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CHAPTER 6

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ABSTRACT
Despite the wide range of available medical therapies for inflammatory bowel disease (IBD) nowadays, treatment of IBD is far from satisfactory and there is an unmet need for effective medical therapeutics in patients with IBD not responding to the conventional strategies. Currently, mesenchymal stromal cells (MSCs) are under investigation as a potential therapeutic option because they possess both immunomodulatory and tissue regenerative properties. However, an optimal treatment strategy of MSC-administration to reach maximal efficacy is not yet known. Therefore, the aim of our current study was to investigate whether timing of human bone marrow-derived MSC (hBM-MSCs) administration and presence of an ongoing inflammatory response are important for treatment efficacy. We induced both mild and severe DSS colitis in mice and treated ‘pre-emptively’ or ‘therapeutically’ with hBM-MSCs. One intraperitoneal therapeutic injection of hBM-MSCs was found to significantly attenuate severe but not mild colitis, accompanied by reduced mucosal IL-6, TNFα and IL-17a levels, resulting in less mucosal damage in the distal colon. Neither in vitro prestimulation of hBM-MSCs with IFNγ nor multiple MSC injections during the colitis, subsequent to a pre-emptive injection, resulted in improvement of efficacy. Furthermore, engraftment of hBM-MSCs at the site of inflammation did not seem to be important to elicit their anti-inflammatory effect suggesting that excreted soluble factors are the key players in MSC-mediated immunosuppression. Taken together, our results indicate that the timing of MSC-administration and the presence of an ongoing severe inflammatory response is crucial to achieve efficacy in DSS-induced colitis.
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Despite the wide range of available medical therapies for inflammatory bowel disease (IBD) nowadays, treatment of IBD is far from satisfactory and there is an unmet need for effective medical therapeutics in patients with IBD not responding to the conventional strategies. Currently, mesenchymal stromal cells (MSCs) are under investigation as a potential therapeutic option because they possess both immunomodulatory and tissue regenerative properties. However, an optimal treatment strategy of MSC administration to reach maximal efficacy is not yet known. Therefore, the aim of our current study was to investigate whether timing of human bone marrow-derived MSC (hBM-MSCs) administration and presence of an ongoing inflammatory response are important for treatment efficacy. We induced both mild and severe DSS colitis in mice and treated ‘pre-emptively’ or ‘therapeutically’ with hBM-MSCs. One intraperitoneal therapeutic injection of hBM-MSCs was found to significantly attenuate severe but not mild colitis, accompanied by reduced mucosal IL-6, TNFα and IL-17a levels, resulting in less mucosal damage in the distal colon. Neither in vitro prestimulation of hBM-MSCs with IFNγ nor multiple MSC injections during the colitis, subsequent to a pre-emptive injection, resulted in improvement of efficacy. Furthermore, engraftment of hBM-MSCs at the site of inflammation did not seem to be important to elicit their anti-inflammatory effect suggesting that excreted soluble factors are the key players in MSC-mediated immunosuppression. Taken together, our results indicate that the timing of MSC-administration and the presence of an ongoing severe inflammatory response is crucial to achieve efficacy in DSS-induced colitis.

INTRODUCTION
Crohn’s disease (CD) and ulcerative colitis (UC), collectively called inflammatory bowel disease (IBD), are chronic diseases characterized by recurrent inflammation of the gastrointestinal tract. An inappropriate immune response to extracellular pathogens in the gut in a genetically predisposed host is thought to be the cause of IBD.1,2 Although a wide range of medical therapies for IBD is available3,4, treatment of IBD is far from satisfactory. In UC a colectomy can be the only effective treatment, however, in CD recurrent disease after surgery is very common. Therefore, there is an unmet need for effective medical therapeutics in patients with IBD not responding to the conventional strategies. Mesenchymal stromal cells (MSCs) are non-haematopoietic precursors of connective tissues and can be found as fibroblast-like cells in the stroma of solid organs and in the bone marrow. MSCs are capable of differentiating in vitro into multiple lineages of the mesenchyme, including osteoblasts, adipocytes and chondroblasts.5-7 Due to their immunomodulatory properties and their ability to actively participate in tissue repair8, MSCs obtained from different sources have emerged as a potential new treatment for IBD. In experimental colitis models, MSCs were reported to ameliorate the inflammatory tissue destruction by downregulating Th1 and Th17 responses in colonic tissue and increasing the regulatory cytokine IL-10.9-12 Several studies, however, indicate that MSCs are not intrinsically immunosuppressive but that activation by proinflammatory cytokines in vivo, e.g. by an ongoing immune response, is necessary to induce their full immunomodulatory capacity. Furthermore, when not primed sufficiently, MSCs might even enhance immune responses.13-15 In addition, in vitro prestimulation of MSCs with proinflammatory cytokines such as IFNγ have been reported to increase efficacy in vivo.9 Therefore, the aim of our current study was to investigate whether the timing of the administration of human bone marrow-derived MSCs (hBM-MSCs) is important for their efficacy in mice with DSS-induced colitis and whether this efficacy is enhanced with multiple injections during the colitis or when hBM-MSCs were prestimulated in vitro with IFNγ. In addition, both mild and severe colitis models were applied to evaluate the importance of an ongoing inflammation in reaching this efficacy.

