Supplementary informations

Endoscopic damage assessment

For continuous monitoring of colitis, a high resolution mouse video endoscopic system was developed. Mice were anaesthetised using intraperitoneal injection of avertine (Sigma Chemical Co, St Louis, Missouri, USA). The experimental endoscopy setup, denoted "*Coloview system*", consisted of a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (all from Karl Storz, Tuttlingen, Germany) to achieve regulated inflation of the mouse colon. The endoscopic procedure was viewed on a colour monitor and digitally recorded. A modified murine endoscopic index score of colitis severity based on the evaluation of colon translucency (0–3 points), granular features of the mucosa (0–3 points), morphology of the vascular pattern (0–3 points), and the presence of loose stools (0–3 points) was applied, as previously described (1), leading to a cumulative score between 0 (no signs of inflammation) and 12 (endoscopic signs of very severe inflammation).

Isolation and culture of HILEC

HILEC were obtained from human intestinal microvascular endothelial cells, which were isolated as previously described (2). Briefly, HIMEC were obtained from involved surgical specimens of patients with CD (n=15) and UC (n=15) (IBD HILEC) and from normal areas of the intestine of patients admitted for bowel resection of colon cancer, polyps, or diverticulosis (NL, n=30). HIMEC were isolated by enzymatic digestion of intestinal mucosal strips followed by gentle compression to extrude endothelial cell clumps, which adhere to fibronectin-coated plates and were subsequently cultured in MCDB131 medium (Sigma) supplemented with FCS, antibiotics, heparin, and endothelial cell growth factor. After 9 days in culture, cells were trypsinized and incubated with the following antibodies: anti-human Podoplanin (clone 18H5; 1 mg per 10^6 cells; Santa Cruz), anti-human VEGFR-3 (clone 54733; 625 ng per 10^5 cells; R&D system), and PE-conjugated mouse anti-

human CD31 antibody (clone WM59; 10 µl per 10^6 cells). HILEC were then sorted on a FACSAria IIU (BD Biosciences), using a FACSDiva software (version 6.1.3; BD Biosciences), by positive selection for Podoplanin and VEGFR-3 gated on a CD31-positive population (see Figure S1A) cells (BD Pharmingen), followed by incubation with Alexa Fluor 647- and Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes). Cultures of HILEC were maintained at 37°C in 5% CO_2, fed twice a week, and split at confluence. HILEC were used between passages 2 and 6.

Fibrin bead and Tubule formation assay

Cytodex-3 microcarrier beads (diameter of 150 µm) were sterilized and prepared for seeding by autoclaving in PBS followed by a series of washes in MCDB-131 medium containing 20% FCS, 0.04g Heparin, 250 Unit/ml Penicillin, 250 µg/ml Streptomycin, and 0.125 µg/ml Amphotericin B (MCDB-/-). One million of NL and IBD HILEC were then added to 5 ml of MCDB-/- and 2500 Cytodex beads and incubated (37°C, 5% CO^2) for a total of 4 h, with gently agitation every 30 min. After this incubation, cell-coated microcarrier beads were transferred to a T-25 flask with 5 ml of complete MCDB131 medium, containing 0.05 mg/ml endothelial cell growth factor (Roche). The flask was then incubated in the standard cell culture position overnight at 37°C, 5% CO^2, allowing cells that have not attached to the beads to adhere to the bottom of the flask. Following the overnight incubation, a fibrinogen solution (5 mg/ml; Sigma), containing 22.8 µg/ml of Aprotinin (Sigma) was prepared in MCDB-/- medium and sterile filtered. HILEC-coated beads were then added to the fibrinogen solution (200 beads/ml) and plated in triplicate in a 24-well plate (500 µl per well), with each well containing 0.625 Unit of Thrombin (Sigma). The fibrin clot was allowed to stand for 5 min before incubating for 20 min at the previously stated conditions. HILEC-coated beads were then cultured in complete MCDB131 medium containing 1% FCS, with or without VEGF-C (10 ng/ml; R&D system), VEGF156 (Cys156Ser; 10 ng/ml; R&D system), or SAR131675 (3) (23 nM dissolved in DMSO; Selleckchem), with the media changed every day for 5 days. At the end of the incubation, fibrin gels were fixed with 2% paraformaldehdye in PBS overnight at 4° C.

