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Stem cell therapy for inflammatory bowel disease
Duijvestein, M.

CHAPTER 5

Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis

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ABSTRACT

Mesenchymal stromal cells (MSCs) are currently under investigation for the treatment of inflammatory disorders, including Crohn's disease. MSCs are pluripotent cells with immunosuppressive properties. Recent data suggest that resting MSCs do not have significant immunomodulatory activity, but that the immunosuppressive function of MSCs has to be elicited by interferon-gamma (IFN-γ). In this article, we assessed the effects of IFN-γ prestimulation of MSCs (IMSCs) on their immunosuppressive properties in vitro and in vivo. To this end, we pretreated MSCs with IFN-γ and assessed their therapeutic effects in dextran sodium sulfate (DSS)- and trinitrobenzene sulfonate (TNBS)- induced colitis in mice. We found that mice treated with IMSCs (but not MSCs) showed a significantly attenuated development of DSS-induced colitis. Furthermore, IMSCs alleviated symptoms of TNBS-induced colitis. IMSC-treated mice displayed an increase in body weight, lower colitis scores, and better survival rates compared to untreated mice. In addition, serum amyloid A protein levels and local proinflammatory cytokine levels in colonic tissues were significantly suppressed after administration of IMSC. We also observed that IMSCs showed greater migration potential than unstimulated MSCs to sites within the inflamed intestine. In conclusion, we show that prestimulation of MSCs with IFN-γ enhances their capacity to inhibit Th1 inflammatory responses, resulting in diminished mucosal damage in experimental colitis. These data demonstrate that IFN-γ activation of MSCs increases their immunosuppresive capacities and importantly, their therapeutic efficacy in vivo.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent cells present in virtually all tissues of the body.[1] In addition to their function as progenitor cells, MSCs have been found to have significant immunosuppressive capacities.[2-4] Recent data suggest that resting MSCs do not have immunomodulatory activity, but when activated by IFN-γ, MSCs develop their full immunosuppressive potential.[5, 6] IFN-γ-activated MSCs secrete large amounts of chemokines to attract T-cells and inhibit T-cell proliferation and proinflammatory cytokine production.[6] Different mechanisms have been proposed that describe how this suppression of T-cell activity might be mediated. In humans, IFN-γ-induced indoleamine 2,3-dioxygenase (IDO) expression appears to be important,[7] whereas the expression of inducible nitric oxide synthase (iNOS) may be more crucial in mice. MSCs lacking the IFN-γ receptor or iNOS are incapable of inhibiting disease progression in a mouse model of graft-versus-host disease (GvHD).[8, 9] However, pretreatment of MSCs with IFN-γ was found to prevent the development of murine GvHD more efficiently than unstimulated MSCs.[9]

Due to their immunomodulatory properties, much interest has focused on investigating MSCs as a potential treatment for inflammatory disorders.[10] In fact, transplantation of MSCs has already been successfully used for treating GvHD.[11-15] We recently demonstrated that MSC administration is safe and feasible in patients with refractory Crohn's disease,[16] and various ongoing studies are aimed at evaluating the proper dose and mode of administration needed for effective results. Alternatively, potentiation of MSC effector mechanisms may improve the efficacy of anti-inflammatory treatment. Given the central role of IFN-γ activation on the immunosuppressive potential of MSCs, we examined the effect of IFN-γ pretreatment of MSCs in two mouse models of colitis.

MATERIAL AND METHODS

Mesenchymal stromal cell isolation

Human bone marrow

MSCs were expanded following a previously described protocol developed by the European Group for Blood and Bone Marrow Transplantation (EBMT).[14] In brief, bone marrow was harvested from patients undergoing orthopedic surgery. Bone marrow mononuclear cells (MNCs) were isolated by Ficoll density gradient centrifugation (density: 1.077 g/cm^3). Cells were then washed and resuspended in low-glucose Dulbecco's Modified Eagle Medium (Invitrogen, Paisley, UK) supplemented with penicillin and streptomycin (Lonza, Verviers, Belgium) and containing 10% fetal bovine serum (FBS, HyClone, Logan, UT) without any additional growth factors. MSCs were plated at a density of 160,000 cells per cm^2. All sampling procedures were performed in accordance with the Helsinki Declaration and were approved by the ethics committee of Leiden University Medical Center (LUMC). All patients provided informed consent.

Mouse bone marrow

MSCs were isolated from 8- to 10-week-old BALB/c mice (Harlan Laboratories, Horst, the Netherlands). Femoral heads and condyles were

removed and bone marrow was flushed. Cells were plated in alpha-minimum essential medium (alpha-MEM, Lonza) supplemented with penicillin and streptomycin (Lonza), 3 mM L-glutamine (Invitrogen) and 10% FBS (Invitrogen). After 24 hours, non-adherent cells were removed. Adherent cells were washed with phosphate-buffered saline (PBS), and medium was changed every 3-4 days. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. All experiments were approved by the Animal Care and Use Committee of the LUMC.

MSC expansion and preparation

Cultures 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. When the cultures reached >80% confluence, cells were trypsinized (trypsin/EDTA; Lonza) and replated at a density of 4000 cells per cm^2. MSCs were passaged no more than five times prior to use. To generate IFN-γ-prestimulated MSCs (hereafter referred to as IMSCs), cells were plated at the same density in media supplemented with either 500 U/mL recombinant human IFN-γ (Sigma-Aldrich, St Louis, Missouri, USA) or murine IFN-γ (PeproTech, Rocky Hill, NJ). Immunophenotyping of cultured MSCs was performed using flow cytometry for the following markers: MHCII, CD73, CD90, CD14, CD34, CD45, CD19 and CD105 (BD Biosciences, Franklin Lakes, NJ, USA). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (version 7.2.5., Treestar, San Carlos, CA, USA).

In vitro differentiation

MSCs were plated at a density of $5x10^4$ cells/cm^2 in 24-well culture plates and kept in complete medium until cells reached 80-90% confluency. For osteogenic differentiation, cells were stimulated for 21 days in standard medium supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerolphosphate and 10^{-8} M dexamethasone and then stained for alkaline phosphatase with Fast Blue (Sigma-Aldrich). For adipogenic differentiation, cultures were stimulated for 21 days with complete medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 100 μM indomethacin, 5 μg/mL insulin, and 10^{-6} M dexamethasone (Sigma-Aldrich). Lipid droplets were visualized by Oil Red O staining. For chondrogenic differentiation, MSCs were grown in serum-free, high-glucose Dulbecco's Modified Eagle Medium (Invitrogen) medium containing 50 μg/mL L-ascorbic-2-phosphate, 40 μg/mL L-proline and 100 μg/mL sodium pyruvate (Sigma-Aldrich) and supplemented with insulin-transferrin-selenium (ITS)+ culture supplement (BD Biosciences), 10 ng/mL transforming growth factor (TGF)-β1 (R&D systems, Abingdon, United Kingdom), 10^{-7} M dexamethasone (Sigma-Aldrich) and 500 ng/mL bone morphogenetic protein (BMP)-2 (R&D systems). Cells were stained with Toluidine blue to confirm chondrogenic differentiation.

RNA isolation and quantitative pcr

RNA was isolated using the RNAeasy mini kit (Qiagen, Hilden, Germany) and cDNA was generated using RevertAid reverse transcriptase (Fermentas, St Leon-Rot, Germany) and random hexamer primers

(Promega, Leiden, The Netherlands). Quantitative pcr reactions were carried out using Invitrogen primers for IDO (forward GATGAAG AAGTGGGCTTTGC, reverse CAGGCAGATGTTTAGCAATGA) and SybrGreen PCR Kit (Qiagen). For relative expression, data were normalized against expression of the household gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of inducible NOS (iNOS)

iNOS levels were measured in cell lysates using the PathScan Total iNOS Sandwich ELISA Kit (Cell Signaling Technology, Bioké, Leiden, the Netherlands) following the manufacturer's instructions.

