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Intraluminal Injection of Mesenchymal Stromal Cells in Spheroids Attenuates Experimental Colitis

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Abstract

Background and Aims: 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 x 10^6 MSCs in spheroids, 2.0 x 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 x 10^6 MSCs in spheroids compared with PBS-treated mice. Systemic inflammation marker serum amyloid A [SAA] was significantly reduced after treatment with 2.0 x 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 proinflammmatory cytokines, when a high dose of MSCs in spheroids was administered.

Key Words: Experimental colitis; mesenchymal stromal cell; spheroid

1. Introduction

Due to their immunomodulatory properties and their ability to actively participate in tissue repair, mesenchymal stromal cells [MSCs] have 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 and human inflammatory bowel disease [IBD] have been published in the past few years.[2,3,4,5,6] 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 tumour 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 [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.[7,12,13,14,15] 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 tumour 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, ie 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.

2. Material and Methods

2.1. 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 αMEM [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 h, non-adherent cells were removed and the medium was refreshed every 3–4 days. The protocol for the experiments was approved by the Committee on Animal Welfare of the Leiden University Medical Center.

2.2. MSC spheroid formation

Spheroids were formed in 96-well culture plates [Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands] of 2500 MSCs per well. Thus, every spheroid consisted of 2500 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 0.5 x 10^6 or 2.0 x 10^6 MSCs in spheroids were injected locally, meaning 200 spheroids consisting of 2500 MSCs per spheroid in case of 0.5 x 10^6 MSCs in spheroids, and 800 spheroids consisting of 2500 MSCs per spheroid in case of 2.0 x 10^6 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-well culture plates per time point were used to generate spheroids, resulting in 1152–1440 spheroids per time point.

2.3. MSC characterisation

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 analysed using a FACSCanto II flow cytometer with Diva Software [BD Biosciences, San Diego, CA, USA] and the data were analysed with FlowJo software [version 8.7.1., Tree Star Inc. Ashland, OR, USA]. *In vitro* differentiation was performed in 24-well culture plates with MSCs at 80% confluency. MSC spheroids were transferred to 24-well 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 dexamethasone, 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 visualised with Oil-red O staining [Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands].

2.4. 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) 0.5 x 10^6 or 2.0 x 10^6 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 three mice that received 2.0 x 10^6 MSCs in spheroids ['normal' group]. In Supplementary Figure 1 [available as Supplementary data at *ECCO-JCC* online] the results of healthy control groups that received 0.5 x 10^6 MSCs in spheroids [n = 3], PBS [n = 3], or without treatment [n = 3] are shown. Mice were anaesthetised with isoflurane and kept upside down for 5 min 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 2.0 x 10^6 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.

2.5. 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 Days 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 deposition, granularity of the mucosal surface, and stool consistency.[18] MEICS was scored blindly and independently by two researchers. At sacrifice, blood was collected by cardiac puncture and centrifuged [10 000g for 10 min] to collect serum which was stored at -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.

2.6. 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-lengths were measured with Olympus CellSens [version 1.7.1, Zoeterwoude, The Netherlands]. All colons were measured twice, and the researcher was blind to the treatment mice had received. The average percentage of mucosal lesions is shown in Figure 2F.

2.7. 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 min 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 min or with proteinase K [50 µg/ml] for 10 min at 37°C, and subsequently incubated overnight at 4°C with primary antibody in PBS containing 0.1% TritonX-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 [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% TritonX-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, USA]. Sections were counterstained with haematoxylin, 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 x 20. 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.

2.8. 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 homogeniser at 4°C in Greenberger lysis buffer [150 mM NaCl, 15 mM Tris, pH 7.4, 1 mM MgCl2, and 1% TritonX-100]. Samples were centrifuged for 15 min [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 were 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.

2.9. MPO determination

Myeloperoxidase [MPO] activity in the homogenates of distal colon was measured as an index for neutrophil infiltration. In brief, 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 min 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 min using a spectrophotometer; 1 µmol H_2O_2 [= 1 unit of MPO] split gives a change in absorbance of 1.13 x 10^{-2}/min. The number of MPO units was determined as ΔA_{450}/[Δtime x 1.13 x 10^{-2}] and corrected for the total amount of protein per sample. Samples were analysed in duplicate.

2.10. 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 polymerase chain reaction [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 normalise the data. Samples were analysed in triplicate.

2.11. 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.

2.12. Statistical Analysis

To compare two groups, parametric or non-parametric 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.

3. Results

3.1. 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], a low expression of the apoptotic marker cleaved caspase-3 [Figure 1A, right panel], and every spheroid was constructed from 2 500 by flow cytometry characterising 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 0.5 x 10^6 or 2.0 x 10^6 MSCs in spheroids intraluminally via an enema at Day 5, when colitis was established. Treatment with 2.0 x 1 0^6 MSCs in spheroids resulted in significantly reduced body weight loss at sacrifice compared with treatment with 0.5 x 1 0^6 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 2.0 x 10^6 MSCs in spheroids compared with PBS [p < 0.05; Figure 2B and C], 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 2.0 x 10^6 MSCs in spheroids compared with PBS [Figure 2E and F].

To quantify mucosal damage during the colitis, a second experiment was performed. Mice were treated with 2.0 x 10^6 MSCs in spheroids or PBS at Day 5 when colitis was established and endoscopy was performed at Days 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 deposition, 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 deposition. The highest MEICS score was reached at Day 10. At Day 12, treatment with 2.0 x 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 [available as Supplementary data at *ECCO-JCC* online].

