNA which resulted in accelerated mucosal healing rather than a direct immunosuppressive effect on T cells[21]. However, all these hypotheses are made with utmost caution since we did not offer direct evidence that supports an interaction between the intraluminally injected spheroids and TNF-α and/or the colonic mucosa. In addition, we did not evaluate other possible mediators such as TSG-6 and transforming growth factor-beta.

Taken together, our results demonstrate that intraluminal injection of *in vitro* generated MSC-spheroids at least partially attenuate DSS-induced colitis. The dose is important since only 2.0×10^6 MSCs in spheroids resulted in significantly less body weight loss and lower disease activity score accompanied by a reduction of systemic inflammation, some colonic cytokines, and neutrophils in the distal colon.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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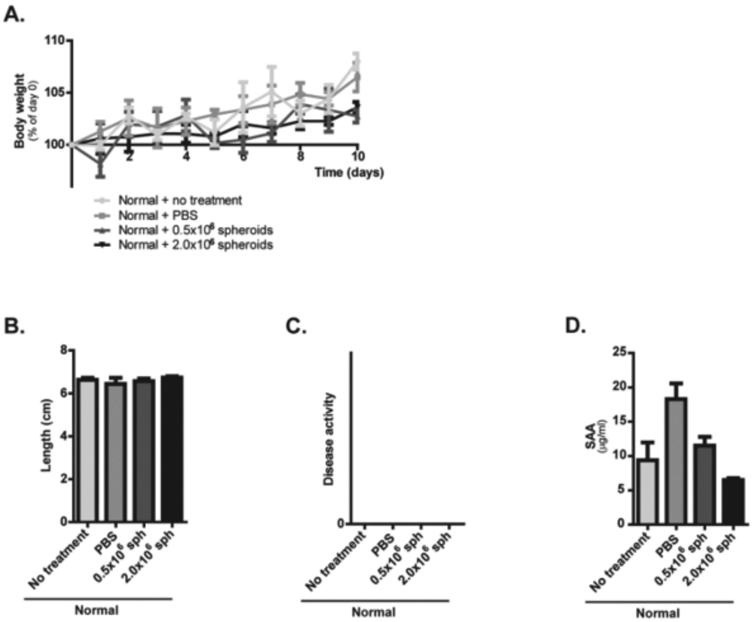
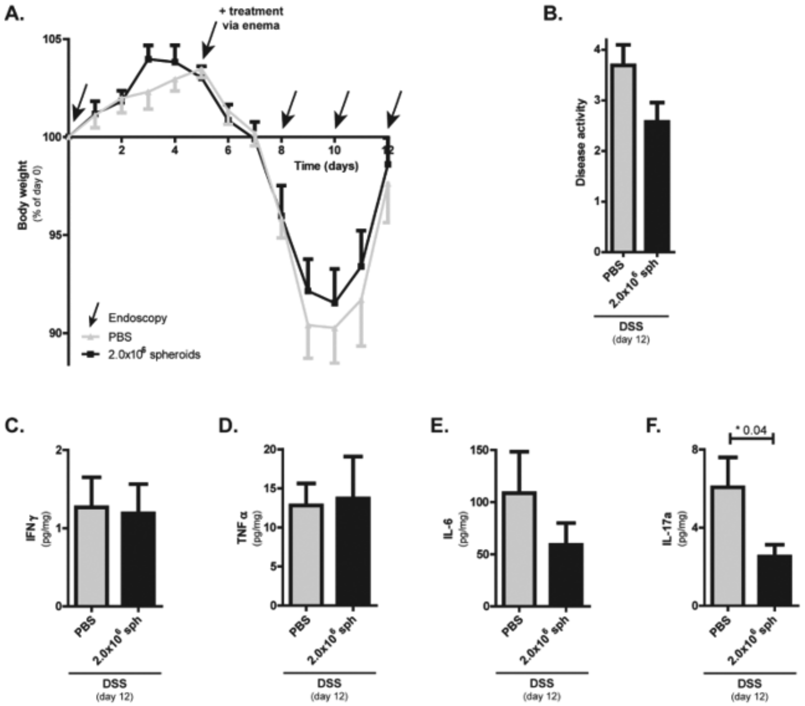
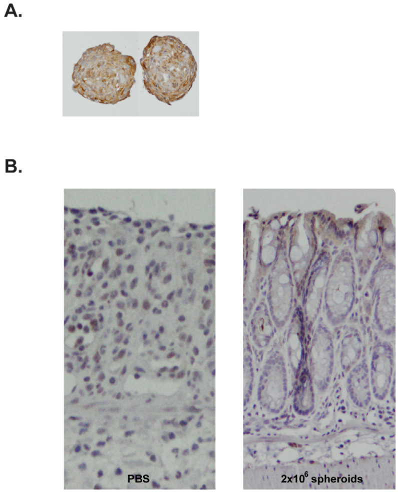