MATERIALS AND METHODS
MSC isolation
Bone marrow-derived MSC (BM-MSC) were obtained by aspiration from three patients undergoing orthopedic surgery. The bone marrow mononuclear cells (MNC) were isolated by Ficoll-Paque density gradient centrifugation (density: 1.077 g/cm3) and plated at 1.3 x

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10^5/cm^2 in DMEM-low glucose (DMEM-LG; Invitrogen Corp., Paisley, UK) supplemented with 10% fetal calf serum (FCS; Greiner Bio-one) and Penicillin/Streptomycin (P/S; Invitrogen Corp., Paisley, UK). All sampling procedures were performed in accordance with the Helsinki Declaration and use for research purposes was approved by the ethics committee of Leiden University Medical Center (LUMC). All patients provided informed consent.

**MSC expansion and preparation**

Cells were grown in 175 cm^2 flasks (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands) in a 37°C humidified incubator containing 5% CO_2, and the medium was refreshed every 3-4 days. When the spindle shaped MSC monolayer reached >80% confluence, cells were detached using trypsin/EDTA (Invitrogen Corp., Paisley, UK) and replated at a density of 4,000 cells per cm^2. In the indicated experiments, MSCs were cultured in the presence of 10 ng/ml recombinant human interferon-gamma (IFN-γ) (Sigma-Aldrich, St. Louis, MO) for 7 days to generate IFN-γ-stimulated MSCs (IFN-γ-hBM-MSCs). MSCs from passages 4 to 5 were used for transplantation experiments. In some studies MSCs were used after lentiviral transduction with a human vector expressing the enhanced green fluorescent protein (eGFP) gene (a kind gift of Jim Swildens, LUMC, The Netherlands). Immunophenotyping of cultured human MSC was performed using the following primary antibodies: CD90, CD73, human leukocyte antigen (HLA)-ABC, CD31, CD34, CD45, CD80, HLA-DR (BD Biosciences, San Diego, CA, USA), and CD105 (Ancell Corp., Bayport, MN, USA). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA) and the data were evaluated with FlowJo software (version 7.6.3., Tree Star Inc. Ashland, OR, USA).

**In vitro differentiation**

For osteogenic differentiation MSCs were grown to 80% confluency in 24-well culture plates and stimulated for 21 days in osteogenic differentiation medium consisting of complete medium supplemented with 0.1 μM dexamethason, 50 μg/ml Vitamin C (both from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), and 5 mM β-glycerophosphate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and stained for alkaline phosphatase activity with Fast Blue (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). For adipogenic differentiation, MSC were stimulated for 21 days in adipogenic differentiation medium consisting of complete medium supplemented with 0.1 μM dexamethason, insulin (10 μg/ml), indomethacin (5μM) and 3-isobutyl-1-methylxanthine (5μM) (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Formation of lipid droplets was visualized with Oilred O staining (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).
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Figure 1 Administration of DSS in the drinking water for 7 days results in a colitis characterized by body weight loss with shortening and thickening of the colon dependent on the dose of DSS supplied. (A): Body weight changes expressed as a percentage of body weight at day 0. (B): Mean weight/length ratio of the colons per DSS dose at sacrifice (day 9). Colons become shorter and thicker when more inflammation is present. *, p = 0.05 1.0% vs 2.0% DSS and **, p = 0.003 control (0.0%) vs 2.0% DSS. (C): Macroscopic image of a colon after 7 days of 2.0% DSS in the drinking water compared to control (0.0% DSS).
Suppression of PBMC proliferation by MSC