3.2. 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 x 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 x 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 x 10^6 MSCs in spheroids vs PBS and p = 0.08 for 2.0 x 10^6 MSCs in spheroids vs PBS; Figure 4C and D].

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 2.0 x 10^6 MSCs in spheroids [IFN-γ, p < 0.05; Figure 5A and B]. In addition, the level of local IL-6 was significantly reduced after treatment with MSC spheroids [p = 0.03 for both 0.5 x 10^6 and 2.0 x 10^6 MSCs in spheroids vs PBS; Figure 5C]. IL-17a was, although not significantly, 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 2.0 x 10^6 MSCs in spheroids significantly reduced both cytokines compared with PBS [Figure 5E and F]. 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].

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 B]. 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].

3.3. 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

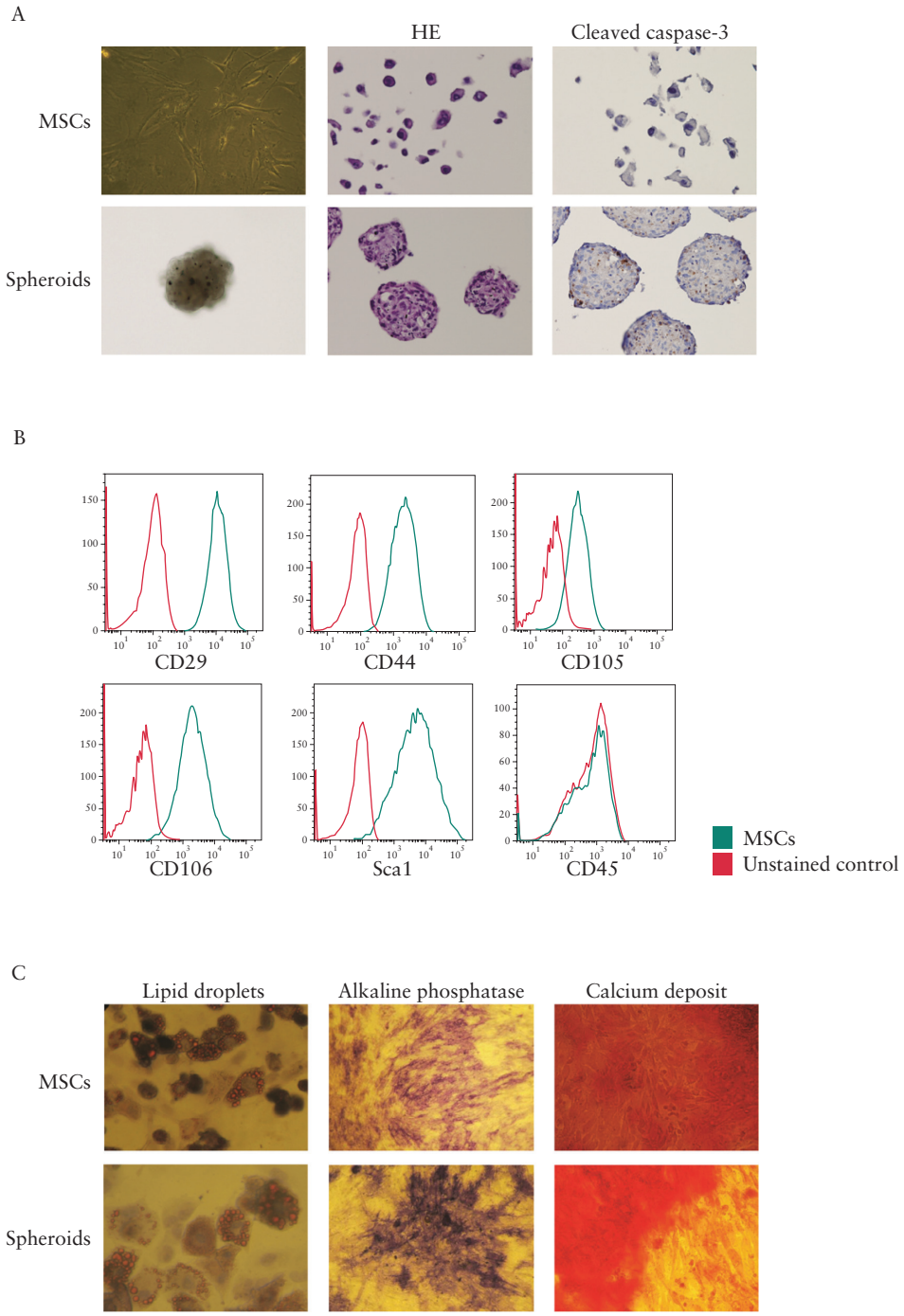


Figure 1. Immunophenotypical characterisation of single cell mesenchymal stromal cells [MSCs] and MSC spheroids. Spheroids were created in 96-well plates with 2500 MSCs per well. [A]: Macroscopic picture [left panel], haematoxylin and eosin [HE] staining [central panel], and cleaved caspase-3 staining [right panel] of spindle shaped MSCs and MSC spheroids. Magnification x 20. [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 x 32.

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].

