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

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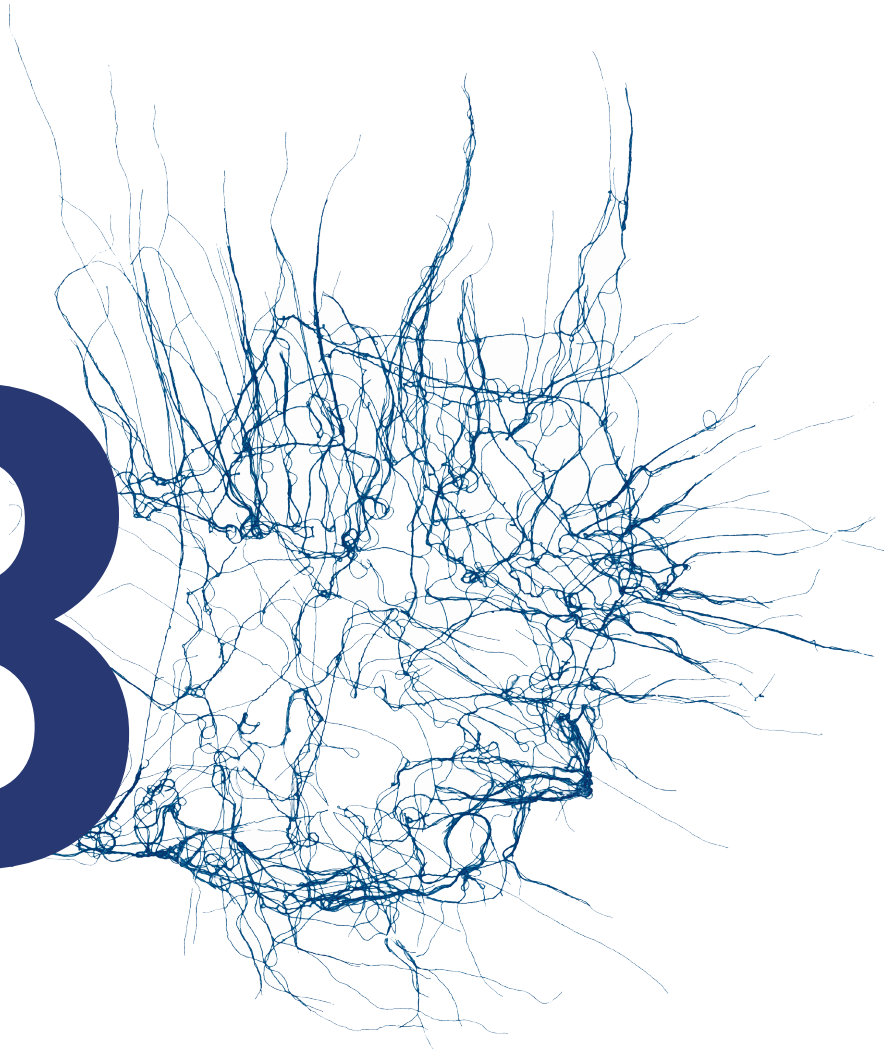

MESENCHYMAL STROMAL CELL-DERIVED EXOSOMES CONTRIBUTE TO EPITHELIAL REGENERATION IN EXPERIMENTAL INFLAMMATORY BOWEL DISEASE

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Cellular and Molecular Gastroenterology and Hepatology. In press.

ABSTRACT

A high dose of mesenchymal stromal cell-derived exosomes is able to counteract epithelial damage *in vitro* and partially reduce colitis *in vivo*. Our results show that MSC-derived exosomes contribute to the resolution of colitis by stimulation of epithelial repair and decreasing epithelial cell apoptosis.

MAIN TEXT

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells, which are studied as a therapeutic agent for inflammatory bowel disease (IBD). While systemic use of MSCs in IBD showed disappointing results thus far, local injection of MSCs stimulates closure of perianal fistulas in Crohn's disease (CD)[1,2]. The adipose tissue-derived MSC-product Cx601 was therefore recently approved by the European Medicines Agency for clinical use in fistulizing CD. Previously, we found that local injections of bone marrow-derived MSCs in the inflamed bowel alleviate experimental colitis in mice[3]. Although their exact working mechanism is unknown, MSCs are thought to work via the modulation of immune responses and stimulation of tissue regeneration via secreted proteins and cell-cell contacts. In addition, recent studies indicated that MSCs also exert effects via MSC-derived exosomes, which are small membrane-enclosed vesicles containing proteins, DNA and (micro)-RNAs[4]. The objective of this study was to evaluate if MSC-derived exosomes contribute to the therapeutic effects of local MSC-therapy. We investigated whether MSC-exosomes stimulate epithelial regeneration and if local application of MSC-exosomes, as a cell-free alternative for MSC-therapy, can alleviate colitis in epithelial-damage driven colitis models.