Cultured MSC were plated in graded doses in 96-well flat-bottom plates (Corning, Life Sciences) and allowed to adhere overnight. Human peripheral blood mononuclear cells (PBMCs) isolated from buffy coats (1 x 10^5/well) were added to the MSC and stimulated with human T-activator CD3/CD28 dynabeads (Invitrogen Corp., Paisley, UK) in a bead:cell ratio 1:5. After 5 days of co-culture, cells were pulsed with [3H]-thymidine (0.5 µCi/well) and incubated for 16 h at 37°C. The cultures were harvested on a glass fiber filter and thymidine incorporation was measured with a liquid scintillation counter (Wallac, Turku, Finland). Data are expressed as mean corrected counts per minute (CCPM) of triplicate co-cultures stimulated with anti-CD28/anti-CD3-coated Dynabeads (one bead/5 cells, Invitrogen) and seeded in Iscove’s modified Dulbecco’s media (Invitrogen) supplemented with 5% human serum (Sanquin, Leiden, The Netherlands), and 5% FCS.

Induction of colitis and cell transplantation

All experiments were approved by the Committee on Animal Welfare (CAW) of the Leiden University Medical Center. 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/6 Jico mice (Charles River Laboratories, The Netherlands) with dextran sulphate sodium (DSS; MW 36,000-50,000 kDa; MP Biomedicals, Illkirch, France) supplied in the drinking water for 7 days. Severity of the colitis is defined by their weight loss, with a maximal tolerated weight loss by the CAW of 20%. In order to be able to evaluate improvement and worsening of the induced colitis and based on DSS dose finding experiments, with characteristic shortening and thickening of the colon (figure 1), we induced ‘mild’ colitis with 1.25% DSS, characterized by a mean weight loss of approximately 5%, and ‘severe’ colitis with 1.75% DSS, attained at a weight loss of approximately 15%. In this setting both intentionally ‘pre-emptive’ treatment with MSCs was assessed, by administration prior to onset of colitis, as well as intentionally ‘therapeutic’ treatment of colitis. In the pre-emptive model, six hours before DSS introduction, all mice were injected intraperitoneally with 0.5 x 10^6 hBM-MSCs in 100 µl PBS or with PBS alone as a control (figure 2A). Some groups of mice received, after this pre-emptive injection, three additional injections of hBM-MSCs at day 2, 4 and 6. Mice were sacrificed 10 days after the start of the experiment, except for 2 animals per treatment group with severe colitis. These mice were sacrificed at day 63 to evaluate the long term effect of hBM-MSC therapy (figure 2B). In the ‘therapeutic’ treatment model, mice received 6 hours after DSS introduction one injection with 0.5 x 10^6 hBM-MSCs or IFNγ-hBM-MSCs in 100 µl PBS or with PBS alone as a control (figure 2C). Mice were sacrificed at day 9. Body weight of all mice was measured daily and
disease progression and recovery were calculated as a percentage of weight loss from initial body weight.

**Histological evaluation of disease activity**

Segments of colon were stored in 4% neutral buffered formalin and were serially dehydrated, cleared in xylene and embedded in paraffin. Serial sections of 4 µm were cut, stained with hematoxylin and eosin (H&E) and blindly scored by an independent researcher. The histological disease activity score (maximum score: 28) was calculated using the following criteria: (a) percentage of involved area (0, normal; 1, 1-10%; 2, 10-50%; 3, >50%), (b) number of follicle aggregates (0, normal (0-1); 1, little (2-3); 2 moderate (4-5); 3, extensive (>5)), (c) presence of edema and (d) presence of fibrosis (0, absent; 1, little; 2, moderate; 3, extensive), (e) localisation of erosion/ulceration (0, absent; 1, lamina propria; 2, submucosa; 3, transmural), (f) percentage of crypt loss (0, normal; 1, 1-10%; 2, 10-50%; 3, >50%), (g) infiltration of granulocytes and (h) mononuclear cells (0, normal; 1, few; 2, moderate; 3, extensive) and (i) histologic changes (0, no evidence of inflammation; 1, low level of inflammation with scattered infiltrating mononuclear cells (1-2 foci); 2, moderate inflammation with multiple foci; 3, high level of inflammation with increased vascular density and marked wall thickening; 4, maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells).9

**Immunohistochemistry**

GFP transduced MSCs were detected with an anti-GFP antibody (1:600, Invitrogen, Bleiswijk, The Netherlands). In brief, slides were incubated in 0.3% H2O2/methanol for 20 minutes at room temperature to block endogenous peroxidase activity. After antigen retrieval, 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, 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). 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).