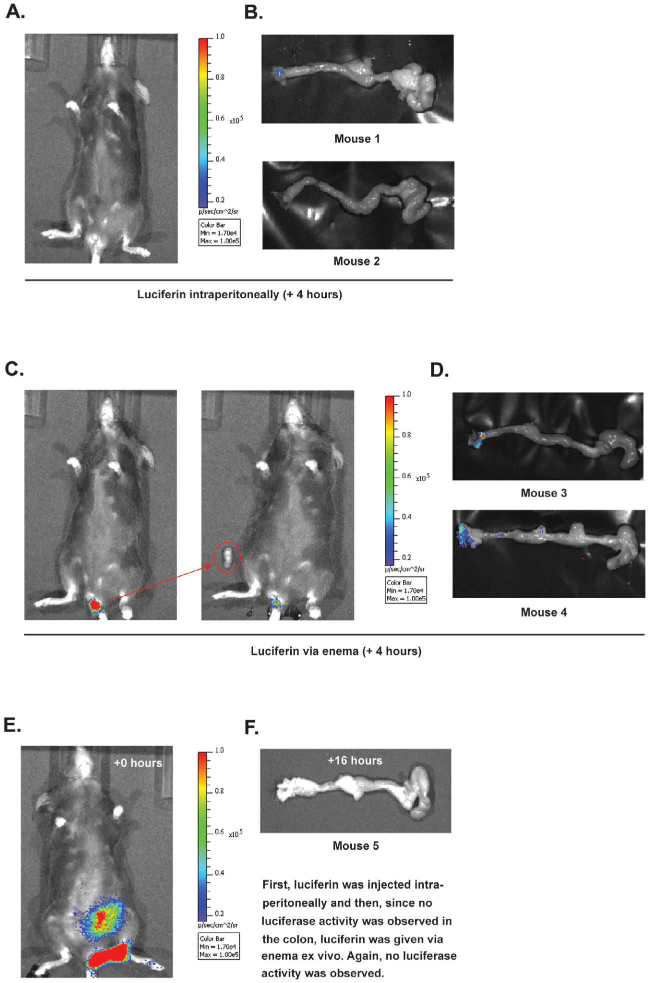
Supplementary FIGURE 1. Intraluminally injected mesenchymal stromal cell (MSC)-spheroids in healthy mice. Mice received $0.5x10^6$ or $2.0x10^6$ MSCs in spheroids, PBS via enema or no treatment at day 5. Mice were sacrificed at day 10. Data are expressed as mean ± SEM. *n* = 3 per group from two separate experiments. A. Body weights were measured daily and expressed as the percentage of body weight at day 0. B. Disease related shortening of the colon. C. Disease activity score based on the presence of loose stool, fecal blood and macroscopic inflammation determined at sacrifice (day 10). D. Measurement of systemic inflammation marker serum amyloid A (SAA).



Supplementary FIGURE 2. Mice sacrificed at day 12 after endoscopy. Dextran sodium sulphate (DSS) was introduced in the drinking water for 7 days. Mice received 2.0x10^6 MSCs in spheroids or PBS via enema at day 5. Mice were sacrificed at day 12 after endoscopic evaluation of colonic inflammation at day 0, 5, 8, 10 and 12. Data are expressed as mean ± SEM. *n* = 9-10 per group. A. Body weights were measured daily and expressed as the percentage of body weight at day 0. B. Disease activity score based on the presence of loose stool, fecal blood and macroscopic inflammation determined at sacrifice (day 12). Cytokine levels of (C) IFN-γ, (D) TNF-α, (E) IL-6 and (F) IL-17A.



Supplementary FIGURE 3. Mesenchymal stromal cell (MSC)-spheroids generated with GFP-positive MSCs. A. *In vitro* generated GFP-positive MSC-spheroids were embedded in paraffin and stained with an anti-GFP antibody. B. At sacrifice (day 10), colons were embedded in paraffin and stained with an anti-GFP antibody. Representative images of the colons of mice with DSS-induced colitis treated with PBS (left) and 2.0×10^6 MSCs in spheroids (right).



Supplementary FIGURE 4. Tracing of mesenchymal stromal cell (MSC)-spheroids in the lumen of the colon after injection via enema. Dextran sulphate sodium (DSS) was introduced in the drinking water and 2.0x10^6 MSCs in spheroids transduced with luciferase were given via enema. Luciferin was given either intraperitoneally or via enema. A. Bioluminescence imaging (BLI) 4 hours after an enema with 2.0x10^6 MSCs in spheroids, luciferin administered intraperitoneally. No signal was observed *in vivo* in both mice. B. Directly after *in vivo* BLI, mice 1 and 2 were sacrificed and the colons were imaged *ex vivo*. Except from a small spot in the distal colon of mouse 1, again no bioluminescent signal was observed. C. BLI 4 hours after an enema with 2.0x10^6 MSCs in spheroids, luciferin administered via enema. Left panel shows *in vivo* imaging of a defecating mouse with anal prolapse with distally luciferase activity indicating the presence of MSCs. Right panel shows the same mouse after defecating. The feces of the mouse is indicated with the red circle. D. Directly after *in vivo* BLI, mice were sacrificed and the colons were imaged *ex vivo*. In both mice 3 and 4, luciferase activity was observed in the distal colon. E. Directly after an enema with 2.0x10^6 MSCs in spheroids and subsequent luciferin by enema high luciferase activity was observed *in vivo* in the abdomen of mouse 5. F. 16 hours later, luciferin was injected intraperitoneally and the mouse was sacrificed to image the colon *ex vivo*. No luciferase activity was observed. Therefore, luciferin was given via enema *ex vivo*. Again, no luciferase activity of MSCs could be traced.