MSC-exosomes were isolated from murine, bone marrow-derived MSCs (cell characterization shown in Supplementary Figure 1A-B), using ultracentrifugation of MSC-conditioned medium (CM), containing 1.2 µg exosomes per ml. The presence of MSC-exosomes was confirmed by the exosome markers Flotillin-1 and Alix on western blot (Supplementary figure 1C) and visualization of 50-150 nm vesicles using transmission electron-microscopy (Supplementary Figure 1D). The uptake of fluorescently-labeled MSC-exosomes by CT26 mouse colonic epithelial cells was confirmed by a prominent red fluorescent signal upon addition of MSC-exosomes to CT26-cells (Figure 1A, Supplementary Figure 2A). To determine the effects of MSC-exosomes on epithelial regeneration, CT26-cells were exposed to dextran sulphate sodium (DSS) (Supplementary Figure 2B). A significantly higher cell number was detected, when DSS-damaged CT26-cells were cultured with a high dose of MSC-exosomes (20 µg/ml), compared to cells cultured with non-CM (Figure 1B). The high dose MSC-exosomes reduced levels of the apoptotic marker cleaved caspase-3 in CT26-cells upon damaging with 2%-DSS (Figure 1C, Supplementary Figure 2C), and 3%-DSS (Supplementary Figure 2D), indicating decreased apoptosis. As epithelial repair is a combination of proliferation and migration, we also assessed the effects of MSC-exosomes on cell migration, using a scratch assay. CT26-cells treated with CM with exosomes showed the fastest wound closure, but also CM without exosomes and a high dose of exosomes induced significantly increased wound healing compared to non-CM (Figure 1D, Supplementary Figure 2E). In addition, human

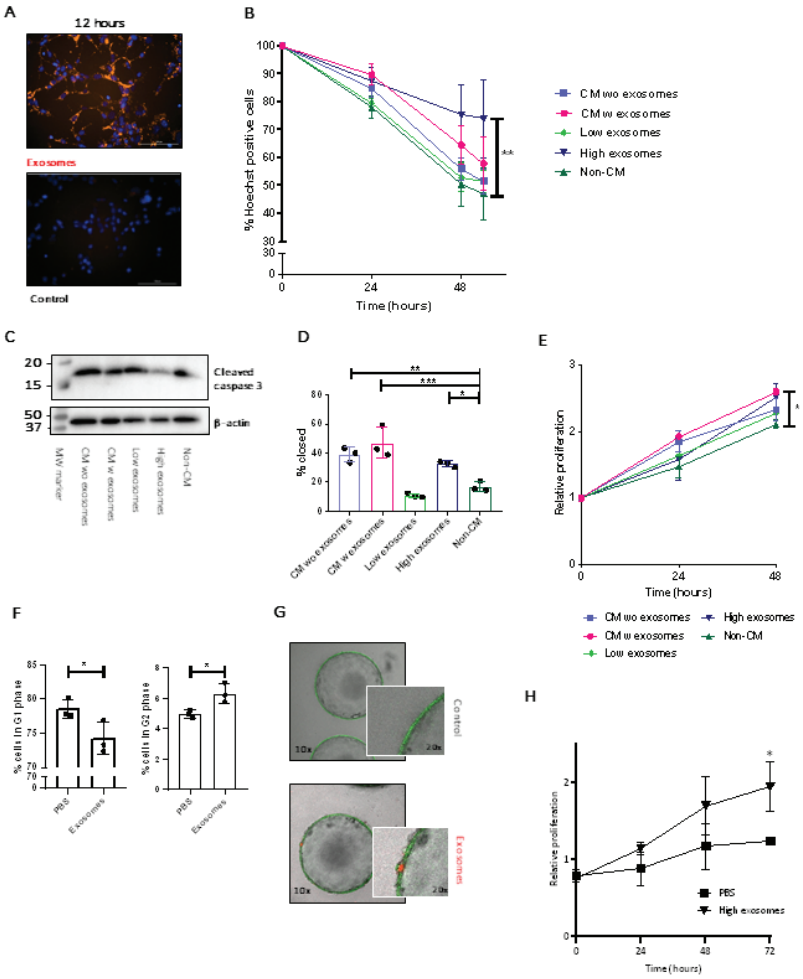


FIGURE 1. MSC-exosomes stimulate epithelial regeneration *in vitro*. A. Fluorescent images of CT26-cells treated with PKH26-labeled exosomes after 12 hours. B. Percentage of Hoechst-positive DSS-damaged CT26-cells after treatment with MSC-conditioned medium (CM) with or without exosomes, low or high concentration of exosomes or non-CM. Data represent an average of three independent experiments in triplicate. One-way Anova, Dunnett's multiple comparison with non-CM. C. Western blot analysis for cleaved caspase-3 in 2%-DSS treated CT26-cells under indicated conditions. Representative blot from three independent experiments. D/E. Relative wound closure after 27 hours (D) and proliferation (E) of CT26-cells stimulated with indicated conditions. Data represent mean of three independent experiments in triplicate. One-way Anova, Dunnett's multiple comparison with non-CM. F. Percentage of CT26-cells in G1- and G2-phase after coculture with exosomes. Representative data are shown from two independent experiments in triplicate. Student's t-test. G. Images of GFP-positive colon organoids cultured with and without PKH26-labeled exosomes for one week. Images are representative from two independent experiments. H. MTS assay of dissociated colon organoids cultured with exosomes. Data represent two independent experiments performed in triplicate for every time point. Student's t-test. *p≤0.05, **p<0.01, ***p<0.001.