4. Discussion

Recently, MSCs were observed to form aggregates in the peritoneum, which produced TSG-6 and thereby attenuated DSS-induced

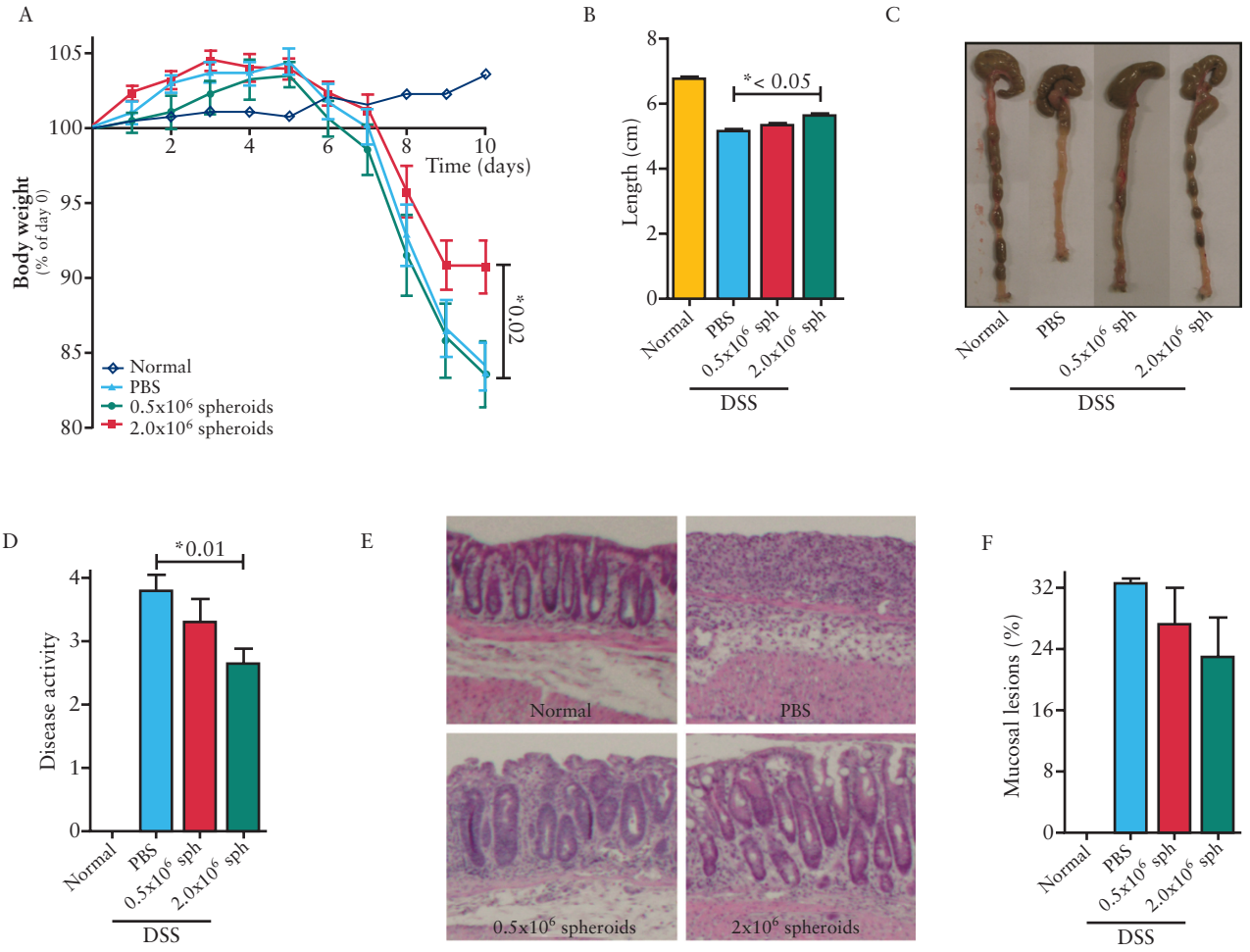


Figure 2: Intraluminally injected mesenchymal stromal cell [MSC] spheroids alleviate dextran sulphate sodium [DSS]-induced colitis. DSS was introduced in the drinking water for 7 days. Mice received 0.5 x 10^6 or 2.0 x 10^6 MSCs in spheroids or phosphate-buffered saline [PBS] via enema at dDay 5. Mice were sacrificed at Day 10. Data are expressed as mean ± standard error of the mean [SEM]; n = 7–14 per group from two separate experiments. The 'normal' group consisted of three healthy mice that received 2.0 x 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 x 10^6 MSCs in spheroids vs both PBS and 0.5 x 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 haematoxylin and eosin [HE]. Magnification x 20. [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 the healthy control group.

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 the healthy state, MSCs tend to spontaneously form spheroids.[16,21] 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 x 10^6 MSCs in spheroids was given. Body weight loss and disease activity score at sacrifice were significantly reduced after treatment with 2.0 x 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.[22,23] 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.[24]

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.[25,26] Locally, SAA can promote IL-6 and TNF-α production by macrophages.[27,28] 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