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

Cultured human MSCs were plated in 96-well flat-bottom plates (Corning) and allowed to adhere overnight. Human PBMCs isolated from buffy coats were stimulated with anti-CD28/anti-CD3-coated Dynabeads (1 bead/5 cells, Invitrogen) and were seeded in Iscove's Modified Dulbecco's Media (IMDM, Invitrogen) supplemented with 5% human serum (Sanquin, the Netherlands), 5% FBS and 100 IU/mL interleukin (IL)-2 (LUMC Pharmacy, Leiden, the Netherlands). Transwell experiments were performed using 0.2μm membrane inserts (Nunc, Denmark). Cell proliferation was measured by ^{3}H-thymidine incorporation.

Induction of colitis and study design

Animals were housed in individually ventilated cages and supplied with drinking water and food ad libitum. Experiments were conducted using 6- to 8-week-old female mice. For dextran sulfate (DSS)-induced colitis, C57BL/6Jlco mice (Charles River, the Netherlands) were supplied with drinking water supplemented with 2.25% (w/v) DSS (MW 36 000-50 000 kDa; MP Biomedicals, Illkirch, France) for seven days. On day 0, mice were injected intraperitoneally with 0.5×10^{6} human MSCs diluted in 100 μL PBS, 0.5×10^{6} human IMSCs diluted in 100 μL PBS or a vehicle control (PBS alone). All mice were sacrificed on day 9 after the start of the experiment (Figure 1A).

Trinitrobenzene sulfonate (TNBS, Sigma-Aldrich) colitis was induced in BALB/c mice (Harlan Laboratories, Netherlands). To study the therapeutic effect of MSCs on established colitis, mice were presensitized through the skin on day 0. On day 7, 2.75 mg TNBS diluted in 40% ethanol was injected intrarectally (4 cm from the anus) using a 22 G catheter (Abbocath, Hospira Benelux, Brussels, Belgium). Six hours after the second TNBS instillation, mice were injected intraperitoneally with 1.0×10^{6} mouse MSCs, 1.0×10^{6} mouse IMSCs or PBS alone. All mice were sacrificed on day 9 (Figure 1B). For survival analysis, an additional cohort of BALB/c mice was treated intrarectally with 3.25 mg TNBS diluted in 40% ethanol on days 0 and 6. In this case, mice were injected intraperitoneally with 1.0×10^{6} mouse MSCs, 1.0×10^{6} mouse IMSCs or PBS alone 6 hrs after the first TNBS instillation (Figure 1C).

Assessment of inflammation

Body weights were recorded daily and wasting disease progression was calculated by percentage of weight loss from initial body weight. Animals

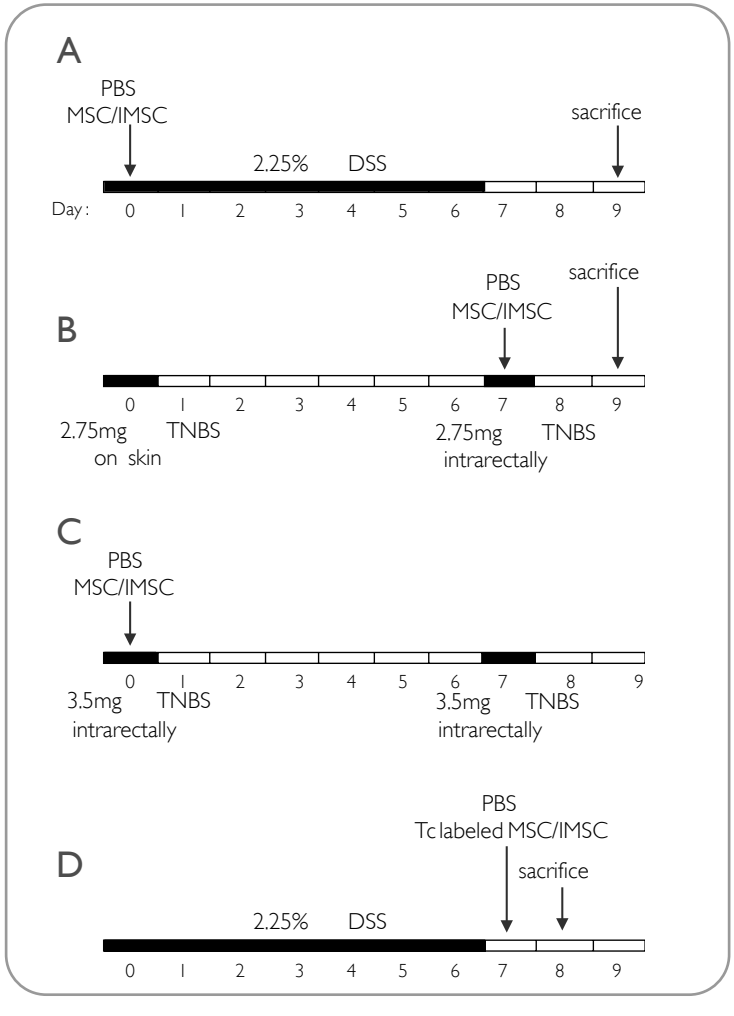

Figure 1. Schematic overview of experimental colitis experiments.

were withdrawn from the study when weight loss was >20% compared to that on day 0 or when weight loss was >10% within 24 hours. Blood samples were collected by cardiac puncture immediately upon sacrifice; serum was separated by centrifugation (10 000 x g for 10 min) from whole blood and stored at -20°C until use. Colons were removed and opened longitudinally. Two independent investigators blinded for treatment allocation scored the colons for the presence of loose stool, visible fecal blood, and macroscopic inflammation using a scale of 0 to 3 per item with a maximum score of 9, as previously described.[17] Tissue weights were recorded and used as an index of disease-related intestinal wall thickening. Colons were subsequently divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine determination, while the second part was stored in formalin and embedded in paraffin for (immuno)histological evaluation.

Histological analysis

Colonic segments were fixed in 10% neutral buffered formalin, serially dehydrated, cleared in xylene, and embedded in paraffin wax. Sections (4 µm thick) were collected on coded slides, stained with hematoxylin and eosin (H&E), and then independently and blindly scored by an experienced pathology resident. The histology damage score was calculated using the following criteria: (1) percentage of area involved, (2) number of follicle aggregates, (3) edema, (4) fibrosis, (5) erosion/ulceration, (6) crypt loss, and infiltration of (7) mononuclear and (8) polymorphonuclear cells. The percentage of area involved and crypt loss were scored on a scale of 0 to 4 (0, normal; 1, <10%; 2, 10%; 3, 10-50%; 4, >50%). Erosions were defined

as 0 if the epithelium was intact, 1 if the lamina propria was involved, 2 if ulcerations involved the submucosa, and 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale of 0 to 3 (0, absent; 1, weak; 2, moderate; 3, severe). The histology damage score ranged from 0 points to a maximum of 26 points.[18]

Immunohistochemistry

T-cells were detected using an anti-CD3 antibody (1:1000, DakoCytomation BV, Eindhoven, the Netherlands). In brief, slides were incubated in 0.3% H_2O_2/methanol for 15 minutes at room temperature. After antigen retrieval, slides were blocked with Teng-T (10 mmol/L Tris, pH 8.0, 5 mmol/L EDTA, 0.15 mol/L NaCl, 0.25% gelatin, 0.05% (v/v) Tween-20) for 30 minutes and followed by overnight incubation at 4°C with primary antibody in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA). Primary antibodies were detected using an avidin-biotin detection system. Secondary antibodies were obtained from DakoCytomation BV (1:200) (anti-rabbit, E0432), diluted in PBS containing .01% Triton-X and 1% BSA and incubated for 1 hour at room temperature. Slides then were incubated for 30 minutes with horseradish-peroxidase (HRP)-conjugated streptavidin-biotin (DakoCytomation BV). Antibodies were detected using the PowerVision Plus Poly-HRP detection system from Immunologic (DPVB + 110 HRP, Duiven, the Netherlands), and peroxidase activity was detected with Fast 3.3'-diaminobenzidine tablets (Sigma-Aldrich). Sections were counterstained with hematoxylin, dehydrated, and mounted in Pertex (Histolab Products AB, Göteborg, Sweden). Slides were analyzed and scored using ImageJ software (http://rsb.info.nih.gov/ij/).