MSC-exosomes, derived from MSCs after stimulation with cytokines of an IBD-signature, showed increased wound closure in human epithelial cells compared to non-CM and CM without exosomes (Supplementary Figure 2F). Murine epithelial cells stimulated with CM with exosomes showed a slight, but significant increase in proliferation in monolayer CT26 cultures with 3,000 (Figure 1E) and 9,000 cells (Supplementary Figure 2G). Cell cycle analysis revealed that MSC-exosomes increased the percentage of epithelial cells in both the S- and G2-phase, at the expense of the G1-phase (Figure 1F, Supplementary Figure 2H). Next, we evaluated the effects of exosomes on three-dimensional mouse colonic organoids. We confirmed that PKH-labeled exosomes were taken up by the epithelial organoids (Figure 1G) and induced organoid proliferation (Figure 1H, Supplementary Figure 3A). However, Ki67 staining of the colonic organoids after 72 hours showed a similar level of proliferating cells (Supplementary Figure 3B). Mucin2 (MUC2) and cytokeratin 20 (CK20) (Supplementary Figure 3C) were downregulated in dissociated colonic organoids cocultured with MSC-exosomes, suggesting that the increase in organoid proliferation by MSC-exosomes was not directly leading to enhanced differentiation of epithelial cells. No differences in expression of the stem cell marker, LGR5, and enteroendocrine marker, chromogranin A (ChgA), were found (Supplementary Figure 3C). Finally, we showed that cyclo-oxygenase 2 (COX-2), described to be upregulated in colonic epithelial cells from IBD patients[5], was significantly downregulated in colonic organoids 72 hours post-treatment with exosomes (Supplementary Figure 3C).

Next, we used the *in vivo* DSS mouse model for colitis to investigate if MSC-exosomes are responsible for the beneficial effects of local MSC-therapy. DSS treated mice were endoscopically injected with MSCs ($2x10^6$), MSC-exosomes (20 µg), CM containing exosomes (~0.24 µg) or solvent control at day 5. *In vitro*, $2x10^6$ MSCs will produce ~9.6 µg of exosomes in 3 days. Local MSC-therapy and to some extent local MSC-exosome-therapy alleviated DSS-induced colitis, as shown by a higher relative body weight, lower murine endoscopic index of colon severity (MEICS), lower macroscopic disease score, increased colon length and decreased epithelial damage, compared to control or CM treated mice. However, local exosome-therapy was less effective compared to MSC-therapy (Figure 2A-E, Supplementary Figure 4). This suggests that MSCs also exert their efficacy through other mechanisms or that continuous local production of exosomes is needed for profound therapeutic effects. Since locally injected MSCs are thought to be licensed *in vivo* by the pro-inflammatory milieu, it might be that cytokine-stimulated MSCs produce more efficient vesicles[6], which is also supported by our human data. The effects of MSC-exosomes might be mediated by microRNAs, since it was shown that for human MSCs, microRNAs involved in processes related to cell death and growth were enriched in exosomes[7]. In other disease models stromal cell-derived exosomes were also reported

to stimulate regeneration of damaged cells[8,9]. In conclusion, our results show that MSC-derived exosomes may contribute to the amelioration of colitis by stimulation of epithelial repair and decreasing epithelial apoptosis.