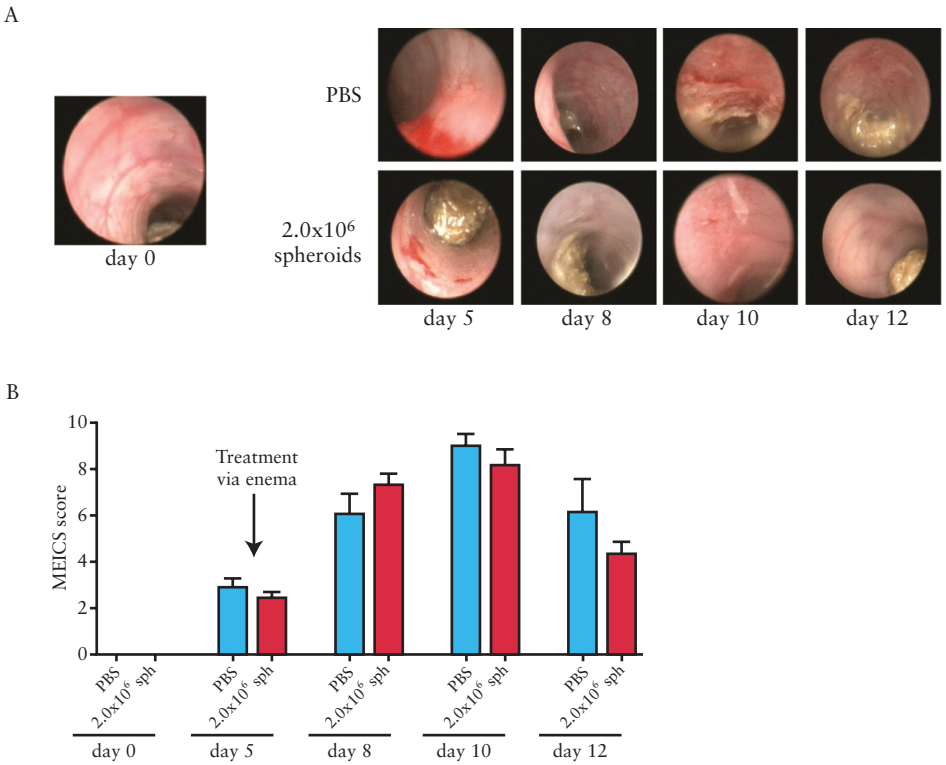
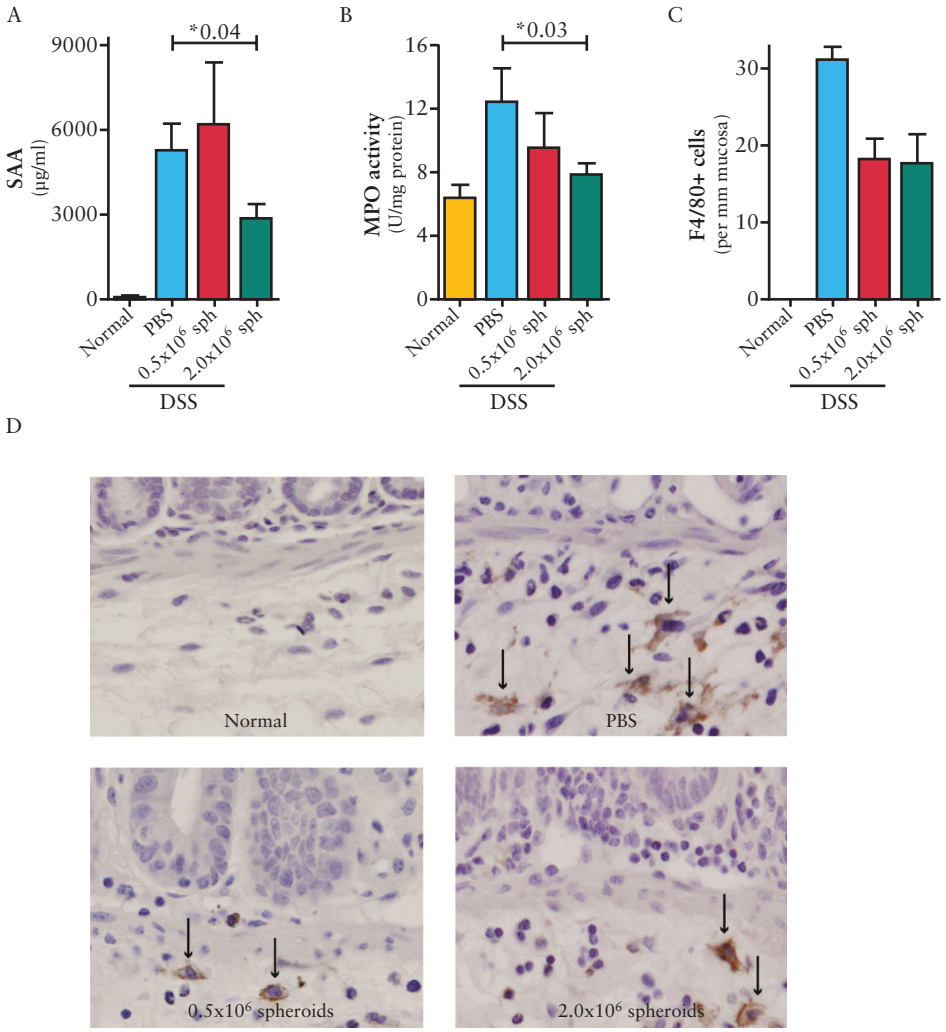


Figure 3. Endoscopic evaluation of colonic inflammation in mice with dextran sulphate sodium [DSS]-induced colitis. DSS was introduced in the drinking water for 7 days. Mice received 2.0 x 10^6 MSCs in spheroids or phosphate-buffered saline [PBS] via enema at Day 5. Endoscopy was performed at Day 0 before DSS introduction and at Days 5, 8, 10, and 12. Mice were sacrificed after the endoscopy at Day 12. Data are expressed as mean ± standard error of the mean [SEM]; $n = 9–10$ per group. [A]: Representative endoscopic images of mice with colitis treated with either 2.0 x 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]

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.[29,30] 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 therein.[31] 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,[32,33] we examined the amount of neutrophils in the distal colons. The amount of neutrophils was significantly lower after intraluminal treatment with 2.0 x 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 colons of treated mice at sacrifice. Both MSCs and 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.[34,35,36] 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.[37] 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.