Cytokine measurements

Cytokine levels were determined in colon mucosa. Colon homogenates were obtained using a Potter-Elvehjem glass homogenizer at 4°C in four volumes of Greenberger lysis buffer (150 mM NaCl, 15 mM Tris, pH 7.4, 1 mM $MgCl_2$, 1% Triton X-100) supplemented with protease inhibitors (Roche, Almere, the Netherlands). Samples were centrifuged at 30 000 x g for 10 minutes at 4°C and stored at −80°C until cytokine determination. Protein content was determined using the BCA Protein Assay (Thermo Scientific Pierce, Etten-Leur, the Netherlands), and cytokine levels in homogenates were measured using the Cytometric Bead Array System (BD Biosciences) following the manufacturer's instructions. Serum amyloid A (SAA) levels in mouse serum samples were measured using a commercial ELISA kit (Invitrogen).

Cell labeling with ^{99m}Tc-tropolon

To analyze the biodistribution and compare homing of MSCs and IMSCs, $1.0x10^6$ human (I)MSCs were labeled with technetium-99m (^{99m}Tc) and intraperitoneally injected into different groups of animals on day 7 (n=3) (Figure 1D). In brief, human MSCs were washed twice with PBS by centrifugation in a two-step washing process (5 minutes at 150 x g). Cell labeling with technetium-99m was performed according to a leukocyte-labeling method that had been modified for stem cells.[19, 20] Ten milliliters of a 50 mg/mL 2-hydroxy-2,4,6-cycloheptatrienone solution (tropolone,

Aldrich Chemical Co., Milwaukee, WI, USA) was first dissolved in ethanol and then diluted in saline to 20% (v/v). A solution of $SnCl_2$ (950 mg/L $SnCl_2.2H_2O$ and 2 g/L sodium pyrophosphate.$10H_2O$) was obtained from a TechneScan® PYP® kit (Tyco Healthcare Mallinckrodt Medical BV, Petten, the Netherlands). KBH_4 (crystalline, Sigma, St. Louis, MO) was dissolved in 4 M NaOH and diluted with 0.1 M NaOH to a final concentration of 10 mg/ml. A lipophilic reactive complex was prepared by adding tropolone to a solution of $SnCl_2$ in a ratio of 2:1, which was then added to a solution of KBH_4 and ^{99m}Tc-sodium pertechnetate (200-500 MBq/mL, Technekow, Tyco Healthcare-Mallinckrodt Medical BV, Petten, the Netherlands). Two hundred microliters of the lipophilic reactive complex was immediately added to the cell pellet (containing $2x10^5$-$2x10^6$ cells) and gently shaken for 1 hour at 20°C. Labeled cell solutions were then centrifuged in a two-step washing process with PBS to remove non-cell-related (radioactive) reactants. To determine labeling efficiency, radioactivity in the final cell pellet and supernatant was measured using a dose calibrator (VDC 101, Veenstra Instruments, Joure, the Netherlands). Labeling yields ranged from 34-58%, and the stability of the complex was >90% for 24 hours in serum at 37°C. Animals were sacrificed 24 hours after injection of radiolabeled (I)MSCs in established colitis models, and relevant organs and tissues were excised, weighed, and measured for radioactivity using a dose calibrator. These values were calculated in relation to the percentage of the total dose of radioactivity administered (%ID/g tissue).

Microarray analysis

Human MSCs obtained from four individual subjects were treated with IFN-γ or left untreated for 6 days. RNA was isolated as described above, labeled using the Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX) and hybridized to Ref12v3 Arrays. Data were quantile normalized and analyzed using Genome studio and Mayday software.[21]

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) using two-sided t-tests and the Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. The Kaplan-Meier log-rank test was used to analyze survival curves. All analyses were performed using the SPSS statistical package (version 16.0, SPSS Inc., Chicago, IL) and GraphPad Prism software (version 5.01). $P < .05$ was considered statistically significant.

RESULTS

Pretreatment of MSCs with IFN-γ increases immunosuppression

Given the significant induction of various immune-related proteins by IFN-γ, we first examined whether MSCs continued to fulfill the minimal criteria for defining MSCs even after IFN-γ exposure.[22] Human MSCs cultured for 6 days in the presence of IFN-γ retained their capacity to adhere to plastic (data not shown). As previously described,[23-25] human MSCs increased the expression of major histocompatibility complex (MHC) class II genes in response to IFN-γ treatment (Figure 2A), whereas the expression of other