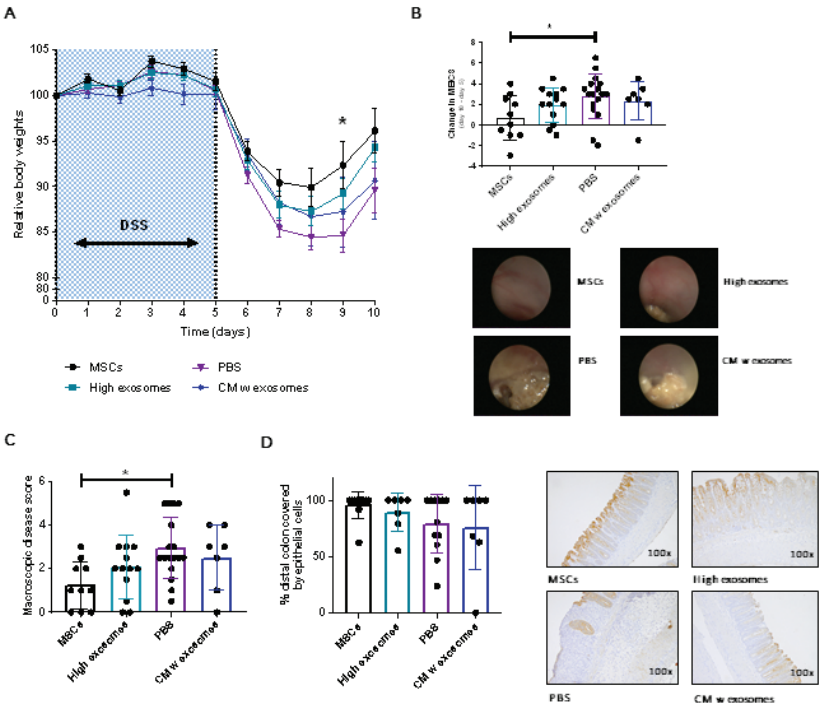
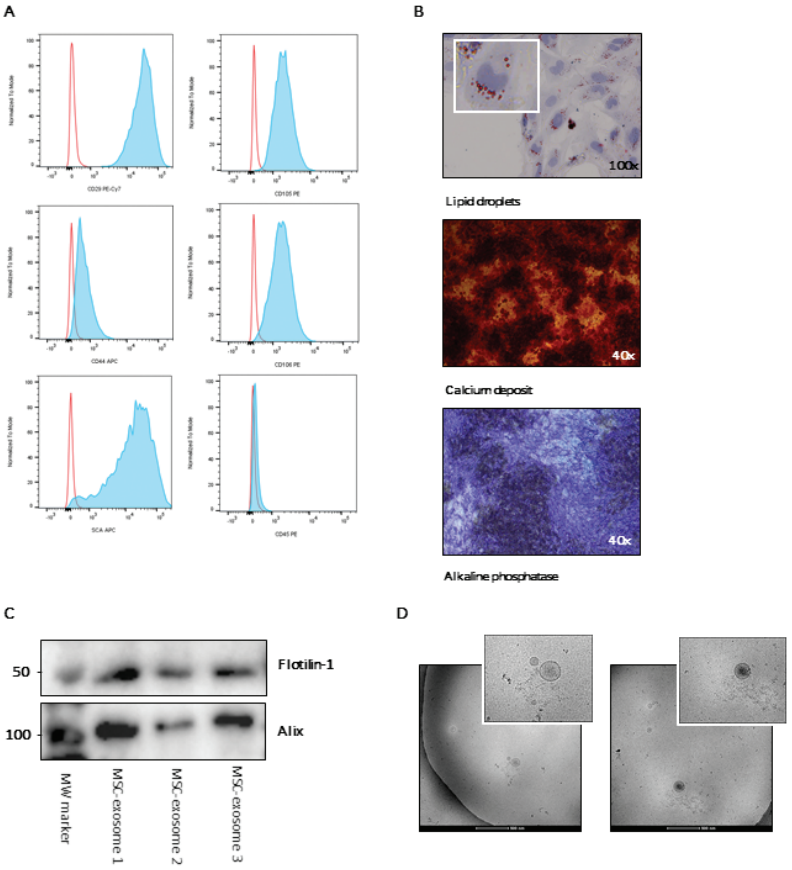


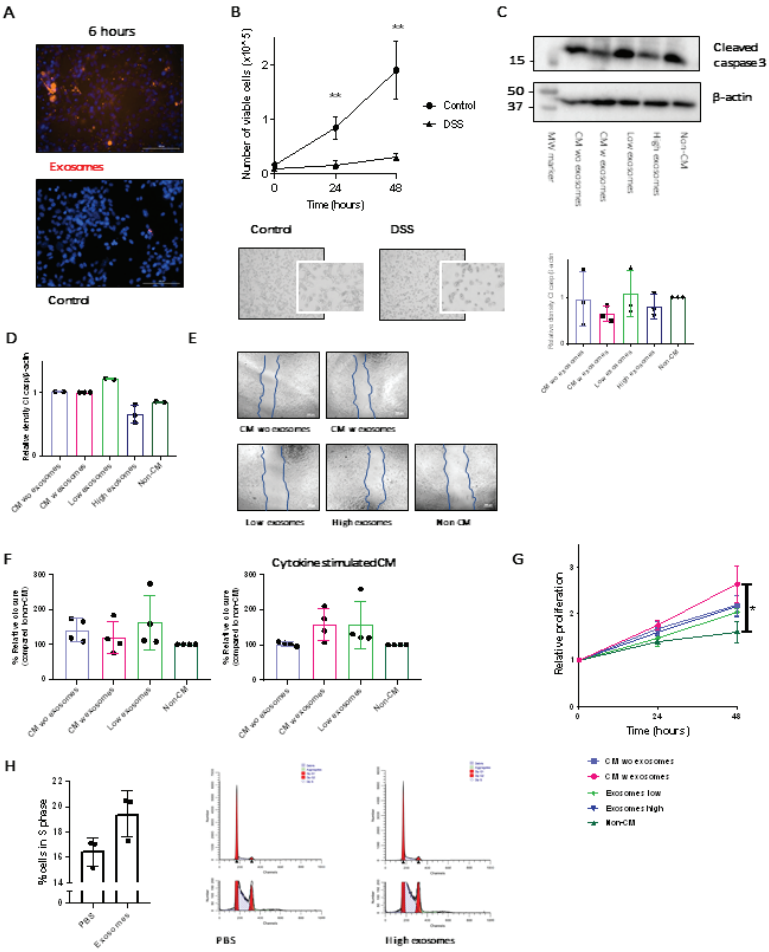
FIGURE 2. Locally applied MSC-exosomes partially alleviate experimental colitis. A. Relative body weights of mice with DSS-induced colitis, endoscopically treated with indicated conditions. Mean ± SEM. One-way Anova, Dunnett's multiple comparison with PBS. B. Difference in murine endoscopic index of colitis severity (MEICS) between day 10 and day 5 for the treatment groups. One-way Anova, Dunnett's multiple comparison with PBS. C. Macroscopic colonic disease score at day 10. One-way Anova, Dunnett's multiple comparison with PBS. D. Percentage of distal colon covered by epithelial cells and representative images showing pan-cytokeratin positive epithelial cells to identify epithelial cells. All data represent data of two independent mouse experiments, n=7-19 mice/group. *p<0.05.