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 as of major importance in the homeostasis of the colonic milieu,[38,39] clinical trials using IL-10 as a treatment for Crohn's disease have failed to show superiority of IL-10 therapy.[40] In addition, MSCs co-cultured *in vitro* with activated NK cells resulted in reduced levels of IL-10.[41] On the other hand, when MSCs were co-cultured *in vitro* with dendritic cells, IL-10 production was elevated.[42] 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 h after MSC spheroid injection to evaluate this hypothesis. In line with the observed IL-10 levels in the homogenates of the distal



Immunohistological staining of F4/80+ cells

Figure 4. Attenuated colitis is accompanied by reduced systemic and local inflammatory responses. Dextran sulphate sodium [DSS] was introduced in the drinking water for 7 days. Mice received 0.5 x 10^6 or 2.0 x 10^6 mesenchymal stromal cells [MSCs] in spheroids or phosphate-buffered saline [PBS] via enema at Day 5. Mice were sacrificed at Day 10. Data are expressed as mean ± standard error of the mean [SEM]; $n = 4$–8 per DSS group and $n = 2$ in the healthy control group, that received 2.0 x 10^6 MSCs in spheroids from two 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 the healthy control group. [D]: Representative immunohistological sections of mouse colons stained with a F4/80-antibody to reveal macrophages [arrows]. Magnification x 20.

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.[43,44,45] 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 scrupulous 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 [see Supplementary data, available at *ECCO-JCC* online]. 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 B, see Supplementary data available at *ECCO-JCC* online]. However, when luciferin was given as an enema 4 h after intraluminal injection of luc-MSC spheroids, a clear signal was observed distally in the colon [Supplementary Figure 4C and D], 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 any more when luciferin was given

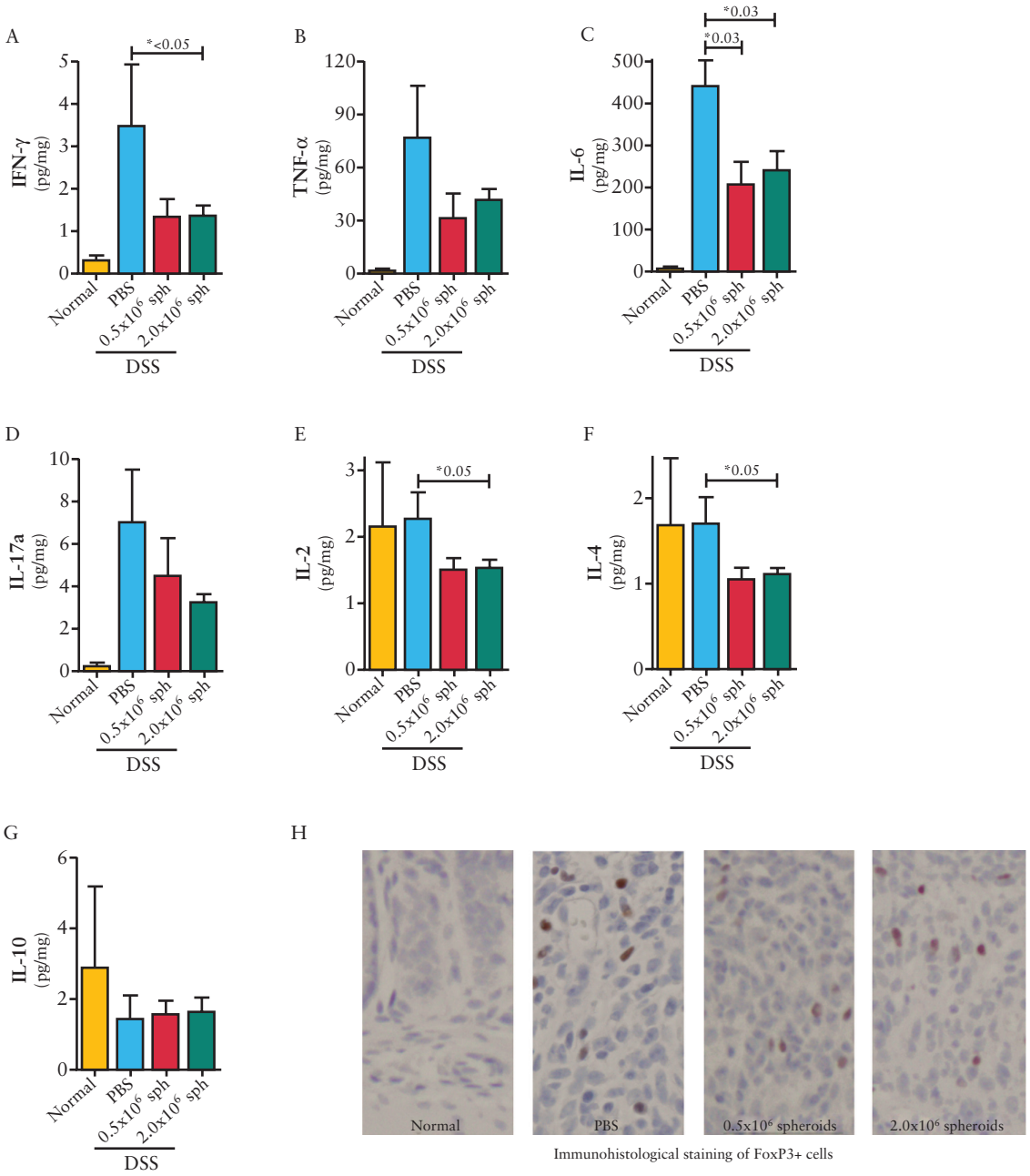


Figure 5. Local treatment with mesenchymal stromal cell [MSC] spheroids alters colonic cytokine production. Dextran sulphate sodium [DSS] was introduced in the drinking water for 7 days. Mice received 0.5 x 10^6 or 2.0 x 10^6 MSCs in spheroids or phosphate-buffered saline [PBS] via enema at Day 5. Mice were sacrificed at Day 10. Data are expressed as mean ± standard error of the mean [SEM]; n = 4–8 per DSS-group and n = 2 in the healthy control group that received 2.0 x 10^6 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 x 20.