The cells were then permeabilized and blocked with 0.3% Triton X-100 for 20 min at RT. Gels were then incubated for 1 hour with phalloidin Alexa Fluor 488 (Molecular Probes) to stain for actin. After 3 washes with PBS, fibrin gels were stained with DAPI for 10 min and imaged (up to 5 beads/well) with a laser scanning confocal microscope (Fluoview FV1000; Olympus). The FluoView software was used to capture the images as well as to quantify the number of sprouts per bead, using a 20x objective. A sprout was defined as a multicellular extension with >1 connected lymphatic endothelial cells that were attached to the microcarrier.

HILEC tube formation was also assessed using Matrigel (BD Biosciences) as previously described (4). Briefly, multiwell dishes were coated with 250 µL of complete medium containing 5 mg/ml Matrigel for 30 min at 37°C, and NL and IBD HILEC were seeded in triplicate at a density of 5×10^4 in complete MCDB131 medium with or without VEGF-C (10 ng/ml; R&D system), VEGF156 (Cys156Ser; 10 ng/ml; R&D system), or SAR131675 (3) (23 nM dissolved in DMSO; Selleckchem). Cells were cultured on Matrigel for 4 h, and inverted phase-contrast microscopy was used to assess and count endothelial tube-like structures. Five high-power fields per condition were examined. All experiments were repeated three times.

Whole mount staining

Colons were removed from untreated (UN) and treated mice subjected to the DSS protocol with the indicated treatments, and immersed in 1% paraformaldehyde fixative solution, overnight at 4°C. Tissues were then washed and incubated with hamster anti-CD31 (1:500; Millipore) and rabbit anti-Lyve-1 (1:500; abcam) antibodies diluted in PBS containing 0.3% Triton X-100, 2% bovine serum albumin, 5% goat serum, 0.01% glycine, and 0.1% sodium azide, overnight at 4°C. Anti-hamster Alexa Fluor 647 and anti-rabbit Alexa Fluor 488 (1:500; Molecular Probes) were used as secondary antibodies, and incubated overnight at 4°C. Samples were then mounted with Vectashield (Vector Laboratories) and imaged with a laser scanning confocal microscope (Fluoview FV1000; Olympus).

Immunofluorescence of human and murine colon tissues

Frozen sections (6 μm) of human and murine colon tissues were fixed in 4% paraformaldehyde solution for 10 min and then permeabilized in 0.1% Triton X-100 solution for 20 min at RT. Tissue sections were then blocked with PBS containing 2% bovine serum albumin, 0.05% Tween and 2% goat (for human colon tissues) or donkey serum (for murine colon tissue) for 30 min at RT. After blocking, human colon sections were incubated with mouse anti-VEGR-3 (1:100; Millipore), and rabbit anti-Lyve-1 (1:200; abcam) antibodies for 2 h. Murine colon sections were incubated overnight at 4°C with rabbit anti-CD31 (1:100; Life Science Biotechnology) and goat anti-Lyve-1 (1:50; R&D systems) primary antibodies. All sections were subsequently incubated for 30 min with anti-mouse Alexa Fluor 647-conjugated, anti-rabbit Alexa Fluor 488-conjugated, anti-goat Alexa Fluor 488-conjugated, and anti-rabbit Alexa Fluor 647-conjugated antibodies (1:1000; Molecular Probes), followed by incubation with DAPI for nuclear staining (1:25000; Invitrogen). Sections were mounted with ProLong Gold mounting medium (Invitrogen) and analyzed with a laser scanning confocal microscope (FluoView FV1000; Olympus).

Proliferation and migration assays

A crystal violet assay was used to assess HILEC proliferation, as described previously (5). Briefly, NL and IBD HILEC at the same passage were seeded in triplicate in 96-well cell culture plates (1×10^3 cells/well) with complete growth medium containing 1% FCS, and either left untreated or treated with VEGF-C (10 ng/ml; R&D system) or VEGF156 (Cys156Ser; 10 ng/ml; R&D system), both in combination with SAR131675 (3) (23 nM dissolved in DMSO; Selleckchem). Medium was changed every other day and after 6 days in culture, cells were stained with 0.2% crystal violet (Sigma) dissolved in ethanol. Uptake of dye by cells on plates was eluted with 33% acetic acid in water. Plates were gently shaken for 20 min and the absorbance at 560 nm was measured by a Versamax microplate reader (Molecular Devices) at a wavelength of 560 nm. The optical density

(OD) of each sample was then compared with a standard curve, in which the OD was directly proportional to known cell numbers.