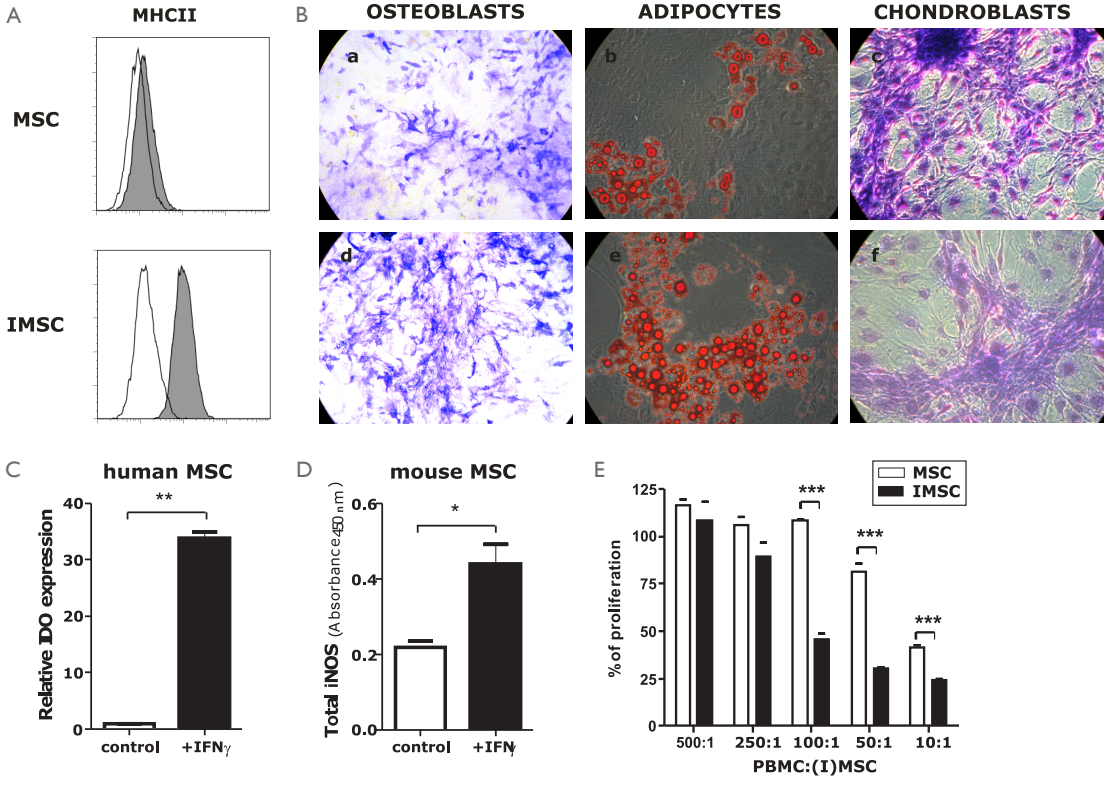

Figure 2. Effect of IFN-γ stimulation on MSC phenotype and function. A. Surface markers for MHC class II on unstimulated (upper panel) and IFN-γ-stimulated human MSCs (lower panel), as analyzed by flow cytometry. Markers are shown (solid histogram) compared to isotype control (line). B. Differentiation capacity of human MSCs (upper panels) and IMSCs (lower panels) into osteoblasts, adipocytes and chondroblasts. Osteogenic differentiation was detected with Fast Blue to show alkaline phosphatase activity (a, d). Lipid droplets in the cell cytoplasm were stained with Oil Red O (b, e). Toluidine blue staining corresponds with chondrogenesis (c, f). Magnifications, 10x (a, b, d, e); 32x (c, f). C. IFN-γ dependent relative IDO expression in human MSCs (left panel). Data are presented as means ± SEM; ** $P < .001$. D. IFN-γ treatment of mouse MSCs stimulates expression of iNOS (right panel). Data are presented as means ± SEM; * $P < .05$. E. Human IMSCs inhibit the proliferation of activated PBMCs in a dose-dependent fashion. PBMCs (100 000 cells) were stimulated with anti-CD3/CD28 beads and cocultured with MSCs (white columns) or IMSCs (black columns) in various ratios (PBMC:(I)MSC). Proliferation was measured by 3H-thymidine uptake in counts per minute and is expressed as a percentage of PBMC proliferation in the absence of MSCs. Data are presented as means ± SEM; *** $P < .001$.

markers in the MSC panel were unaffected (Figure 3). Furthermore, IMSCs maintained their in vitro differentiation capacity to form osteoblasts, adipocytes and chondroblasts (Figure 2B). In addition, we confirmed that IFN-γ stimulation leads to elevated IDO expression in human MSCs by RT-PCR (Figure 2C), and in increased iNOS levels in mouse MSC lysates (Figure 2D). At a functional level, human IMSCs significantly inhibited PBMC proliferation at lower PBMC:(I)MSC ratios compared to untreated MSCs, indicating that IMSCs have higher immunomodulatory capacities (Figure 2E).

IMSCs attenuate the development of DSS-induced colitis

Given the augmented immunosuppressive properties of IMSCs in vitro, we examined the anti-inflammatory effects of human IMSCs in vivo using a DSS-induced colitis model. Mice were intraperitoneally injected with human MSCs, IMSCs or PBS at the start of DSS induction (day 0). On day 9 (upon sacrifice), IMSC-treated mice showed significantly reduced weight loss compared to PBS-treated mice (Figure 4A). No differences in body weight were observed between MSC- and PBS-treated animals. Colons were assessed for the presence of loose stool, bleeding and macroscopic inflammation (Figure 4B). IMSC-treated (but not MSC-treated) mice had a significantly decreased disease score when compared with PBS-treated mice (Figure 4C). The PBS-treated group clearly exhibited a severe mucosal mononuclear cell infiltrate and disruption of crypt architecture (epithelial ulcerations and loss of goblet cells), whereas DSS-induced lesions were partially prevented in the IMSC-treated group (Figure 4D). These observations were also reflected by the histology scores (Figure 4E). These data consistently demonstrate that IMSCs are effective in the prevention of DSS-induced colitis.

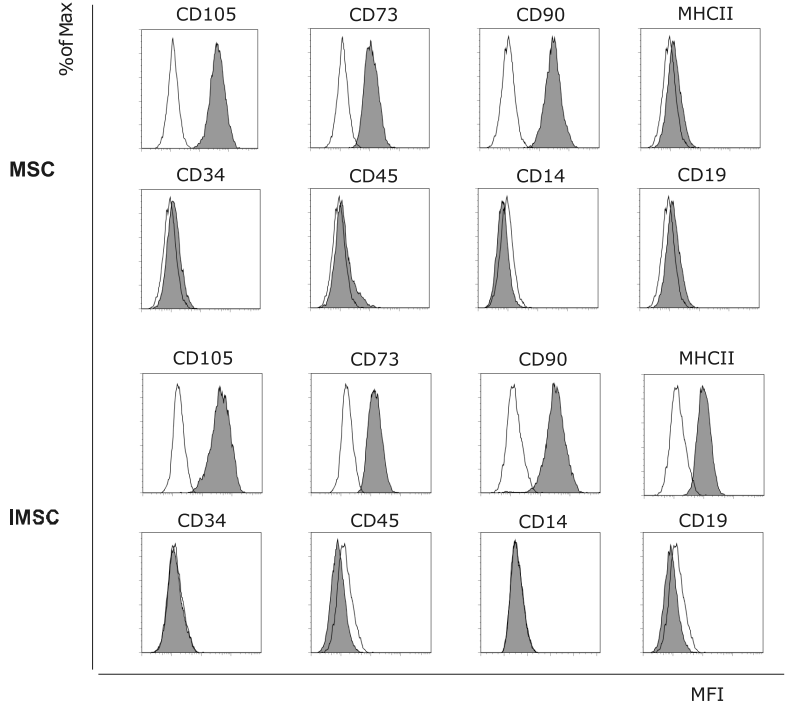

Figure 3. Effect of IFN-γ stimulation on MSC phenotype. Surface markers on unstimulated (upper panels) and IFN-γ-stimulated human MSCs (lower panels) were analyzed by flow cytometry. Markers are shown (solid histogram) compared to isotype control (line).