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.[46,47] 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 that of 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 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 players in the suppressed differentiation.[45] In addition, spheroid formation was reported to induce increased expression of

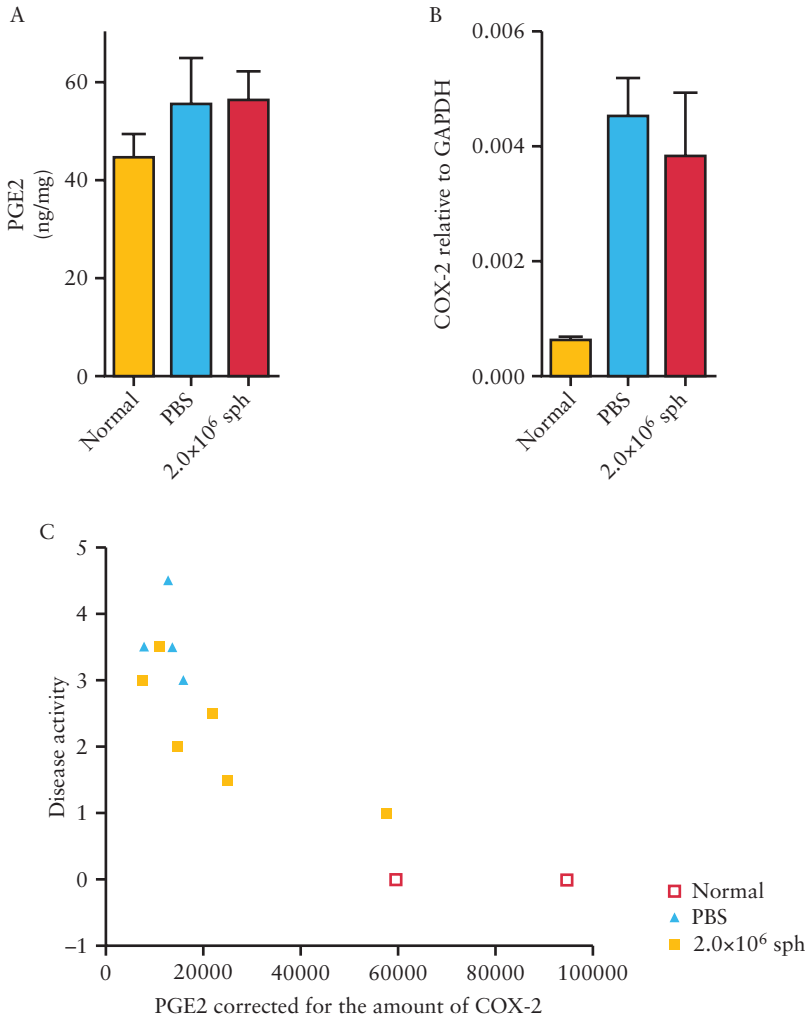


Figure 6. PGE2 and COX-2 RNA in the homogenates of the distal colon. Dextran sulphate sodium [DSS] was introduced in the drinking water for 7 days. Mice received 2.0 x 10^6 mesenchymal stromal cells [MSCs] in spheroids or phosphate-buffered saline [PBS] via enema at Day 5. Mice were sacrificed at Day 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 normalised 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 ± standard error of the mean [SEM]; $n = 4$–6 per DSS-group and $n = 2$ in the healthy control group that received 2.0 x 10^6 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.

COX-2-dependent PGE2 both *in vitro* and *in vivo*.[21] 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.[48,49,50,51] Wound healing was even impaired in COX-$2^{-/-}$ mice with DSS-induced colitis as a result of an inability to increase colonic PGE2.[47] 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.[46] 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 attenuates DSS-induced colitis. The dose is important, since only 2.0 x 10^6 MSCs in spheroids resulted in significantly less body weight loss and