**Cytokine measurements**

Homogenates were prepared from distal colon (n = 5 per treatment group) 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). Total protein content was determined using the BCA
A. Mild colitis (1.25% DSS)
DSS + hBM-MSCs day 0 (n = 30)
DSS + hBM-MSCs day 0, 2, 4 and 6 (n = 10)
DSS + PBS (n = 20)
Control (no DSS and no treatment) (n = 10)

Severe colitis (1.75% DSS)
DSS + hBM-MSCs day 0 (n = 36)
DSS + hBM-MSCs day 0, 2, 4 and 6 (n = 10)
DSS + PBS (n = 20)
Control (no DSS and no treatment) (n = 10)

B. C57BL/6 mice
8 weeks old

I.p. injection of 0.5 x 10^6 hBM-MSCs (100 µl) or 100 µl PBS

DSS in drinking water for 7 days

Normal drinking water until sacrifice

C57BL/6 mice
8 weeks old
**C.**

**Mild colitis (1.25% DSS)**

- DSS + hBM-MSCs (n = 5)
- DSS + IFNγ-hBM-MSCs (n = 5)
- DSS + PBS (n = 5)

**Severe colitis (1.75% DSS)**

- DSS + hBM-MSCs (n = 5)
- DSS + IFNγ-hBM-MSCs (n = 5)
- DSS + PBS (n = 5)

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**FIGURE 2** Design of the in vivo experimental studies. Mild (1.25% DSS) or severe (1.75% DSS) colitis was induced by adding DSS to the drinking water for 7 days. (A): Preemptive model. Eight weeks old female C57BL/6 mice were intraperitoneally injected with 0.5x10^6 hBM-MSCs or PBS at day 0. Six hours later DSS was introduced in the drinking water for 7 days. In case of four injections, successive injections with 0.5x10^6 hBM-MSCs were given at day 2, 4 and 6. Mice were sacrificed at day 10 except for 2 animals per treatment group with severe colitis. (B): These mice were sacrificed at day 63 to evaluate the long term effect of hBM-MSC therapy. (C): Therapeutic model. DSS was added to the drinking water at day 0. Six hours later, mice were intraperitoneally injected with 0.5x10^6 hBM-MSCs, IFNγ-hBM-MSCs or PBS. Mice were sacrificed at day 9.
Protein Assay (Thermo Scientific Pierce, Etten-Leur, The Netherlands) and cytokine levels were measured using the Cytometric Bead Array (BD Biosciences, San Diego, CA, USA) following the manufacturer’s instructions. Cytokine levels measured were corrected for the amount of total protein per mg colon.