Migration assay was assessed as previously reported (2), with some modifications. By using a BD BioCoat Matrigel invasion chamber with polycarbonate filters (8-μm pore size; BD Biosciences), starved (MCDB-131 for 18 h) NL and IBD HILEC (20 x 10^3/filter) or fully differentiated monocyte-derived MΦ (150 x 10^3/filter) were plated in the upper chamber in MCDB-131 medium or in RPMI 1640 supplemented with VEGFC (10 ng/ml; R&D system) and mF431C1 (300 μg/ml, Imclone) respectively, whereas the lower chamber contained MCDB-131 medium with VEGF-C (10 ng/ml; R&D system), or VEGF156 (Cys156Ser; 10 ng/ml; R&D system) for HILEC and RPMI 1640 alone for MΦ. In some wells, 25 nM of the chemical compound SAR131675 (Selleckchem) were added with HILEC. After 8 h, medium was removed from both chambers, and cells that had migrated onto the lower surface of the porous membrane were washed twice in PBS and stained with Diff Quick (Medion Diagnostics), according to the manufacturer's instruction. Triplicates of migrated cells were counted in the entire membrane using a 20x objective.

Immunoprecipitation and Western Blotting

Human. Starved (MCDB-131 for 18 h) sub-confluent HILEC were left unstimulated or stimulated with VEGF-C (10 ng/ml; R&D system) or VEGF156 (Cys156Ser; 10 ng/ml; R&D system), both in combination with SAR131675 (23 nM dissolved in DMSO; Selleckchem) for 48 h in complete growth medium containing 1% FCS. The cells were lysed with extraction buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, pH 8.0, 150 mM NaCl, 1% Triton X-100, supplemented with a cocktail of protease inhibitors (Sigma). The protein concentration of lysates was measured using the Bio-Rad protein assay (Bio-Rad Laboratories) and extracts were pre-cleared with lysis buffer-containing protein-G agarose beads for 1 hour at 4°C. Pre-cleared lysates (1 mg) were incubated with an antibody against human VEGFR-3 (1 μg/ml in lysis buffer;

R&D system) overnight at 4°C. After washing with lysis buffer, the immunoprecipitates were resuspended in SDS-sample buffer and immunoblotted, as described below.

Immunoblotting was performed as previously described (2). Proteins (80 μg) were separated on a 10% Tris-glycine gel and electrotransferred to a nitrocellulose membrane (Bio-Rad Laboratories). Nonspecific binding was blocked with Tris-buffered saline (TBS) containing 5% non-fat dried milk and 0.1% Tween 20, followed by overnight incubation at 4°C with the mouse anti-phosphotyrosine antibody (clone 4G10; 1:1000; Millipore). Membranes were washed for 1 h with TBS containing 0.1% Tween 20 and then incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:3000; GE Healthcare). The membranes were then incubated with Immobilon Western Chemilum (Millipore) for 1 minute, after which bands were detected by Chemidoc (Bio-Rad Laboratories), using Quantity One software. The filter was then stripped with buffer Restore (Pierce) and reprobed with the rabbit anti-human VEGFR-3 (1:500; Zymed Laboratories) followed by an anti–actin antibody (clone C11; 1:1000; Santa Cruz Biotechnology) for control protein loading.

Mouse. Colonic samples from normal and colitic mice with the indicated treatments and at the indicated time points were mechanically homogenized in lysis buffer for protein extraction. Insoluble material was removed by centrifugation for 30 min at 14,000 *rpm* at 4°C. The concentration of proteins in each lysate was measured using the Bio-Rad protein assay (Bio-Rad Laboratories). Immunoprecipitation and immunoblotting were performed as described above, using the following antibodies: anti-human VEGF-C (1:500; R&D system), anti-mouse VEGF-C (1:1000; abcam); anti-mouse VEGFR-3 (R&D Systems) to immunoprecipitate, anti-mouse VEGFR-3 (clone 31C1; 1:500; ImClone Systems) to blot, anti-phosphotyrosine (clone 4G10; 1:1000; Millipore), and anti–actin antibody (clone C11; 1:1000; Santa Cruz Biotechnology) as loading control.