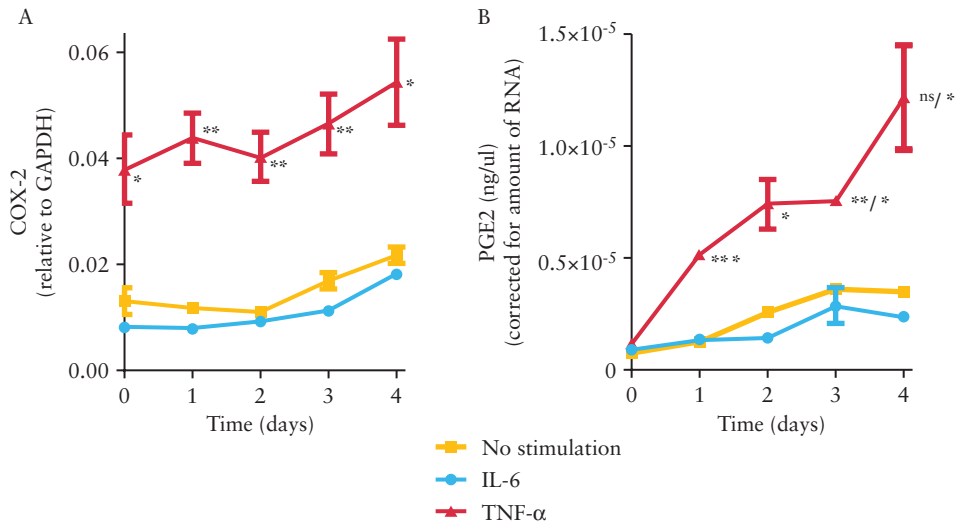


Figure 7. TNF-α-stimulation of mesenchymal stromal cell [MSC] spheroids enhances COX-2 dependent PGE2 secretion *in vitro*. Spheroids were created in 12-15 96-well plates with 2500 MSCs per well, resulting in 1152–1440 MSC spheroids per time point. Culture medium was centrifuged to get rid of debris and stored. RNA was isolated from the MSC spheroids and cDNA was prepared. COX-2 RNA expression was measured in triplicate and normalised to GAPDH and PGE2 levels in duplicate. Data are expressed as mean ± standard error of the mean [SEM]. [A]: MSC spheroids were created without stimulation, with IL-6-stimulation [500 U/ml], or with 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 or TNF-α or without stimulation (Day 0: non-significant [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).

lower disease activity score accompanied by a reduction of systemic inflammation, some colonic cytokines, and neutrophils in the distal colon.

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Conflict of Interest

The authors confirm that there are no conflicts of interest.

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Authors Contributions

IM: concept and design of the study, acquisition of the data, analysis and interpretation of the data, drafting the article, final approval of the version to be submitted. MCB: acquisition of the data, analysis and interpretation of the data, revising the article critically for important intellectual content, final approval of he version to be submitted. EJ-M: acquisition of the data, revising the article critically for important intellectual content, final approval of the version to be submitted. MM-O: acquisition of the data, revising the article critically for important intellectual content, final approval of the version to be submitted. JJR: acquisition of the data, revising the article critically for important intellectual content, final approval of the version to be submitted. DvdH: acquisition of the data, revising the article critically for important intellectual content, final approval of the version to be submitted. DWH: concept and design of the study, revising the article critically for important intellectual content, final approval of the version to be submitted. AM-J: concept and design of the study, revising the article critically for important intellectual content, final approval of the version to be submitted. HWV: concept and design of the study, revising the article critically for important intellectual content, final approval of the version to be submitted.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

References

1. Bernardo ME, Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 2013;**13**:392–402.
2. Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009;**136**:978–89.
3. Liang L, Dong C, Chen X, *et al*. Human umbilical cord mesenchymal stem cells ameliorate mice trinitrobenzene sulfonic acid [TNBS]-induced colitis. *Cell Transplant* 2011;**20**:1395–408.
4. Zhang Q, Shi S, Liu Y, *et al*. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. *J Immunol* 2009;**183**:7787–98.
5. Duijvestein M, Vos AC, Roelofs H, *et al*. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010;**59**:1662–9.
6. Forbes GM, Sturm MJ, Leong RW, *et al*. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol* 2014;**12**:64–71.
7. Krampera M. Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia* 2011;**25**:1408–14.
8. Krampera M, Cosmi L, Angeli R, *et al*. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**:386–98.
9. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* 2007;**110**:91–100.
10. Polchert D, Sobinsky J, Douglas G, *et al*. IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur J Immunol* 2008;**38**:1745–55.