**Flow cytometry**
Mice were sacrificed on day 9 after colitis induction with DSS (n = 5 mice per treatment group). Colonic tissue was harvested, minced with fine scissors and placed in a solution of 5 mM EDTA and 1 mM dithiothreitol in Hanks balanced salt solution for 20 min at 37°C under slow rotation to detach epithelial cells. To collect lamina propria cells pieces of colonic tissue were placed into a solution of 2% collagenase I A (Sigma-Aldrich) in PBS and shaken at 37°C for 1 h. The cell suspension was then triturated through a nylon mesh and centrifuged in PBS at 300 g for 10 min at 4°C. Red blood cells in the cell pellet were lysed with lysis buffer (AZL, Leiden, The Netherlands), and the cells were washed in PBS and subsequently resuspended in medium containing IMDM (Lonza, Verviers, Belgium) supplemented with 2.5% FCS and P/S. Total cell numbers were determined with a Sysmex cell counter (Sysmex America, Inc. Mundelein, Illinois, US). In addition, peripheral blood was drawn via cardiac puncture and mononuclear cells were isolated with density centrifugation. Total cell numbers were determined with a Sysmex cell counter (Sysmex America, Inc. Mundelein, Illinois, US). The resulting single-cell suspensions were stained for flow cytometry with primary antibodies for 30 minutes at 4°C in the dark and the cells were washed with PBS/1% human Albumin (Sanquin, Leiden, The Netherlands) before analysis using a FACSCanto II (BD Biosciences, San Diego, CA, USA). The following antibodies were used: F4/80 (AbD Serotec, Düsseldorf, Germany), CD90, B220, Ly6G, NK1.1, CD49b, CD11c, I-A(b), CD11b and CDLy6C (BD Biosciences, San Diego, CA, USA). The analysis of the acquired data was done with FlowJo software version 7.6.1 (Tree Star Inc. Ashland, OR, USA). Monocytes were defined as Ly6Chi or Ly6Clo in a CD11b positive population that was negative for CD90, B220, CD49b, NK1.1, Ly-6G, F4/80, CD11c and I-A(b). Macrophages/dendritic cells were identified in a CD11b positive population expressing F4/80, CD11c and I-A(b) but no CD90, B220, CD49b, NK1.1, Ly-6G.

**Statistical analysis**
Numerical values were expressed as means ± standard error of the mean (SEM). To compare two groups, parametric analyses were performed using an unpaired Student t-test. All analyses were performed using GraphPad Prism software (version 5.01). P ≤ 0.05 was considered statistically significant.
RESULTS

Characterisation of isolated hBM-MSCs
Culture-expanded MSC expressed CD90, CD73, CD105 and HLA-ABC, but did not express the haematopoietic surface markers CD31, CD34, CD45 and CD80 (figure 3A). Furthermore, hBM-MSCs of all donors were able to differentiate into osteoblasts and adipocytes (figure 3B) and inhibited PBMC proliferation in a dose-dependent fashion (figure 3C). Stimulation with IFNγ did not alter the morphology or differentiation capacity of hBM-MSCs. However, upon IFNγ-stimulation, HLA-DR expression on the surface of hBM-MSCs was upregulated (figure 3A).

Administration of hBM-MSCs prior to induction of colitis does not attenuate subsequent colitis
Efficacy of MSCs in an intentionally ‘pre-emptive’ setting was assessed in mice were injected intraperitoneally with 0.5x10⁶ hBM-MSCs or PBS six hours prior to colitis induction, i.e., the start of DSS introduction (figure 2A). One injection with hBM-MSCs resulted in an average decrease in body weight of 5.4±1.5% at sacrifice compared to 6.8±1.8% in PBS-treated mice with mild colitis (p = 0.56) (figure 4A). Mice with severe colitis lost on average 13.0±1.1% in body weight when treated with hBM-MSCs, again not significantly different from mice treated with PBS (14.4±1.3%; p = 0.41) (figure 4B). To examine whether multiple injections during the establishment of colitis could increase the efficacy of hBM-MSCs, ten mice received after the pre-colitis induction injection at day 0 three more injections of 0.5x10⁶ hBM-MSCs at day 2, 4 and 6 (figure 2A). Although in the mild colitis group mice treated with four successive injections of hBM-MSCs had a reduced weight loss compared to a single injection and PBS-treated mice (2.5±1.4% vs 5.4±1.5% and 6.8±1.8%, respectively), this difference was not statistically significant (figure 4A). The body weight of mice with severe colitis decreased 13.9±1.2% in case of treatment with multiple injections which was similar to a single MSC injection and PBS-treated mice (13.0±1.1% and 14.4±1.3%; respectively) (figure 4B).

In the H&E-stained sections of the colon, morphological changes were predominantly observed in the distal colon of the DSS-colitis mice compared to healthy controls. No significant differences in histological disease activity scores between hBM-MSC and PBS treatment were observed in either mild or severe colitis (figure 4C and D). Approximately 14 days after DSS introduction, mice with severe colitis had a body weight comparable to day 0 (data not shown). After seven weeks all mice increased on average 20.0% in body weight and at sacrifice all colons were comparable in length to controls
without colitis (figure 5A and B). Histological evaluation showed no crypt lesions or signs of inflammation (figure 5C). No evidence for malignancy was found in the colons of any of these animals.