Enzyme-Linked Immunosorbent Assay

VEGF-C and VEGFD were measured in mucosal biopsy extracts using enzyme-linked immunosorbent assays (ELISA), according to the manufacturer's instructions (R&D Systems). Biopsy specimens collected from the actively inflamed mucosa of patients with UC and CD (IBD) and from normal areas of the colons of control individuals undergoing bowel resection for colon cancer, polyps or diverticulosis (NL). Biopsy samples were homogenized on ice in extraction buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100) supplemented with a cocktail of protease inhibitors. Samples were centrifuged at 900*g* for 15 min, then the supernatants were collected and stored at −80°C. The protein concentration was measured using the Bio-Rad protein assay as per manufacturer's protein.

***In vivo* depletion of MΦ**

8–12-week-old female mice undergoing two cycles of DSS treatment were injected with adenoviruses encoding hVEGFC, and, were indicated, with intrarectal clodronate liposomes (CDL) (ClodronateLiposomes.org, Amsterdam, The Netherlands) or PBS, as described in (6) (n=4-6 mice/group). Additional control groups were either left untreated (DSS + AdVEGFC) or with DSS alone. Briefly, mice were anesthetized by i.p. injection of 200 µl 2,5% Avertin per mouse. Once anesthetized, mice were injected with 100 µl CDL intrarectally using a micropipette. This was performed on days -4 of DSS administration and every 3 days for the entire experiment. For IL-10 ko mice (data not shown) CDL injection started before the development of colitis, at 10 weeks of age and every 3 days until sacrifice. Note that PBS-encapsulated liposomes were not used as a negative control, as preliminary studies indicated that uptake of these liposomes by colonic macrophages (MΦ) caused a partial reduction in the percentage of MΦ in the colon and could affect the physiology of the remaining colonic MΦ (data not shown and ClodronateLiposomes.org,

Amsterdam, The Netherlands). The efficiency of MΦ depletion was determined by FACS analysis, as described for evaluation of LPS-beads positive cells.

Preparation, stimulation, and transfection of MΦ

Lamina propria MΦ were isolated from mice at day 40 of DSS treatment, and from 20-weeks old IL-10 ko mice (data not shown), both groups injected with adenoviruses encoding hVEGF-C or anti-VEGFR3 antibody. Healthy animals were used as control (n=4-6 mice/group). Briefly, according to a published protocol (7), colons were cut longitudinally and washed in PBS containing 1% fetal bovine serum and 1 mM DTT for 15 minutes at RT. Enzymatic digestion was performed in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.5 mg/ml collagenase type VIII (Sigma-Aldrich), 20 mg/ml DNase I (Roche Diagnostics), 100 U/ml penicillin and 100 µg/ml streptomycin for 60 minutes at 37°C with gentle shaking. Cell suspension was passed through a 100 µm filter and then clarified over 70 µm nylon mesh. Colonic macrophages were enriched by positive selection with anti-CD11b Dynal-beads (Invitrogen) following the manufacturer's instructions, and then cultured in 24-well plates (13 x 10^6 per well) in 1 ml of complete RPMI 1640 medium. After a 4-h incubation, both cells and supernatants were collected, as described in (8) for gene and protein expression analysis.

For bone marrow (BM)-derived MΦ, cells were flushed from the femurs and tibia of 7- to 12-weeks-old C57Bl6J healthy mice and treated with ammonium chloride potassium buffer to lyse red blood cells. Cells were then plated onto sterile petri dishes and cultured in RPMI 1640 medium with 20% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, and 1X GlutaMAX (Invitrogen) plus 20 ng/ml of murine recombinant macrophage colony-stimulating factor (M-CSF, R&D Systems) for 7 days, with a complete change of medium every 2-3 days. On day 7, after removal of nonadherent cells, MΦ (> 90% purity) were detached using 5mM EDTA treatment, washed, counted, and replated at a density of 10^6 cells/ml for 24 h with medium (untreated control), IL-4 (10

ng/ml; R&D Systems), LPS (1 μg/ml; Sigma-Aldrich) or VEGFC (100 ng/ml; R&D Systems), this last in the presence or in the absence of the same anti-VEGFR-3 antibody used *in vivo* (mF431C1, 300 μg/ml).