Acknowledgement

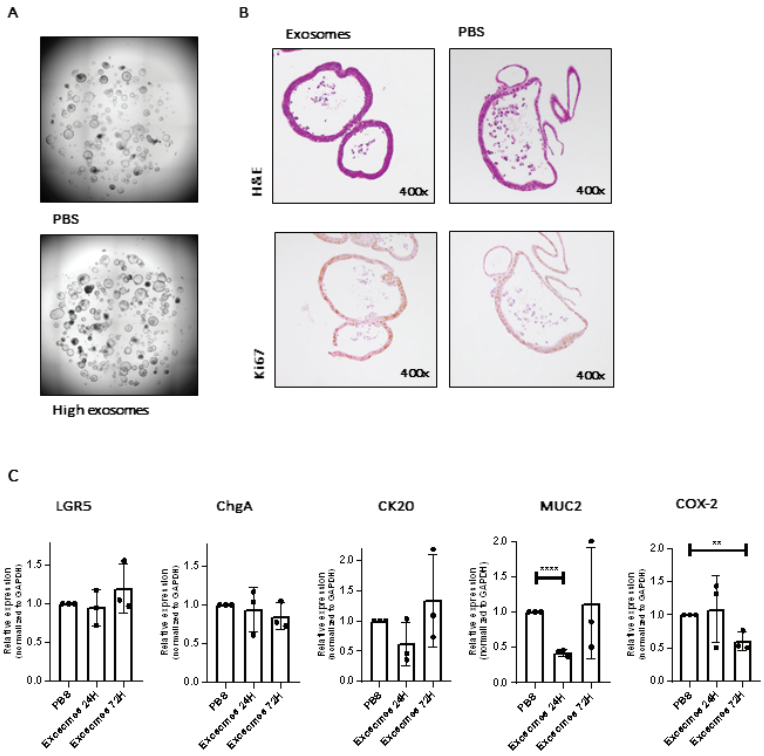
We would like to thank the staff of the Central Animal Facility of the Leiden University Medical Center for animal care and the group of Prof. Clevers, and especially Dr. van Es, from the Hubrecht Institute and Dr. Muncan from the Tytgat Institute for providing WNT3a, Noggin and R-spondin cell lines.



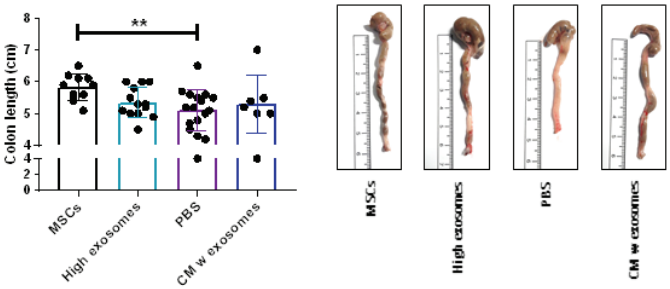
Supplementary FIGURE 1. Characterization of murine MSCs and MSC-exosomes. A. Flow cytometry for cell surface markers CD29, CD105, CD44, CD106, SCA-1, and CD45. B. MSCs differentiation staining for adipocytes (lipid droplets) and osteoblasts (calcium deposit and alkaline phosphatase activity). C. Western blot for exosomal markers Flottilin-1 and Alix. D. Transmission electron microscopy images of exosomes isolated from MSCs.



Supplementary FIGURE 2. *In vitro* models for IBD and the effects of MSC-derived exosomes in these models. A. Fluorescent images of CT26-cells treated with PKH26-labeled exosomes after 6 hours. B. Number of viable cells in DSS exposed CT26-cells over time. Pictures of 4%-DSS treated CT26-cells for 3 hours. Data represent three independent experiments in triplicate. Student's t-test. C. Western blot analysis for cleaved caspase-3 in CT26-cells incubated with 3%-DSS for 6 hours and treated with CM with or without exosomes, low or high dose of exosomes or non-CM for 24 hours. Representative blot from three independent experiments. Graph of the relative density of the cleaved caspase-3 bands corrected for the loading control β-actin. D. Densitometric analysis of the cleaved caspase-3 bands corrected for the loading control. E. Representative images to **FIGURE 1D** of wound healing assay in CT26-cells after 27 hours. F. Relative wound closure after 40 hours of DLD1-cells stimulated with indicated conditions. Cytokine (IFN-γ, OSM, TNF-α and IL-17) stimulated CM was used the exosomes used in the second graph. Representative data from four independent experiments performed in triplicate. G. MTS assay of 9,000 CT26-cells cultured with CM with or without exosomes, low or high exosomes or non-CM. Data represent mean of two independent experiments in triplicate for every time point. One-way Anova, Dunnett's multiple comparison with non-CM. H. Percentage of CT26-cells in S-phase after culture with and without exosomes. Two representative images from ModFit cell cycle analysis after coculture of CT26-cells with PBS or 20 µg exosomes. Representative data from two independent experiments. *p<0.05,**p<0.01.