**FIGURE 3** Immunophenotypical characterization of the hBM-MSCs. (A): Surface markers for CD90, CD73, CD105, HLA-ABC (positive) and CD31, CD34, CD80 and HLA-DR (negative) on hBM-MSCs as analysed by flow cytometry. Surface markers did not differ between the three donors. When stimulated with IFNγ, hBM-MSCs expressed HLA-DR. (B): Differentiation capacity of hBM-MSCs into osteoblasts (alkaline phosphatase) and adipocytes (lipid droplets). Magnification x10. (C): hBM-MSCs and IFNγ-hBM-MSCs inhibit the proliferation of activated PBMCs in a dose-dependent fashion. Data are expressed as mean ± SEM.

**Colitis is alleviated by administration of hBM-MSCs after colitis induction**

Next, we examined whether administration of hBM-MSCs after colitis induction would increase the therapeutic efficacy MSCs. Mice were injected intraperitoneally with one injection of 0.5x10^6 hBM-MSCs or PBS six hours after the induction of DSS in the drinking water (figure 2C). Previous studies already showed that stimulation of human MSCs with proinflammatory cytokines such as IFNγ increases the immunosuppressive capacities of MSCs. Therefore, we also treated mice with IFNγ-hBM-MSCs.

In the mild colitis model, no major differences in body weight at sacrifice were observed between one of the treatment groups and PBS (figure 6A), although mice treated with
Clinical and histological evaluation of mice with severe colitis 9 weeks after the induction of DSS. Data are expressed as mean ± SEM; n = 2 in all groups. (A): Average body weight gain of mice sacrificed at day 63 expressed as a percentage of body weight at day 0. (B): Macroscopic image of representative colon harvested on day 63 upon sacrifice. (C): Microscopic image of an H&E-stained paraffin slide of a representative colon. Magnification x20.
(IFNγ-)hBM-MSCs showed a clear trend towards higher body weights compared to mice that received PBS. Particularly mice with severe colitis showed consistently less weight loss when treated with hBM-MSCs compared to PBS, from day 7 until the end of experiment (p < 0.05; figure 6C). DSS-induced colitis resulted in changes in the crypt architecture (goblet cell depletion and ulcerations of the epithelium) and infiltration of mononuclear cells in the lamina propria was observed (figure 6C). These lesions were significantly less present after treatment with hBM-MSCs in mice with severe colitis compared to PBS treated mice, as reflected by the histological disease activity score.

**Treatment with hBM-MSCs affect local cytokine levels and monocyte composition in severe colitis.**

To investigate the immunomodulatory effects of hBM-MSCs on experimental colitis, cytokine concentrations were determined in homogenates of the distal colons of mice with severe colitis. Colitis induction by DSS resulted in (dose-dependent) mucosal upregulation of the proinflammatory cytokines IL-6, TNFα and IL-17a in the distal colon (figure 7). Administration of hBM-MSCs in these mice resulted in a clear trend towards lower levels of these cytokines, particularly IL-6 and IL-17a, whereas treatment with IFNγ-hBM-MSCs had hardly any effect (figure 8). After hBM-MSCs injection the IL-17a levels were even decreased to levels observed in healthy control mice (figure 7 and 8A). The reduction in the cytokine profile in the intestinal mucosa suggests that hBM-MSCs may affect (pro)inflammatory subtypes of monocytes. Interestingly, the ratio of the anti-inflammatory Ly6Clo- over the Ly6Cchio-monocytes in the colonic mucosa was remarkably higher in the hBM-MSC-treated mice as opposed to the IFNγ-hBM-MSCs and PBS treated mice, whereas in the peripheral blood the Ly6C^lo^/Ly6C^hi^ ratios were highly comparable (figure 8B).