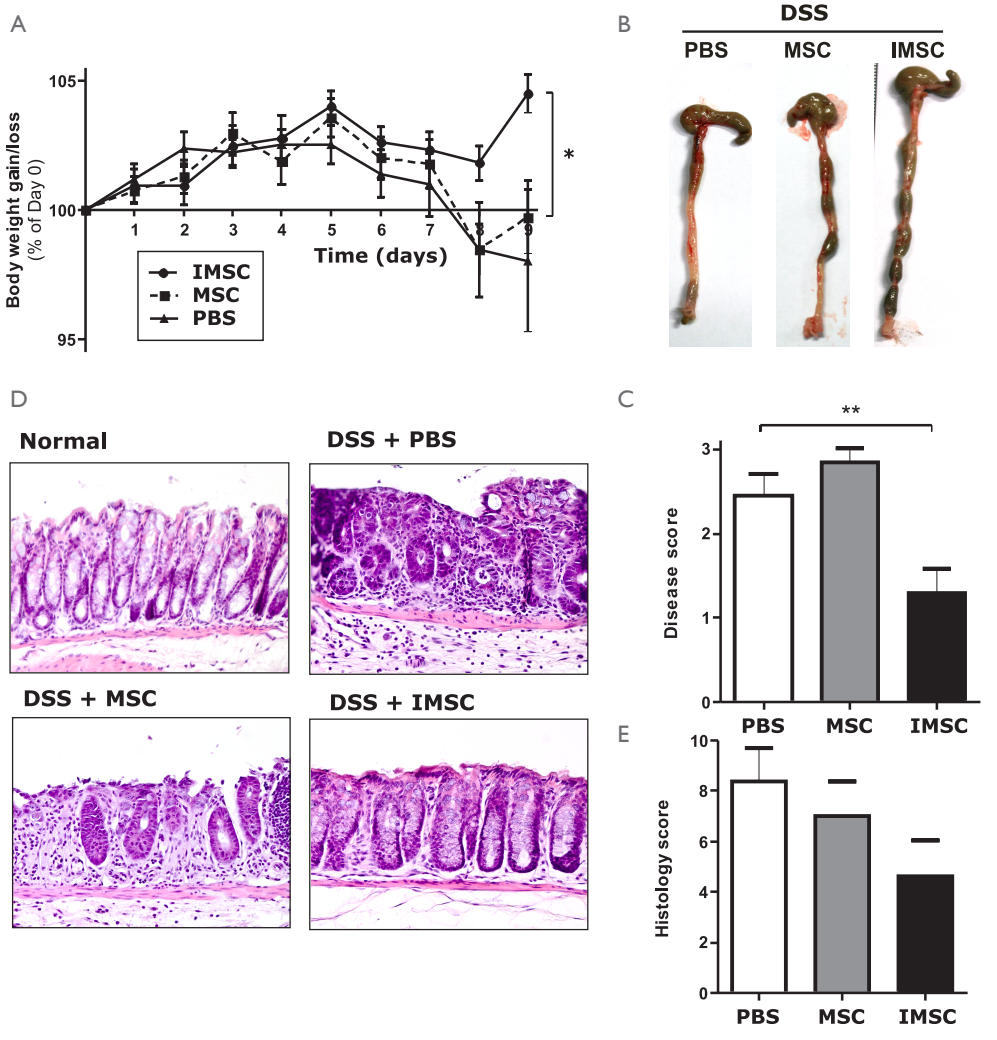

Figure 4. IMSCs prevent wasting disease progression in DSS-induced colitis. Mice received 2.25% DSS dissolved in drinking water for seven days. On day 0, mice were injected with human MSCs, IMSCs or PBS alone. Clinical evolution was monitored by body weight changes, macroscopic and microscopic scores. All mice were sacrificed on day 9. Data represent mean ± SEM; n=11 mice per group. A. Body weight changes during the course of the experiment. The values of body weight change are expressed as a percentage of initial body weight on day 0. *P < .05 compared to PBS treatment. B. Macroscopic images of representative mouse colons harvested on day 9. C. Macroscopic disease scores based on the presence of loose stool, bleeding and macroscopic inflammation determined on day 9 upon sacrifice. **P < .001. D. Histological evidence that IMSCs prevent DSS-induced pathology. Photomicrographs (20x magnification) of an H&E-stained paraffin section of a representative mouse colon from each treatment group. E. Histology scores were derived from microscopic analyses of longitudinal colon sections from each mouse.

IMSCs alleviate TNBS-induced colitis

We next examined the effects of IMSC administration on acute Th1-mediated colitis in mice. The therapeutic effect of mouse IMSCs was assessed after skin sensitization on day 0, followed by intrarectal TNBS challenge on day 7. Six hours after TNBS challenge, mice received an injection of mouse MSCs, IMSCs or PBS. Body weight, colon weight and length, as well as macroscopic signs indicative of colonic inflammation were determined upon sacrifice (Figure 5A, page 78). TNBS-induced colitis resulted in a body weight loss of 4.9% in PBS-treated animals. MSC-treated mice displayed an average decrease of 1.3% in body weight, whereas IMSC-treated mice had gained 2.5% (Figure 5B). This body weight increase was reflected in a lower disease score (Figure 5C) and a decreased colon weight-to-length ratio (Figure 5D). Histological grading showed that the IMSC group exhibited diminished severity of colitis (Figure 5E), primarily as a result of lower levels of inflammation, less crypt loss, and reduced number of granulocytes and mononuclear cells. Histochemistry demonstrated prominent infiltration of mononuclear cells into the affected colons, particularly in severely damaged regions. Immunohistochemistry showed that CD3+ cells (T-cells) were predominantly located in regions of inflammation (Figure 5F), and that IMSC treatment appeared to reduce the number of T-cells.

For survival analysis, a rectal enema of TNBS was applied to BALB/c mice on days 0 and 7. Mouse MSCs, IMSCs or PBS alone was injected intraperitoneally 6 hours after the first TNBS administration. Injection of either MSCs or IMSCs similarly increased the survival of BALB/c mice after TNBS-induced colitis (Figure 6).

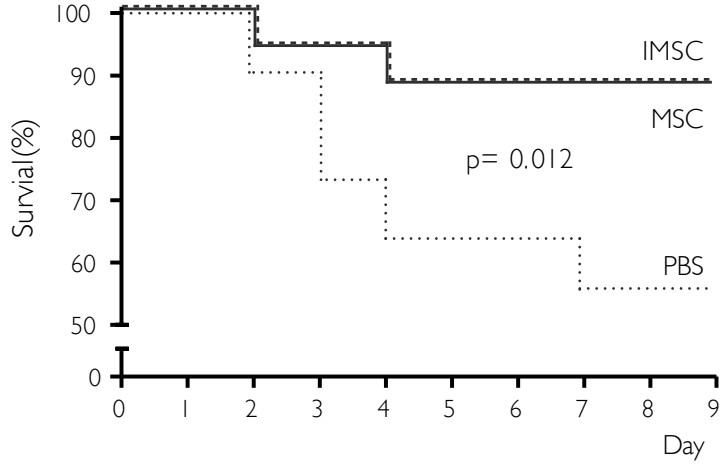

Figure 6. Treatment with mouse MSCs and IMSCs improves the survival of Balb/c mice with TNBS-induced colitis. Colitis was induced by intrarectal injection of TNBS in Balb/c mice. Mice were injected intraperitoneally with MSCs, IMSCs or PBS alone on day 0, starting 6 h after intrarectal TNBS administration. Survival (Kaplan-Meier analysis) was compared between (I)MSC- and PBS-treated animals. n=11 mice per group; *P = .012.

IMSCs reduce systemic markers of inflammation

We next examined the influence of IMSCs on serum amyloid A (SAA) levels. SAA is a systemic marker of inflammation similar to C-reactive protein (CRP) in humans. We detected high SAA levels in serum isolated from PBS-treated mice, which corresponded to the severe colitis seen in these animals. In contrast, treatment with mouse IMSCs resulted in significantly lower SAA serum levels, confirming the decreased inflammatory responses observed in these animals (Figure 7A).