When indicated, fully differentiated monocyte-derived MΦ were transfected with STAT6 or scramble small interfering (si) RNA (all from Santa Cruz Biotechnology) using lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Seventy-two hours after the transfection, cells were either left untreated or stimulated with VEGFC (100 ng/ml; R&D Systems) for 24 h. The efficiency of the STAT6 silencing was assessed by Western blotting (data not shown).

For both lamina propria- and BM-derived MΦ, the expression of M2 and M1 MΦ-associated genes was examined by Real Time PCR for Ym1, Arg-1, Fizz1, Cox2, and iNOS, and by FACS for MHCII and CD80. For FACS analysis, cells were incubated with FITC-conjugated anti-MHC II (Southern Biotechnology Associates) or FITC-conjugated anti-CD80 (ABD Serotec), and data were analyzed using the FACSDiva software (BD Biosciences). The concentration of IL-10, IL-6, TNF-α and IFN-γ on supernatants were measured using Mouse Ready-SET-Go! ELISA kits from eBioscience. cAMP was measured by enzyme immunoassay (Cayman Chemicals). Anti-phospho-STAT6 (Tyr641) and anti-STAT6 antibodies, both from Cell Signaling, were used to assess STAT6 activation and expression by Western Blotting.

Primer Pair sequences for Real-Time analyses

Real Time PCR analyses were performed starting with cDNA obtained from MΦ described above. The primer pairs used are summarized in the table below:

GENE Product	*Forward PRIMER*	*Reverse PRIMER*
mArg-1	5'-cagaagaatggaagagtcag-3'	5'-cagatatgcagggagtcacc-3'
mFIZZ1	5'-tcccagtgaatactgatgaga-3'	5'-ccactctggatctcccaaga -3'
mYm1	5'-gggcatacctttatcctgag-3'	5'-ccactgaagtcatccatgtc-3'
mCOX2	5'-agacatggagtcataggctctg-3'	5'-ccattttccttcttgtggagca-3'

miNOS	5'-gccaccaacaatggcaaca-3'	5'-cgtaccggatgagctgtgaatt-3'

The reactions were performed on ViiA™7 Real-Time PCR System (Applied Biosystems). GAPDH expression was used as housekeeping gene. Data were calculated using the $2^{\wedge-\Delta Ct}$ method.

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Supplementary Figure Legends

Figure S1. **VEGFR-3 signaling is important for HILEC proliferation, migration and organization *in vitro*.** (**A-D**) Medium supplemented with 100 ng/ml VEGF-C promoted the proliferation (**A**), migration (**B**) and capillary formation (**C, D**) of both control (NL) and IBD HILEC, but with a significantly higher response in IBD HILEC compared to NL cells. VEGF156 mutant showed similar results. Following 96 h of VEGF-C/VEGF156 treatment, the blockade of VEGFR-3 (SAR131675) activation significantly inhibited both basal and VEGF-C- or VEGF156-induced proliferation, migration and tube formation, with similar effects in NL and IBD HILEC. (**C**) Representative confocal images showing the formation of capillary networks from HILEC-coated microcarrier beads embedded within 5.0 mg/ml fibrin tissue on day 4 and stained for DAPI (blue) and F-actin (green). Dotted lines represent beads and tubular structures. Magnification: 40x. *P<0,05 vs. untreated; **P<0,001 vs. untreated; # P<0,01 vs. other conditions as indicated. (**E**) Immunoprecipitation performed on cell lysates from VEGF-C- or VEGF156- stimulated NL (left panel) and IBD (right panel) HILEC showed increased VEGFR-3 phosphorylation (pTyr), which in turn was inhibited upon SAR131675 treatment.