11. Duijvestein M, Wildenberg ME, Welling MM, *et al*. Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. *Stem Cells* 2011;**29**:1549–58.
12. Li W, Ren G, Huang Y, *et al*. Mesenchymal stem cells: a double-edged sword in regulating immune responses. *Cell Death Differ* 2012;**19**:1505–13.
13. Romieu-Mourez R, Francois M, Boivin MN, Stagg J, Galipeau J. Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-gamma, TGF-beta, and cell density. *J Immunol* 2007;**179**:1549–58.
14. Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006;**107**:2570–7.
15. Chan JL, Tang KC, Patel AP, *et al*. Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma. *Blood* 2006;**107**:4817–24.
16. Sala E, Genua M, Petti L, *et al*. Mesenchymal stem cells reduce colitis in mice via release of TSG6, independently of their localization to the intestine. *Gastroenterology* 2015;**149**:163–76.
17. Lee RH, Pulin AA, Seo MJ, *et al*. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;**5**:54–63.
18. Becker C, Fantini MC, Neurath MF. High resolution colonoscopy in live mice. *Nat Protoc* 2006;**1**:2900–4.
19. Melgar S, Karlsson A, Michaelsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2005;**288**:G1328–38.
20. Moolenbeek C, Ruitenberg EJ. The 'Swiss roll': a simple technique for histological studies of the rodent intestine. *Lab Anim* 1981;**15**:57–9.
21. Bartosh TJ, Ylostalo JH, Bazhanov N, Kuhlman J, Prockop DJ. Dynamic compaction of human mesenchymal stem/precursor cells into spheres self-activates caspase-dependent IL1 signaling to enhance secretion of modulators of inflammation and immunity [PGE2, TSG6, and STC1]. *Stem Cells* 2013;**31**:2443–56.
22. Rosenberg L, Nanda KS, Zenlea T, *et al*. Histological markers of inflammation in patients with ulcerative colitis in clinical remission. *Clin Gastroenterol Hepatol* 2013;**11**:991–6.
23. Cellier C, Sahmoud T, Froguel E, *et al*. Correlations between clinical activity, endoscopic severity, and biological parameters in colonic or ileocolonic Crohn's disease. A prospective multicentre study of 121 cases. The Groupe d'Etudes Thérapeutiques des Affections Inflammatoires Digestives. *Gut* 1994;**35**:231–5.
24. Baars JE, Nuij VJ, Oldenburg B, Kuipers EJ, van der Woude CJ. Majority of patients with inflammatory bowel disease in clinical remission have mucosal inflammation. *Inflamm Bowel Dis* 2012;**18**:1634–40.
25. Eckhardt ER, Witta J, Zhong J, *et al*. Intestinal epithelial serum amyloid A modulates bacterial growth in vitro and pro-inflammatory responses in mouse experimental colitis. *BMC Gastroenterol* 2010;**10**:133.
26. Gutfeld O, Prus D, Ackerman Z, *et al*. Expression of serum amyloid A, in normal, dysplastic, and neoplastic human colonic mucosa: implication for a role in colonic tumorigenesis. *J Histochem Cytochem* 2006;**54**:63–73.
27. Song C, Hsu K, Yamen E, *et al*. Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. *Atherosclerosis* 2009;**207**:374–83.
28. Anthony D, McQualter JL, Bishara M, *et al*. SAA drives proinflammatory heterotypic macrophage differentiation in the lung via CSF-1R-dependent signaling. *FASEB J* 2014;**2**:3867-77.
29. Ghia JE, Galeazzi F, Ford DC, Hogaboam CM, Vallance BA, Collins S. Role of M-CSF-dependent macrophages in colitis is driven by the nature of the inflammatory stimulus. *Am J Physiol Gastrointest Liver Physiol* 2008;**294**:G770–7.
30. Watanabe N, Ikuta K, Okazaki K, *et al*. Elimination of local macrophages in intestine prevents chronic colitis in interleukin-10-deficient mice. *Dig Dis Sci* 2003;**48**:408–14.
31. Dieleman LA, Ridwan BU, Tennyson GS, *et al*. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology* 1994;**107**:1643–52.
32. Bian Z, Guo Y, Ha B, Zen K, Liu Y. Regulation of the inflammatory response: enhancing neutrophil infiltration under chronic inflammatory conditions. *J Immunol* 2012;**188**:844–53.
33. Anthony D, Seow HJ, Uddin M, *et al*. Serum amyloid A promotes lung neutrophilia by increasing IL-17A levels in the mucosa and gammadelta T cells. *Am J Respir Crit Care Med* 2013;**188**:179–86.
34. Maggini J, Mirkin G, Bognanni I, *et al*. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS One* 2010;**5**:e9252.
35. Asami T, Ishii M, Fujii H, *et al*. Modulation of murine macrophage TLR7/8-mediated cytokine expression by mesenchymal stem cell-conditioned medium. *Mediators Inflamm* 2013;**2013**:264260.
36. Nemeth K, Leelahavanichkul A, Yuen PS, *et al*. Bone marrow stromal cells attenuate sepsis via prostaglandin E[2]-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–9.
37. Wang M, Zhang G, Wang Y, *et al*. Crosstalk of mesenchymal stem cells and macrophages promotes cardiac muscle repair. *Int J Biochem Cell Biol* 2014;**58C**:53–61.
38. Pigneur B, Escher J, Elawad M, *et al*. Phenotypic characterization of very early-onset IBD due to mutations in the IL10, IL10 receptor alpha or beta gene: a survey of the Genius Working Group. *Inflamm Bowel Dis* 2013;**19**:2820–8.
39. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;**75**:263–74.
40. Kelsall B. Interleukin-10 in inflammatory bowel disease. *N Engl J Med* 2009;**361**:2091–3.
41. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**:74–85.
42. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–22.
43. Tatara R, Ozaki K, Kikuchi Y, *et al*. Mesenchymal stromal cells inhibit Th17 but not regulatory T-cell differentiation. *Cytotherapy* 2011;**13**:686–94.
44. Ghannam S, Pene J, Moquet-Torcy G, Jorgensen C, Yssel H. Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. *J Immunol* 2010;**185**:302–12.
45. Duffy MM, Pindjakova J, Hanley SA, *et al*. Mesenchymal stem cell inhibition of T-helper 17 cell- differentiation is triggered by cell-cell contact and mediated by prostaglandin E2 via the EP4 receptor. *Eur J Immunol* 2011;**41**:2840–51.
46. Matsuura M, Okazaki K, Nishio A, *et al*. Therapeutic effects of rectal administration of basic fibroblast growth factor on experimental murine colitis. *Gastroenterology* 2005;**128**:975–86.
47. Morteau O, Morham SG, Sellon R, *et al*. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. *J Clin Invest* 2000;**105**:469–78.
48. Singer II, Kawka DW, Schloemann S, Tessner T, Riehl T, Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology* 1998;**115**:297–306.
49. Frey MR, Edelblum KL, Mullane MT, Liang D, Polk DB. The ErbB4 growth factor receptor is required for colon epithelial cell survival in the presence of TNF. *Gastroenterology* 2009;**136**:217–26.
50. Frey MR, Hilliard VC, Mullane MT, Polk DB. ErbB4 promotes cyclooxygenase-2 expression and cell survival in colon epithelial cells. *Lab Invest* 2010;**90**:1415–24.
51. Hobbs SS, Goettel JA, Liang D, *et al*. TNF transactivation of EGFR stimulates cytoprotective COX-2 expression in gastrointestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2011;**301**:G220–9.
52. Tessner TG, Cohn SM, Schloemann S, Stenson WF. Prostaglandins prevent decreased epithelial cell proliferation associated with dextran sodium sulfate injury in mice. *Gastroenterology* 1998;**115**:874–82.