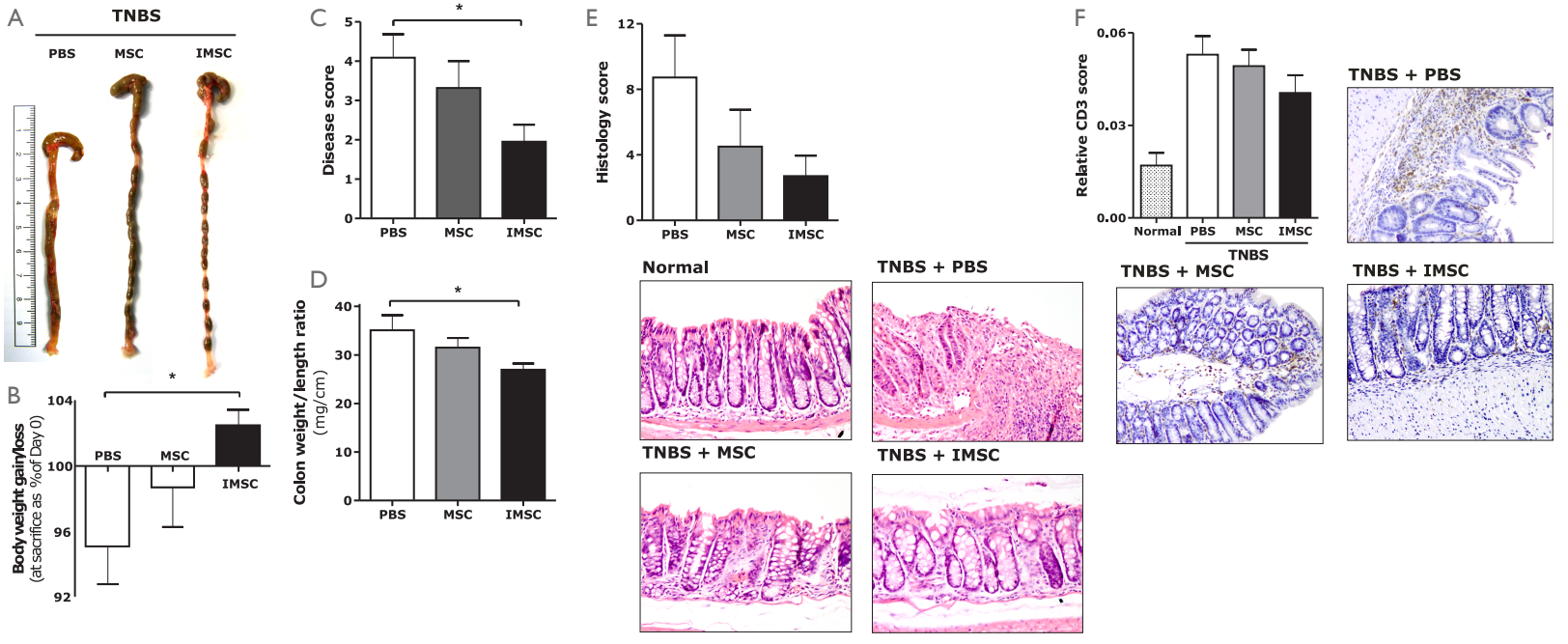

Figure 5. IMSCs alleviate TNBS-induced colitis. Mice were sensitized through the skin on day 0, challenged intrarectally on day 7, and received mouse MSC, IMSC or PBS injections 6 hrs after challenge. Disease progression was assessed by changes in daily body weight as well as macroscopic and microscopic scores on day 9 upon sacrifice. Data are presented as mean ± SEM; n=11 mice per group. A. Macroscopic images of representative mouse colons. B. Animal body weight change on day 9 upon sacrifice. The values of body weight are expressed as a percentage of initial body weight on day 0. *P < .05. C. Macroscopic disease score based on the presence of loose stool, bleeding and macroscopic inflammation determined on day 9 upon sacrifice. *P < .05. D. Assessment of colonic weight-to-length ratio upon sacrifice as an index of disease-related intestinal wall thickening. *P < .05. E. Representative histological sections of the mouse colon upon sacrifice and histological grading of colonic colitis scores. IMSCs decrease colitis activity in TNBS-treated mouse colons. F. CD3-positive T-cells in vivo. Representative colon sections stained with anti-CD3, revealing T lymphocytes. Magnification, 20x. CD3-positive T-cells expressed as the ratio of CD3 relative to hematoxylin counterstain.

IMSCs alter cytokine profiles in colon homogenates

To investigate the effect of (I)MSC treatment on immunomodulatory profiles, we analyzed cytokine concentrations in colon homogenates. As previously described, distinct cytokine patterns were observed in DSS- and TNBS-induced colitis models.[26] In both models, TNF-α and IL-6 levels were significantly elevated, reflecting ongoing general inflammation. Human and mouse IMSC treatment reduced TNF-α and IL-6 levels, while MSC treatment had no effect (Figures 7B and 7C). Acute DSS-induced colitis is mainly mediated by the innate immune system and results in a strong colonic induction of IFN-γ. Furthermore, IL-17A has been shown to be a pathogenic cytokine during DSS-induced colitis, as IL-17A knockout animals show an attenuated decrease in body weight as well as decreased disease activity.[27] Indeed, IFN-γ and IL-17A expression were both increased during induction of DSS colitis (Figures 7D and 7E, left panels). Human MSC administration did not alter IFN-γ and IL-17A levels. In contrast, IMSC administration reduced both IFN-γ and IL-17A expression essentially to the levels seen in control (non-induced) animals, consistent with the decrease in colonic inflammation observed in these mice.

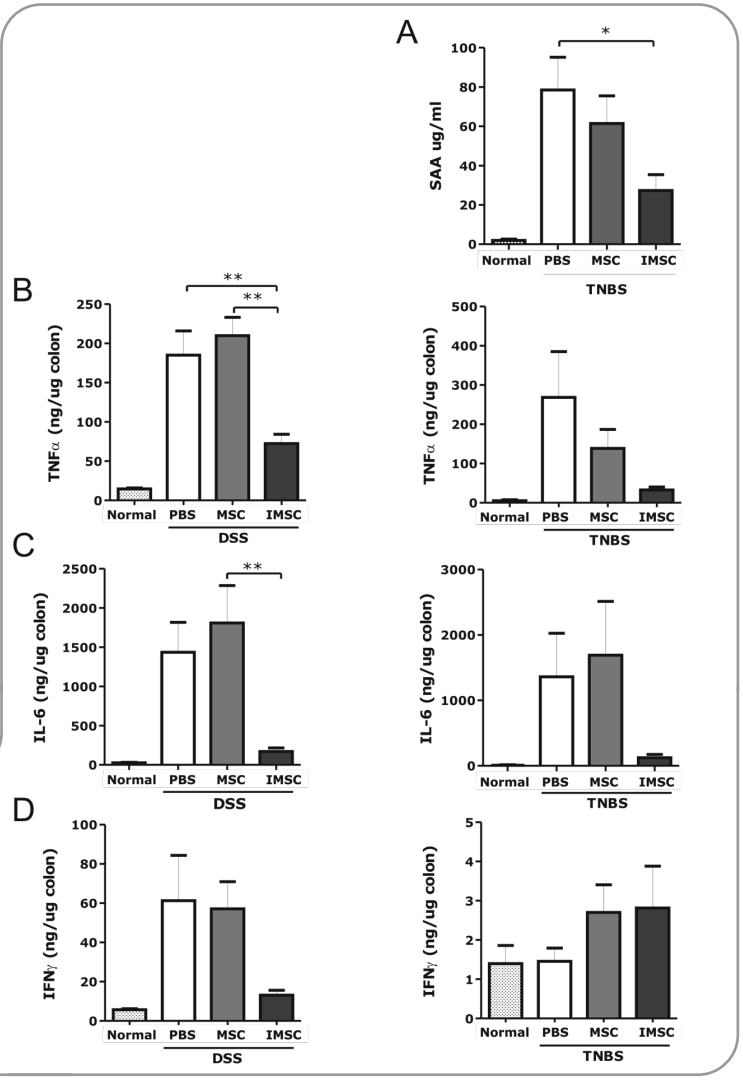

Figure 7. IMSCs decrease systemic and local inflammatory responses in experimental colitis models. Serum and protein extracts were obtained from the colons at the acute phase of disease (day 9), and cytokine levels were measured. Data are presented as mean ± SEM; n=11 mice per group; *P < .05, **P < .005. A. Serum amyloid A (SAA) levels were measured by ELISA and found to be reduced in mice with TNBS-induced colitis after IMSC treatment. B-G. Cytokine levels of TNF-α, IL-6, IFN-γ, IL-10, IL-17A and IL-4 in colon homogenates of DSS- (left) and TNBS-induced (right) mice.