**hBM-MSCs cluster at the serosal side of the colon**

To assess whether MSCs migrate to the inflamed colon, hBM-MSCs were transduced with GFP and intraperitoneally injected at day 0 of DSS introduction. Ten days later, mice were sacrificed. We were able to trace the injected MSCs at that time point as immunohistochemistry of the colons revealed GFP-positive cells at the serosal side of the colon. There they clustered to form spheroid-like structures as illustrated in figure 9. MSCs were never observed within the colonic (sub)mucosa, indicating that they did not transmigrate into the damaged luminal mucosal wall.
**DISCUSSION**

Although encouraging results on MSC-treatment in human IBD have been published\textsuperscript{18,19}, the exact moment and frequency of administration to reach an optimal efficacy is not clear yet. The present study indicates that the time of administration is crucial to achieve efficacy of hBM-MSCs, at least in DSS-induced colitis. One injection of hBM-MSCs prior to colitis induction did not alleviate mild or severe colitis. However, ‘therapeutic’ administration of hBM-MSCs, i.e., injection after colitis induction, ameliorated severe colitis indicating that an initiated/ongoing severe inflammation is needed before MSCs exert their immunosuppressive capacity. Supporting this hypothesis were the observations that hBM-MSCs injected after the start of DSS did not result in a significantly reduced weight loss and lower histological disease activity score in mice with mild colitis model. In contrast, in mice of the ‘pre-emptive’ study, i.e., MSC injection prior to DSS-colitis induction, treated with three extra injections at day 2, 4 and 6, the efficacy of hBM-MSCs did not increase although these latter injections were given during the establishment of the colitis with ongoing inflammation. Although MSCs do not seem to elicit alloreactive lymphocyte responses and have a low expression of MHC-I without the presence of co-stimulatory molecules\textsuperscript{20-22}, they do express MHC-II intracellularly.\textsuperscript{7} Nauta et al.\textsuperscript{23} showed in a murine transplantation model, with sublethally irradiated recipients, that allogeneic bone marrow engraftment was hampered when co-transplanted with allogeneic or third-party MSCs, whereas syngeneic MSCs promoted engraftment. Interestingly, memory T cells were generated suggesting that MSCs are not intrinsically immunoprivileged and that they can trigger an immune response in vivo after multiple injections of the same MSC-donor. This notion is supported by Eliopoulos et al.\textsuperscript{24} who showed that splenocytes from mice that received implants with human MSCs for 15 days, had a strong IFN\textgreek{g} response after coculture in vitro with the same human MSCs for 24 hours. This elevation of IFN\textgreek{g} was not observed after coculture with syngeneic mouse MSCs or when human MSCs were cocultured with splenocytes of mice that did not receive an implant with human MSCs prior to the in vitro coculture. Taken together, these results suggest that allogeneic and xenogeneic MSCs may provoke a specific anti-donor T cell response in immunocompetent mice with generation of memory T cells leading to a possible enhanced rejection of these cells after multiple injections. Although these results argue against the potential of an ‘off-the-shelf’ repeated treatment of allogeneic MSCs, the possibility of an unwanted immune response after administration of allogeneic or xenogeneic MSCs in mice does not per definition resemble the situation in humans. Multiple clinical trials have proven allogeneic MSC treatment to be safe and feasible.\textsuperscript{25-28} Recently, Forbes et al.\textsuperscript{18} published the results of an open-label study investigating the efficacy of allo-
FIGURE 6 DSS-induced colitis is alleviated by administration of hBM-MSCs after colitis induction. DSS was introduced in the drinking water for 7 days. Six hours after this introduction mice received 0.5x10^6 hBM-MSCs or PBS. Mice were sacrificed at day 9. Data are expressed as mean ± SEM; n = 5 in all groups. See figure 2 for study design. Body weight changes of mice with (A): mild and (B): severe colitis. The values of body weight change are expressed as a percentage of body weight at day 0. In mild colitis: *, p = 0.05 IFNγ-hBM-MSCs vs PBS and p = 0.051 hBM-MSCs vs PBS. In severe colitis: day 7 *, p = 0.05, day 8 *, p = 0.02 and day 9 *, p = 0.02 hBM-MSCs vs PBS. (C): Microscopic images of H&E-stained paraffin slides of representative colons of mice with severe colitis. Magnification x20. (D): Histology scores of colons with mild and severe inflammation harvested on day 9. Scores were determined after microscopic analysis of the colon slides from each mouse. * p = 0.05 hBM-MSCs vs PBS.

geneic MSCs in moderate to severe refractory CD.18 Fifteen patients received four infusions of 2x10^6 cells/kg at weekly intervals. Three weeks after the last infusion of MSCs clinical response was observed in 80% of the patients and clinical remission in even 53% of the pati-
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**FIGURE 7** Adding DSS to the drinking water results in elevated levels of IL-6, TNFα and IL-17a in the distal colon. Colon homogenates were prepared from the distal colons with mild and severe colitis and cytokine levels were measured. Data are expressed as mean ± SEM; n = 5 in colitis groups and n = 2 in control group. Local cytokine levels of (A): IL-6, (B): TNFα and (C): IL-17a in the distal colon of mice with mild and severe colitis.