Figure S2 (Related to Figure 2). Systemic delivery of VEGF-C significantly improves colitis both clinically and endoscopically. Mice undergoing two cycles of DSS treatment and 15-week-old IL-10 ko mice with established colitis were injected with adenoviruses encoding hVEGF-C (n=8/time point), or anti-VEGFR3 antibody (mF431C1, n=8/time point). (**A**) Colonic samples from normal and colitic mice with the indicated treatments and at the indicated time points were mechanically homogenized in lysis buffer for protein extraction. Immunoprecipitation and immunoblotting were performed as described in methods, and representative blots are presented. Actin is used as loading control. (**B-D**) Systemic delivery of VEGF-C significantly reduced the severity of colitis, based on endoscopic colitis score (**B**) and Disease Activity Index (**C, D**), whereas mF431C1 worsened the colitis, compared to control mice (only DSS and Il-10 ko). UN: no DSS. Wt: IL-10 wild-type. Black dotted lines represent the two DSS cycles. Red and green dotted lines indicate the first VEGF-C and mF431C1 administration, respectively. Values and bars represent the mean per experimental group ± SEM The *P<0,05; **P<0,001.

Figure S3 (Related to Figure 3 and 5). Systemic delivery of VEGF-C increases LV dimension and lymphatic drainage in colitic mice. Mice undergoing two cycles of DSS treatment, were sacrificed at the end of the experiment (day 40) and colons were collected for the evaluation of the expression of

VEGF-C and VEGFR-3. (**A**) Representative frozen sections from the colons of control (UN, n=5) or DSS-treated (colitic, n=5) mice double-stained with anti-Lyve-1 and anti-VEGFR-3 antibodies. (**B**) VEGFR-3+ lymphatic vessels were quantified by stereological point counting of 10 regions per section and the numbers were normalized per total section area expressed in square millimeters. The results are presented as the mean vessel density per group ± SEM. *P<0,05. (**C**) The average fluorescence intensity (FI) per vessel was analyzed on VEGFR-3+ LVs (10-15 vessels per section). FI is expressed as relative units normalized per vascular area expressed in square micrometers. *P<0,05. (**D**) Representative blots showing VEGF-C protein levels in control (UN) and DSS-treated (colitic) mice, with Actin as loading control. (**E**) Whole mounts of proximal, distal and rectal colonic segments from DSS-treated mice with acute and chronic colitis were stained with antibodies against LYVE-1 and CD31 to measure LV dimension. LV diameter was quantified at the indicated time points and reported as mean values ± SEM, for each group (n=8 mice/group with 5 fields/colonic segment). *P<0,05; **P<0,001 vs DSS alone. (**F**) Ten micrograms of Evans blue dye was injected into the colonic mucosa of healthy (IL-10 Wt, n=5/time point), and colitic (IL-10 ko) AdVEGF-C- (n=5/time point) and mF431C1-treated mice (n=5/time point). IL-10 ko animals (n=5/time point) were used as a control group. Evans blue was then extracted 16 hours after the dye injection from distal colons of comparable weight. The graph shows the total dye remaining in the colon expressed in µg. Black dotted lines represent the two DSS cycles. Red and green dotted lines indicate the first VEGF-C and mF431C1 administration, respectively. Data represent the mean per group ± SEM. *P<0,05; **P<0,001 vs IL-10 ko.

Figure S4 (Related to Figure 6). Systemic delivery of VEGF-C accelerates migration of both CD11c- and CD11c+ macrophages. A single intramucosal injection of carboxylated crimson fluorescent LPS-coated beads was performed at day 5 after the second DSS cycle (chronic inflammation) and at day 21 after the first AdVEGF-C/mF431C1 administration in DSS-treated and IL-10 ko mice. Colitic animals treated with an adenovirus overexpressing GFP (AdGFP) and injected with LPS-coated beads were used as control. (**A**) Uptake of coated beads by each subpopulation was quantified in the colon of DSS-treated animals 12 h after the injection and the percentage of LPS-bead+ cells is reported. Data represent the mean per group ± SEM. (**B, C**) The phenotype of cleared LPS-coated beads^{+} cells in the DLNs 3 days after LPS injection was analyzed. The percentage of the indicated subpopulations, according to their CD11b and CD11c expression levels, is reported in the

graphs for both the DSS (**B**) and the IL-10 ko model of chronic colitis (**C**). Values are expressed as mean per group ± SEM. n=5 mice per group. *P<0,05; **P<0,001.