TNBS-induced colitis is T-cell-mediated and mainly driven by a Th1 response.[28, 29] In this model, a lack of IL-17 signaling results in a more severe disease phenotype, likely due to uncontrolled induction of Th1 responses.[30] Interestingly, treatment of TNBS colitis with mouse IMSCs resulted in the induction of IL-17 (Figure 7E, right panel) as well as IL-4 (Figure 7G, right panel), indicating induction of Th2 and Th17 responses, respectively. These responses may inhibit Th1 skewing and thereby Th1-driven pathogenesis as well. In addition, an increase in colonic IL-10 levels was observed, suggesting the presence of an active immunoregulatory component (Figure 7F, right panel).

IMSCs migrate to sites within the diseased intestine

Previously, MSCs were thought to specifically migrate to sites of tissue damage or inflammation.[31] However, in these studies the focus was primarily on specific tissues of interest and the number of MSCs present at those sites in relation to the total number of MSCs administered was not correlated. We therefore administered human MSCs and IMSCs radiolabeled with technetium-99m to both control animals and animals with established colitis. The biodistribution data show that at 24 hours post-injection, the majority of radioactivity was present in the kidneys, urinary bladder, and a small portion in the liver (Table 1). Of note, we

	MSC				IMSC			
	No colitis		Colitis		No colitis		Colitis	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Colon	0.19	0.08	0.19	0.09	0.13	0.05	0.26	0.11
Coecum	0.15	0.06	0.1	0.05	0.1	0.05	0.19	0.09
Small intestine	1.24	0.51	1.58	0.71	0.61	0.25	2.48	1.01
MLN	0.12	0.05	0.19	0.09	0.13	0.06	0.1	0.05
Spleen	0.74	0.3	1.01	0.45	0.7	0.31	0.58	0.26
Kidney	8.22	3.35	10.09	4.51	5.13	2.09	7.63	3.11
Heart	0.09	0.01	0.03	0.02	0.01	0.01	0.04	0.02
Lungs	0.2	0.08	0.14	0.07	0.15	0.06	0.15	0.06
Liver	3.17	1.29	4.53	2.03	1.89	0.77	2.58	1.05
Femur	0.05	0.02	0.05	0.02	0.03	0.01	0.03	0.01

Table 1. Biodistribution of 99mTc-labelled human (I)MSCs 24 hrs after injection in healthy controls (no colitis) and DSS-treated (colitis) mice. All data are presented as the % (mean ± SEM) of the total injected dose (ID) per gram of excised organ.

found radioactivity across a wide array of tissues, arguing against a highly specific trafficking of MSCs (Figure 8A). Strikingly, when we compared cell trafficking under steady state conditions and during inflammation, we observed a clear difference between MSCs and IMSCs. IMSC migration to the intestine was significantly increased during colitis induction, whereas MSC distribution was unaffected, suggesting that IMSCs gain homing potential to sites of inflammation (Figure 8B).

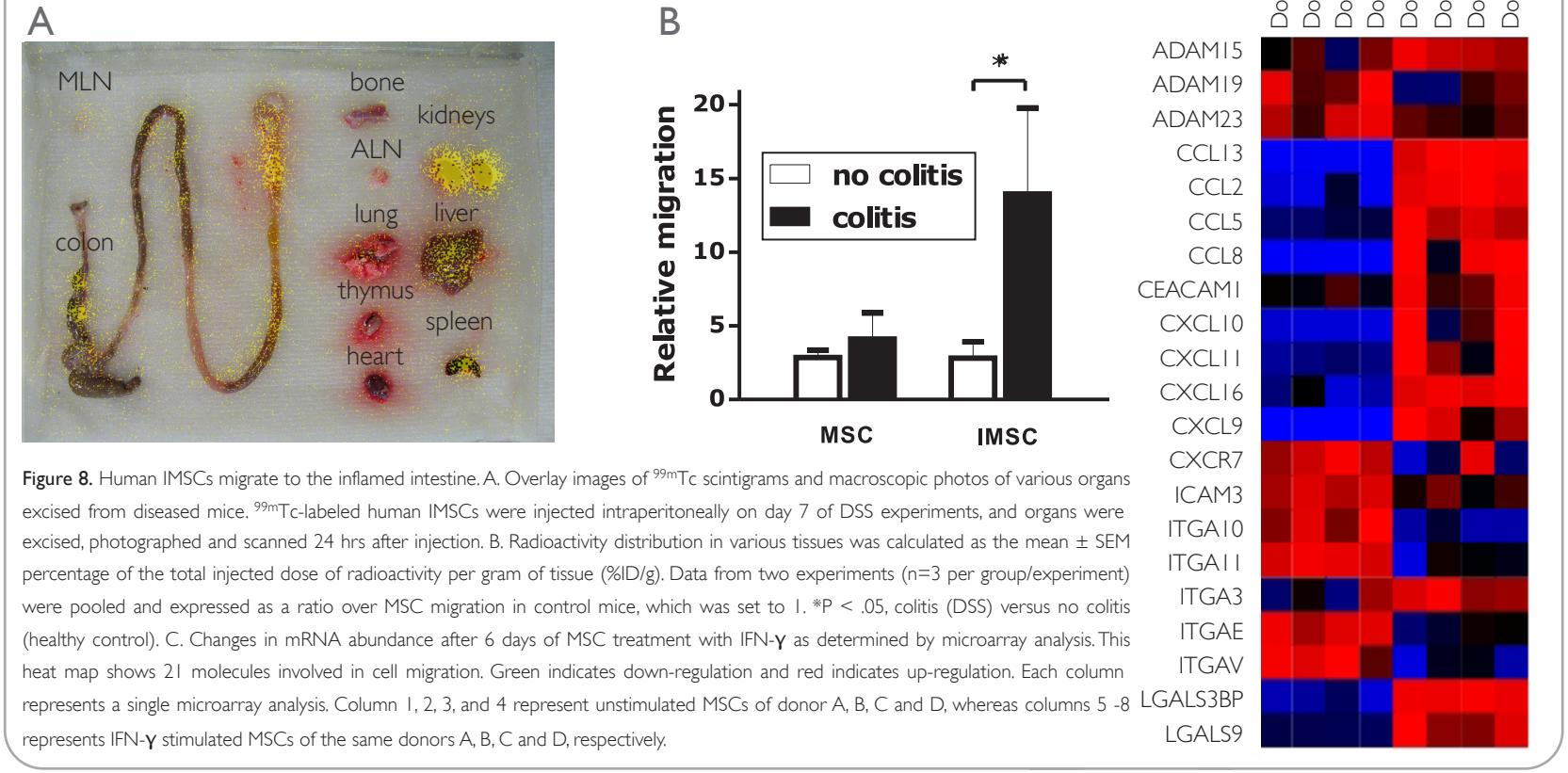

Figure 8. Human IMSCs migrate to the inflamed intestine. A. Overlay images of ^{99m}Tc scintigrams and macroscopic photos of various organs excised from diseased mice. ^{99m}Tc-labeled human IMSCs were injected intraperitoneally on day 7 of DSS experiments, and organs were excised, photographed and scanned 24 hrs after injection. B. Radioactivity distribution in various tissues was calculated as the mean ± SEM percentage of the total injected dose of radioactivity per gram of tissue (%ID/g). Data from two experiments (n=3 per group/experiment) were pooled and expressed as a ratio over MSC migration in control mice, which was set to 1. *P < .05, colitis (DSS) versus no colitis (healthy control). C. Changes in mRNA abundance after 6 days of MSC treatment with IFN-γ as determined by microarray analysis. This heat map shows 21 molecules involved in cell migration. Green indicates down-regulation and red indicates up-regulation. Each column represents a single microarray analysis. Column 1, 2, 3, and 4 represent unstimulated MSCs of donor A, B, C and D, whereas columns 5 -8 represents IFN-γ stimulated MSCs of the same donors A, B, C and D, respectively.