**FIGURE 8** Treatment with hBM-MSCs affect local cytokine levels and monocyte composition in severe colitis. Data are expressed as mean ± SEM; n = 5 in all groups. (A): Colon homogenates were obtained from the distal colons after sacrifice at day 9 and local cytokine levels of IL-6, TNFα and IL-17a were measured. *, p = 0.05 hBM-MSCs vs IFNγ-hBM-MSCs and **, p = 0.004 hBM-MSCs vs PBS. (B): Ratio Ly6C^hi/Ly6C^hi-monocytes in distal colon (left panel) and peripheral blood (right panel).
FIGURE 9 hBM-MSCs cluster at the serosal side of the colon. hBM-MSCs were transduced with GFP and injected at day 0. Mice were sacrificed at day 10. Immunohistochemistry showed that GFP-positive cells were found at the serosal side of the colon where they clustered to form spheroid-like structures. (A): Anti-GFP staining of the distal colon of a mouse with severe colitis. Magnification x20 and zoom in. (B): Histological image of same spheroid as in (A) but further in serial slides. Magnification x20. (C): Microscopic image of H&E-stained paraffin slide with matching anti-GFP staining. Magnification x20 and zoom in.

ents. In contrast, a previous study from our group using a single dose of autologous MSC in CD was found to be relatively ineffective.19 Earlier studies have shown that stimulation of human MSCs with proinflammatory cytokines such as IFNγ increases the immunosuppressive capacity of MSCs.16,17 On the other hand, MSCs can enhance immune responses when IFNγ levels are not sufficient13-15 and they can even become antigen presenting cells by upregulating of MHC class II.29-31 In our hands, in vitro stimulation with IFNγ before injection did not result in an increased efficacy of hBM-MSCs compared to unstimulated hBM-MSCs. It could be that the optimal administration time point of immunostimulated MSCs is different from unstimulated MSCs and that therefore we were not able to increase the effectiveness of hBM-MSCs in DSS-induced colitis.

Since Ly6Chi-monocytes are rapidly recruited from peripheral blood to the inflamed colon after DSS introduction, promoting the proliferation of effector cells that produce IL-6, IL-23
and TNFα, Ly6C\textsuperscript{hi}-monocytes may be crucial in the establishment of colitis\textsuperscript{32,33}. Moreover, DSS-induced colitis was attenuated when migration of these monocytes to the inflamed colon was hampered\textsuperscript{33}. In our hands, levels of IL-6, TNFα and IL-17a, produced by IL-23 induced Th17-cells\textsuperscript{34}, were elevated in the distal colons at sacrifice and reduced after treatment with hBM-MSCs. Interestingly, the relative number of Ly6C\textsuperscript{lo}-monocytes in the colonic mucosa, responsible for promoting tissue repair processes and homeostasis in a non-inflammatory situation\textsuperscript{35}, was increased after treatment with hBM-MSCs suggesting their contribution to the conversion of the proinflammatory milieu to a more regulatory one. Whether MSCs need to migrate to the colon to induce immunosuppression is not clear. We were able to trace hBM-MSCs ten days after intraperitoneal injection at the serosal side of the colon where they formed spheroids. Although MSCs are capable of transmigrating through endothelial cells\textsuperscript{36}, we never observed transmigration into the colonic wall. In addition, in line with previously findings\textsuperscript{9,37}, the amount of cells we found was very low as we did not observe spheroids in all mice that received GFP-positive MSCs. The importance of engraftment is therefore doubtful and recent data suggests that the secretion of soluble factors is the key factor in MSC-mediated immunosuppression\textsuperscript{38,39}.

In conclusion, we showed that injection of hBM-MSCs after but not prior to DSS-colitis induction alleviated severe colitis by reducing the production of proinflammatory cytokines resulting in less colonic damage. This was not observed in mild colitis. These findings suggests that the time of administration and an ongoing inflammation are crucial to achieve efficacy of hBM-MSCs in DSS-induced colitis.

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