Figure S5 (Related to Figure 7). Effect of rectal administration of CDL on local MΦ in the intestine. Healthy (no DSS) and DSS-treated mice received intramucosal injections of CDL or PBS alone starting at Day 0 and every two days for the entire experiment of chronic inflammation. (**A**) Representative FACS plots showing macrophages (MΦ) and dendritic cells (DCs) (left panels), and cd11c+ and cd11c- cells (right panel) within the MΦ cell population at day 21. (**B**) Representative FACS plots of VEGFR3 expression in MΦ and DCs at the indicated experimental conditions. (**C**) Representative immunofluorescence images of VEGFR-3-expressing MΦ in the colon of colitic mice at day 21. n=5 mice per group.

Figure S6 (Related to Figure 7 and 8). MΦ depletion using clodronate liposomes (CDL) and role of the VEGFC/VEGFR-3 signaling on MΦ plasticity and activation during DSS-induced colitis. (**A**) Mice undergoing two cycles of DSS treatment were injected with adenoviruses encoding hVEGF-C (n=4/time point), and, were indicated, with intrarectal CDL or PBS (n=4/time point), as described in methods. CDL administration significantly reduced the protective effect of VEGFC, based on histological score (**A, left panel**), and DAI score (**A, right panel**), compared to control mice (only DSS + VEGFC). Black dotted lines represent the two DSS cycles. *P<0,05. (**B, C**) Increased production of pro-inflammatory cytokines (**B**) and cAMP (**C**) in colonic MΦ isolated from healthy (UN), and DSS-treated mice in the presence of AdVEGFC or mF431C1 and kept in culture for 24 h. Concentration of cytokines in the supernatant of colon MΦ were measured by ELISA. Data are mean values per group (n=4-6/group). **P<0,01 vs. DSS alone. **(D)** Bone marrow-derived MΦ were cultured and stimulated *in vitro* with LPS (M1), IL-4 (M2), VEGFC and VEGFC + mF431C1 for 24 h. The expression of the indicated genes was then examined with Real-Time PCR or FACS. The data shown are the means ± SEM of three independent experiments. **P<0,01 vs. untreated (UN). §P<0,001 vs. VEGFC alone.

Figure S7 (Related to Figure 9). VEGFC modulate resolving MΦ plasticity and migration in a STAT-6 dependent manner. (A, B) Bone marrow-derived MΦ were cultured, transfected with a STAT6 specific siRNA or a STAT6 scramble, and stimulated *in vitro* with VEGFC for 24 h. The expression of the M2 (**A**) and M1 (**B**) MΦ-associated genes was then examined with Real-Time PCR or ELISA. The data shown are the means ± SEM of three independent experiments. **P<0,01 vs VEGFC alone. (C) Bone marrow-derived MΦ described in (**A**) were subjected to migration through

matrigel-coated filters for 8 h and the number of migrated cells per filter is reported. Values are means ± SEM of triplicates of three independent experiments. **P<0,01. (D) Cells described in Figure 9D were injected in the rectal mucosa of DSS-treated mice (n=4/group), and colon length expressed in cm was evaluated. Results are presented as the mean value per group ± SEM. **P<0,01.