IFN-γ pretreatment alters expression of trafficking molecules

In order to examine more elaborately the mechanisms involved in the altered biodistribution of IMSC, gene expression profiles were generated from both MSC and IMSC. As expected, a large number of IFN-γ regulated genes was induced after treatment, including human leukocyte molecules and IDO (data not shown). We then focused on molecules associated with cell migration and adhesion. As has been described previously,[6] IFN-γ treatment of MSCs resulted in upregulation of a number of pro-inflammatory chemokines (Figure 8C). Chemokine receptor expression however, did not change dramatically after 6 days of treatment, with the exception of an increased level of CXCR7. This suggested that treatment with IFN-γ does not result in profound changes in chemokine responsiveness of the MSCs and that this may not be the underlying cause of the altered trafficking observed. In contrast, the expression pattern of adhesion molecules was strikingly altered in IMSCs compared with MSCs from the same individual. Expression of a number of integrins was decreased, including ITGA10, ITGA11, ITGAE and ITGAV. In contrast, lectins LGALS3BP and LGALS9 were increased, as was ADAM15, a matrix metalloproteinase also involved in adhesion of cells to epithelium.

DISCUSSION

MSCs can be used as a potential treatment for various immune disorders. Early phase I-II studies reported that MSCs are a safe and feasible treatment option in humans,[16,32] and trials are ongoing to demonstrate clinical efficacy. Exact treatment dose, timing and frequency of administration, as well as the optimal source of MSCs, are currently under investigation. In addition, many in vitro and animal studies are performed to further elucidate the mechanism of immune modulation by MSCs.

Recent studies demonstrated that the immunosuppressive ability of MSCs is not intrinsic but instead induced by proinflammatory cytokines.[6] Exposing MSCs to inflammatory signals was found to significantly potentiate the immunosuppressive effects of MSCs on T-cells, monocytes/macrophages and dendritic cells (DCs).[7, 9, 33, 34] These data were further validated in vivo using animal models, where IFN-γ pretreatment improved MSC efficacy for the treatment of GvHD.[9] Here, we demonstrate that IFN-γ stimulation potentiates MSCs to provide an effective treatment for both DSS- and TNBS-induced colitis, two experimental models for inflammatory bowel disease IBD.

Previous research revealed beneficial effects of MSCs on experimental colitis.[35, 36] In these model systems, the therapeutic effect was mediated, at least in part, by suppression of inflammatory cytokines/mediators, increased infiltration of regulatory T-cells, and expression of the anti-inflammatory cytokine IL-10.[37] Although the MSCs used in these studies were not pretreated with IFN-γ, it is possible that they were activated in vivo, as MSCs were administered after the onset of inflammation. The proinflammatory environment in experimental colitis is comprised of an influx of mononuclear cells and high levels of TNF-α and IL-6. Both TNF-α and IFN-γ treatment of MSCs induced IDO expression.[33] Since only IMSCs are efficacious in the prevention of DSS-induced colitis, one could

speculate that at time of MSC injection (on day 0), the negligible levels of proinflammatory cytokines were insufficient to activate MSCs and initiate MSC-induced suppression. In fact, the absence of a protective effect of MSCs derived from fat tissue injected one day or one week prior to DSS colitis induction was recently described.[35]

As IFN-γ is known to upregulate both MHC class I and II genes,[22-25] it would be important to determine whether this upregulation makes cells more susceptible to rejection in an immune-competent host, particularly in the case of allogeneic cells. However, we administered both human and mouse (I)MSCs to immunocompetent mice and did not observe any apparent induction of immunologic responses. In vitro, pre-treatment of MSCs by IFN-γ does not enhance T-cell proliferation. On the contrary, PBMC proliferation is significantly inhibited at lower PBMC:(I)MSC ratios compared to untreated MSCs. Furthermore, systemic levels of the acute phase protein SAA were not increased by administration of IMSC. Similarly, in previous studies,[35-39] positive treatment effects of human MSCs in experimental disease models was observed without apparent graft rejection, attributed to the inherent capacities of MSCs to reduce inflammatory cell infiltration, down-regulate inflammatory cytokine production, regulate immune tolerance by increasing the production of anti-inflammatory cytokines (e.g., IL-10) and promoting the generation/activation of Tregs. Nevertheless, dedicated studies into the immunogenic status of (I)MSC are required to formally exclude induction of unwanted immune responses and to determine potential for clinical application.

MSCs have been described to distribute broadly following systemic infusion (IV) and to be recovered from injured tissues such as skin, muscle and gut.[31, 40] In agreement with this, using ^{99m}Tc-labeling, we also found activity in almost all tissues after intraperitoneal administration of human (I)MSCs. Interestingly, when focusing on the intestines specifically during colitis, enhanced migration of IMSCs but not MSCs was seen. Recently, Ko et al. demonstrated that enhancing binding of MSCs to adhesion molecule VCAM-1 (vascular cell adhesion protein) enhances their migration to the inflamed colon. Strikingly, only under these conditions, MSCs had a curative effect.[41] These results are similar to those obtained by us in the present study as we show that MSC homing to the intestines is upregulated after IFN-γ stimulation and that only IMSCs were able to alleviate colitis symptoms. However, even under inflammatory conditions, only a small proportion of IMSC can be detected in the intestine, suggesting that additional mechanisms of immune suppression may be active.

Previous studies have shown that IFN-γ primed MSCs secrete pro-inflammatory chemokines, resulting in the recruitment of T-cells.[6] Our study corroborates these findings and also points out the need for MSC–T-cell proximity in order to achieve potent immunosuppression. Also in agreement with previous literature, we did not observe much alteration in chemokine receptor expression. However, a clear alteration was seen in the pattern of adhesion molecule expression. Most strinkingly, a number of integrins was downregulated, while various lectins were upregulated. In addition, ADAM15 expression was increased, which has been associated

with increased adhesion of fibroblast-like cells. The molecule forms homotypic cell-cell interactions and is expressed in human intestinal epithelium, thereby being a possible targeting molecule to this site. In summary, the complete balance between adhesion molecule expressions is strongly altered by IFN-γ treatment. As this balance influences the likelihood of a cell trafficking to a certain location, these alterations may underlie the altered trafficking observed after IFN-γ stimulation. However, further studies will be required to evaluate the individual contributions of all differentially expressed adhesion molecules.

CONCLUSION

We conclude that IFN-γ potentiates MSCs to suppress inflammatory responses in experimental colitis models. We show that IMSCs inhibit Th1 inflammatory responses, leading to reduced T-cell activation and less proinflammatory cytokine secretion, resulting in diminished mucosal damage in two pre-clinical colitis models. We hypothesize that preactivation of MSCs with IFN-γ could lead to a more rapid clinical response and a dose reduction of cells, which could have profound effects on current treatment development programs.

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