Supplementary FIGURE 3. Gene expression and proliferation in MSC-exosome treated organoids. A. Representative images of colon organoids cultured without and with MSC-exosomes for 72 hours. B. Hematoxylin & eosin and Ki67 staining of colon organoids cultured with exosomes or PBS for 24 and 72 hours. C. Relative mRNA expression of LGR5, chromogranin A (ChgA), cytokeratin 20 (CK20), cyclo-oxygenase-2 (COX-2) and mucin (MUC2) in dissociated colon organoids cocultured with PBS or exosomes for 24 and 48 hours. Data represent mean of three independent experiments performed in triplicate. Student's t-test. **p<0.01, ****p<0.0001.



Supplementary FIGURE 4. Colon length. Representative pictures of colons from the different treatment groups. Data represent data of two independent mouse experiments, n=7-19 mice/group.

SUPPLEMENTARY METHODS

MSC isolation and characterization

All animal experiments were approved by the Central Authority for Scientific Procedures on Animals and the Animal Welfare Body of the Leiden University Medical Center (AVD116002017860). MSCs were isolated from the bone marrow of Tg(s100a4-cre)1Egn mice (Jackson laboratory, Bar Harbor, ME, USA) as described previously[10]. In brief, mice were sacrificed and femurs were removed. The bone marrow was flushed and filtered to remove debris. After centrifugation, bone marrow derived cells were cultured in Alpha MEM medium with Glutamax (Gibco #32561-029, Gaithersburg, MD, USA) supplemented with 1% penicillin/streptomycin (Gibco #15140-122) and 10% fetal calf serum (Gibco #10270-106). Non-adherent cells were removed after 24 hours and the medium was refreshed every 3-4 days. MSCs were characterized by flow cytometry analysis for surface markers (anti-CD105-PE, clone MJ7/18 (#562759), anti-CD106-PE, clone 429 (#561613), anti-CD44-APC, clone IM-7 (#561862) (all BD Biosciences, San Diego, CA, USA), anti-CD45-PE, clone 30-F11 (#12-0451-82), anti-CD29-PECy7, HMb1-1 (#25-0291-80), anti-SCA-APC, clone D7 (#17-5981-81) (all eBioscience, Vienna, Austria)) using the LSR II flow cytometer (BD Biosciences) and analysed with FlowJo software (Tree Star Inc., Ashland, OR, USA) and by *in vitro* osteogenic and adipogenic differentiation[3]. MSCs were used in experiments between passage 4-9. Human MSCs were derived from the bone marrow of healthy volunteers, obtained with informed consent for clinical application and research, and cultured and analysed as described previously[2].

MSC conditioned medium, exosome isolation and PKH26-labeling

CM was obtained by culturing confluent MSCs in FCS-free medium for 3 days. To mimic the IBD environment, human MSCs were also stimulated in FCS-free medium supplemented with pro-inflammatory cytokines (1 μg/ml interleukin-17 (IL-17, #167200-17-B), 1 μg/ml oncostatin M (OSM, #167300-10-B), 1 μg/ml interferon-γ (IFN-γ, #167300-02-B) and 1 μg/ml tumor necrosis factor-α (TNF-α, #167300-01A-B), all Peprotech, London, UK) for 3 days. CM was centrifuged at 300 g and 2,000 g for 10 minutes to remove cell debris and the supernatant was used for experiments (CM with exosomes). For the isolation of exosomes the CM was concentrated by ultrafiltration over a 100-kDA molecular weight cut-off filter (Amicon Ultra-15 tubes, Merck Millipore #UFC910024, Burlington, MA, USA) at 5,000 g for 40 minutes (Heraeus multifuge X1R, ThermoFisher Scientific, Waltham, MA, USA). The flow-through contained the CM without exosomes. The pellet was resuspended in PBS and consequently centrifugated at 100,000 g for 8 hours (Optima XE-90 ultracentrifuge, Beckman Coulter, Pasadena, CA, USA), after which pelleted exosomes were visible.

Murine MSC-exosomes were labelled using PKH26 (Sigma-Aldrich #PKH26GL, Saint Louis, MO, USA) according to the manufacturer's protocol. In short, exosomes were incubated for 4 minutes with PKH26. After this period the staining was stopped with vesicle-free FCS and exosomes were harvested with centrifugation (4 hours at 100,000 g) and washed once with PBS. To exclude non-specific fluorescent staining, a mock control containing vesicle-free FCS was included in the entire staining procedure.

Characterization of MSC-exosomes

The concentration of MSC-exosomes was determined by the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). MSC-exosomes were characterized for exosome-markers by western blot and electron microscopy. Western blot analysis was performed using 25 µg protein loaded on a 10% SDS-PAGE gel[11] and transferred to PVDF membranes (0.45 µm pore size, Whatman #10401196, Maidstone, UK). Ponceau staining (Sigma-Aldrich #3504) was used as to confirm equal loading. Blots were incubated with primary antibodies to rabbit anti-Alix, clone 3A9 (Bio-rad Laboratories #MCA2493, Hercules, CA, USA) and mouse anti-Flotillin-1, clone EPR6041 (Abcam #1333497, Cambridge, UK). In addition, MSC-exosomes were visualized using transmission electron microscopy. From a purified exosome suspension 3 µl was put on a copper EM grid that was glow-discharged in air. Using an EM-GP plunge freezer (Leica, Wetzlar, Germany) excess of the suspension was removed by blotting for 1 second using filter paper (Whatman) at 95% humidity at room temperature and the grid was plunged into liquid ethane at -183 °C. Samples were transferred to a Tecnai F20 Transmission Electron Microscope (ThermoFisher Scientific) using a Gatan cryo holder and images were recorded at 15.000x magnification on a Gatan 2kx2k CCD camera (Gatan, Pleasanton, CA, USA) behind a 2001 energy filter operating at 20 eV slit width.

In vitro two-dimensional colitis models

To verify if MSC-exosomes could be taken up by epithelial cells, CT26-cells were cocultured with PKH26-labeled MSC-exosomes and fluorescent images were obtained after 6 and 12 hours using the Cytation5 live cell imaging system (Biotek, Winooski, VT, United States). DSS (MW 36.000-50.000 kDA, MP Biomedicals #160110, Brussels, Belgium) was used to induce epithelial damage in CT26-cells by incubating cells with 2-4% DSS in FCS-free RPMI 1640 (Gibco #21875-034) for 3, 6, 12 or 24 hours. The effect of MSC-exosomes on DSS damaged CT26-cells was assessed by adding MSC-CM with exosomes (containing ~1.2 µg/ml exosomes), MSC-CM without exosomes, non-CM, 2 µg/ml (low) exosomes or 20 µg/ml (high) exosomes in non-CM to the damaged epithelial cells. The number of cells over time was measured by counting living cells using Hoechst staining (Cell Signaling #33342, Danvers, MA, USA) with the imaging function of the Cytation5 and Gen5 software

(Biotek) up to 54 hours. The percentage of Hoechst positive cells was given relative to Hoechst positive cells at 0 hours. Proteins from CT26-cells treated with different exosome conditions were extracted after 24 hours. 25 µg protein was loaded on a 15% SDS-PAGE gel and after transfer western blot was performed for rabbit anti-cleaved caspase-3, clone5A1E (Cell Signaling #9661S) and rabbit anti-β-actin, clone I-19 (Santa Cruz Biotechnology #1616, Dallas, TX, USA) as a loading control. For densitometric analysis the density of cleaved caspase-3 bands was corrected for β-actin.

To assess the effect of both murine and human exosomes on the migration of epithelial cells, a wound healing assay was performed. CT26 (mouse) or DLD1-cells (human) were seeded in 48-well plates (25,000 cells/well) and after overnight incubation, a wound was created in the confluent cell layer using a 200 µl pipet tip. MSC-CM with exosomes, MSC-CM without exosomes, non-CM, 2 µg/ml (low) exosomes or 20 µg/ml (high) exosomes in non-CM was added to the damaged epithelial cells and photos were made after 15, 27, 65 and 73 hours for CT26-cells and 40 hours for DLD-1 cells, using the Cytation5 to evaluate wound closure. Wound closure was determined by the average of 5 wound size measurements per image and was calculated relative to the wound size at start of the experiment. Proliferation of non-damaged CT26-cells was determined by a MTS assay. In short, 3,000 or 9,000 CT26-cells were seeded and stimulated with the previous mentioned conditions. MTS substrate (CellTiter #G3580, Promega, Madison, WI, USA) was added to the wells at day 0, 1, 2 and the absorbance was measured at 490nm using the Cytation5. Absorbance is given relative to absorbance at day 0.

Cell cycle analysis

CT-26 cells (250,000 or 500,000 cells/well) were cultured in 6-wells plates in the absence or presence of 20 µg/ml exosomes in non-CM. After 24 hours cells were harvested, fixated with methanol[12] and stained with 10 µM DAPI (Sigma-Aldrich #D9542) to analyse the percentage of cells in each phase of the cell cycle. A three-laser LSRII flow cytometer (BD Biosciences) was used for data acquisition, after approval of the Cytometer Set-up and Tracking system. The 488 nm blue laser was used to generate FSC and SSC signals. The 405 nm violet laser was used to generate DAPI fluorescence, using a 450/50 nm band pass filter. A 450/50-pulse width vs. 450/50-pulse area was used to select for single cells. At least 10,000 single cell events were collected. A data file contained all events, including debris and cell aggregates. Data were analysed using WinList 8.0 to select for single cells and to generate a DNA histogram remotely linked to ModFit LT 4.1 (Verity Software House, Topsham, ME). A trapezoid S-phase model was used giving a best fit with the data.

In vitro three-dimensional colitis models

Colonic organoids were generated to create a three-dimensional *in vitro* model to study the effect of exosomes on epithelial cells[13]. Colonic crypts were isolated from both wildtype C57BL/6J mice (Jackson Laboratory) and C57BL/6-Tg(UBC-GFP)30Scha/J mice (Jackson Laboratory). In brief, the mice were sacrificed by cervical dislocation and colon were removed. The colonic tissue was transferred to 20 mM Ethylenediaminetetraacetic acid (Merck Millipore #324503) in Hanks' Balanced Salt Solution (Gibco #14175-053) and incubated for 30 minutes at 37°C with repeated vortexing to release the colonic crypts. The crypts were washed in Ad-DF^{+++}, consisting of Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12, Gibco, #12634-010)) supplemented with 1% glutamax (#35050-038), 1% penicillin/streptomycin (#15140-122) and 1% HEPES (#15630-056, all Gibco). After centrifugation, the colonic crypts were plated in 20 µl matrigel (growth factor reduced matrigel, #356231, Corning, NY, USA) in 48-well culture plates. After polymerization of the matrigel, 250 µL of complete growth medium was added, consisting of Ad-DF^{+++}, supplemented with B27 (Invitrogen #11530536, Carlsbad, CA, USA), N-acetylcysteine (Sigma-Aldrich #A9165-5), nicotiamide (Sigma-Aldrich #n0636), A83-01 (Tocris #2939, Bristol, UK), p38 inhibitor (Sigma-Aldrich #s7067), epidermal growth factor (Invitrogen #PMG8041), Noggin CM (20%), R-spondin CM (10%) (both kindly provided by the Tytgat Institute, Amsterdam, the Netherlands) and Wnt3a CM (50%) (kindly provided by the Hubrecht Institute, Utrecht University, the Netherlands). During the first 4 days medium was supplemented with Rho-K inhibitor (Sigma-Aldrich #Y0503). Colonic organoids were maintained in a humidified incubator at 37°C containing 5% CO_2. Every 3-4 days the medium was replaced.

To verify if MSC-exosomes could also be taken up by colonic organoids, mechanically disrupted organoids were cultured with 60 µg exosomes PKH26-labeled exosomes for one week. The Leica SP8 microscope with a white light laser source was used to capture both the GFP-positive organoids (488-509 nm) and the PKH26-labeled exosomes (551-567 nm). To determine the effects of MSC-exosomes on colonic organoids, 5 wells with organoids were cultured with either 60 µg exosomes in PBS or PBS without exosomes, after induction of epithelial damage by mechanical disruption. Images were obtained after 24, 48 and 72 hours. Organoids were processed for paraffin embedding or mRNA was isolated using the nucleospin RNA kit (Macherey-Nagel #740955250, Düren, Germany) after 24 and 72 hours. cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scentific) according to the manufacturers' protocol. Quantitative polymerase chain reactions (qPCR) were performed using SYBR green (Biorad #1708886, Hercules, CA, USA) and primers (all Invitrogen) for a stem cell marker (leucine-rich repeat-containing G-protein coupled receptor 5; Fw

TGGTGGCTTTGACCGTGTT, Rv CGATTACCCCAATTAGCAGCTTT), differentiation markers (mucin2; Fw CCCAGAGAGTTTGGAGAGCA, Rv CTCCTCACATGTGGTCTGGT, chromogranin A; Fw GGCCCAGCAGCCGCTGAAGCAGCA, Rv CTCTGCGGTTGGCGCTGCCCTCCTC, cytokeratin 20; Fw CGCATCTCTGTCTCCAAAGC, Rv TTCTGCATTGCCAGTTTCCC) and the prostaglandin pathway (cyclo-oxygenase 2; Fw CCGTGCTGCTCTGTCTTAAC, Rv TTGGGAACCCTTCTTTGTTC). Glyceraldehyde 3-phospate dehydrogenase (Fw AACTTTGGCATTGTGGAAGG, Rv ACACATTGGGGGTAGGAACA) was used as a housekeeping gene. Data shown are relative to target mRNA expression levels in PBS stimulated organoids after respectively 24 and 48 hours. For proliferation assays, 96-wells plates were used and MTS substrate was added to the wells with organoids with or without exosomes after 0, 1, 2 and 3 days.

***In vivo* colitis model**

Experimental colitis was induced in female C57BL/6Jico mice by adding 2%-DSS to the drinking water for 7 days. DSS containing drinking water was refreshed every other day. Mice were endoscopically treated using a high resolution miniaturized colonoscope system (Stöpler, Karl Storz, Tuttlingen, Germany), as described previously[3], with MSCs ($2x10^6$ cells), MSC-exosomes (20 µg) or 200 µl MSC-CM containing ~1.2 µg/ml exosomes (n=7-19 mice/group) at day 5. In the control group, mice received local injections with 200 µl PBS. At the moment of treatment (day 5) during endoscopy the murine endoscopic index of colitis severity (MEICS)[14] was scored. Five days after treatment, endoscopy and MEICS scoring were performed again and mice were euthanized. The colon length was measured as well as the macroscopic disease score[15] for each mouse. The mouse experiment was performed twice and all parameters, except for the MEICS during treatment, were scored blinded to treatment groups.

Immunohistochemical analysis

Colonic organoids treated with or without exosomes were immunohistochemically stained for the proliferation marker rabbit anti-Ki67, clone SP6 (Abcam #16667) as described before[10]. To evaluate epithelial damage in the colon, epithelial cells were stained with mouse anti-pan-cytokeratin, clone PCK-26 (Sigma-Aldrich #C5992) and the % of distal colon covered by pan-cytokeratin positive cells was scored blinded to treatment groups.

Statistical analysis

Data are presented as mean ± standard deviation (SD), except for Figure 2A, where data are presented as mean ± standard error of the mean (SEM). Unpaired Student's t-tests were used to compare two groups. Differences between more than 2 groups were measured using an one-way ANOVA or Kruskal-Wallis tests followed by multiple comparison tests

(compared to the control). All analyses were performed using GraphPad Prism software. P-values ≤ 0.05 were considered statistically significant. All authors had access to the study data and had reviewed and approved the final manuscript.

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