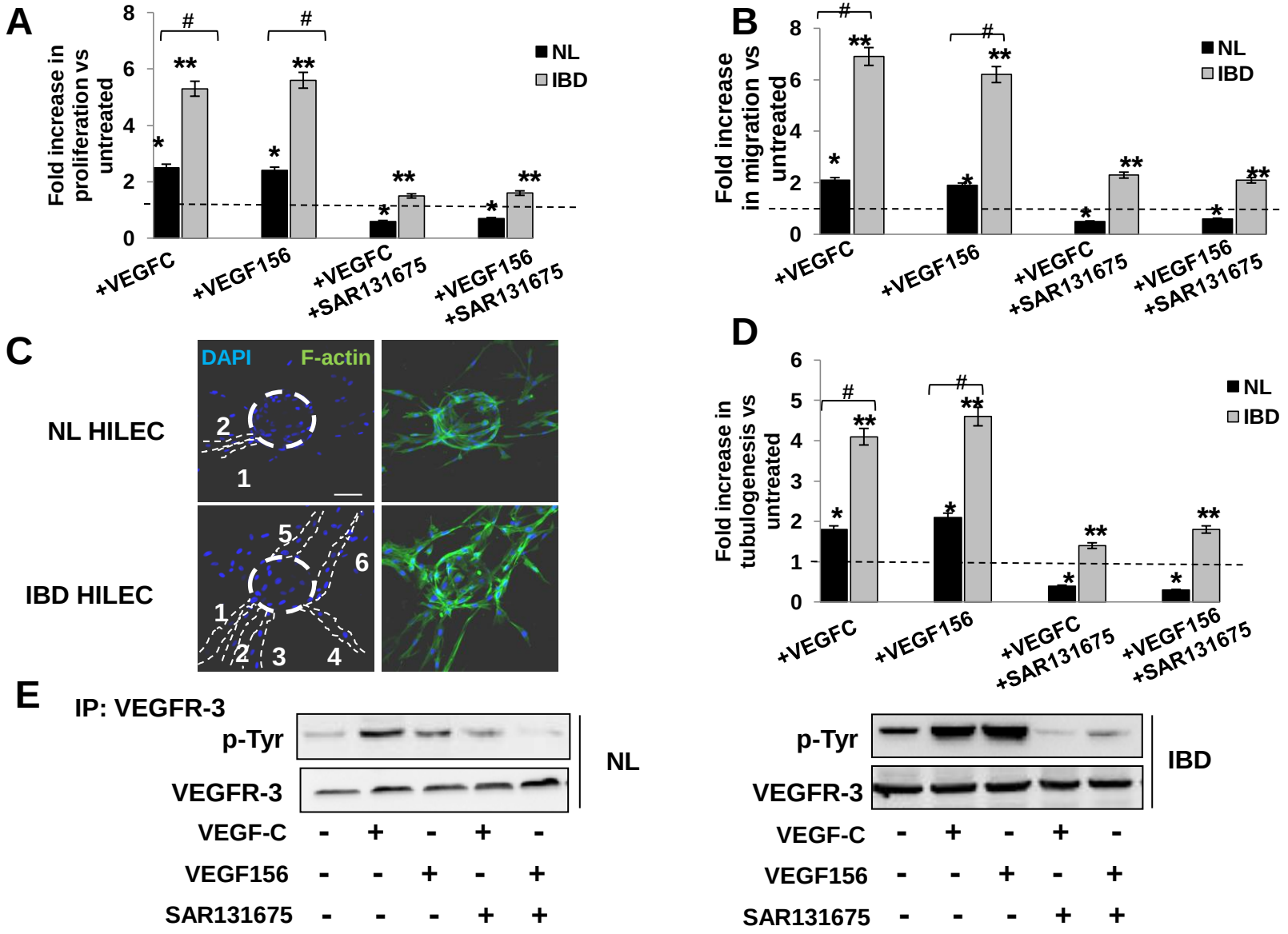

Figure S1. **VEGFR-3 signaling is important for HILEC proliferation, migration and organization *in vitro*.** (**A-D**) Medium supplemented with 100 ng/ml VEGF-C promoted the proliferation (**A**), migration (**B**) and capillary formation (**C, D**) of both control (NL) and IBD HILEC, but with a significantly higher response in IBD HILEC compared to NL cells. VEGF156 mutant showed similar results. Following 96 h of VEGF-C/VEGF156 treatment, the blockade of VEGFR-3 (SAR131675) activation significantly inhibited both basal and VEGF-C- or VEGF156-induced proliferation, migration and tube formation, with similar effects in NL and IBD HILEC. (**C**) Representative confocal images showing the formation of capillary networks from HILEC-coated microcarrier beads embedded within 5.0 mg/ml fibrin tissue on day 4 and stained for DAPI (blue) and F-actin (green). Dotted lines represent beads and tubular structures. Magnification: 40x. *P<0,05 vs. untreated; **P<0,001 vs. untreated; # P<0,01 vs. other conditions as indicated. (**E**) Immunoprecipitation performed on cell lysates from VEGF-C- or VEGF156- stimulated NL (left panel) and IBD (right panel) HILEC showed increased VEGFR-3 phosphorylation (pTyr), which in turn was inhibited upon SAR131675 treatment.

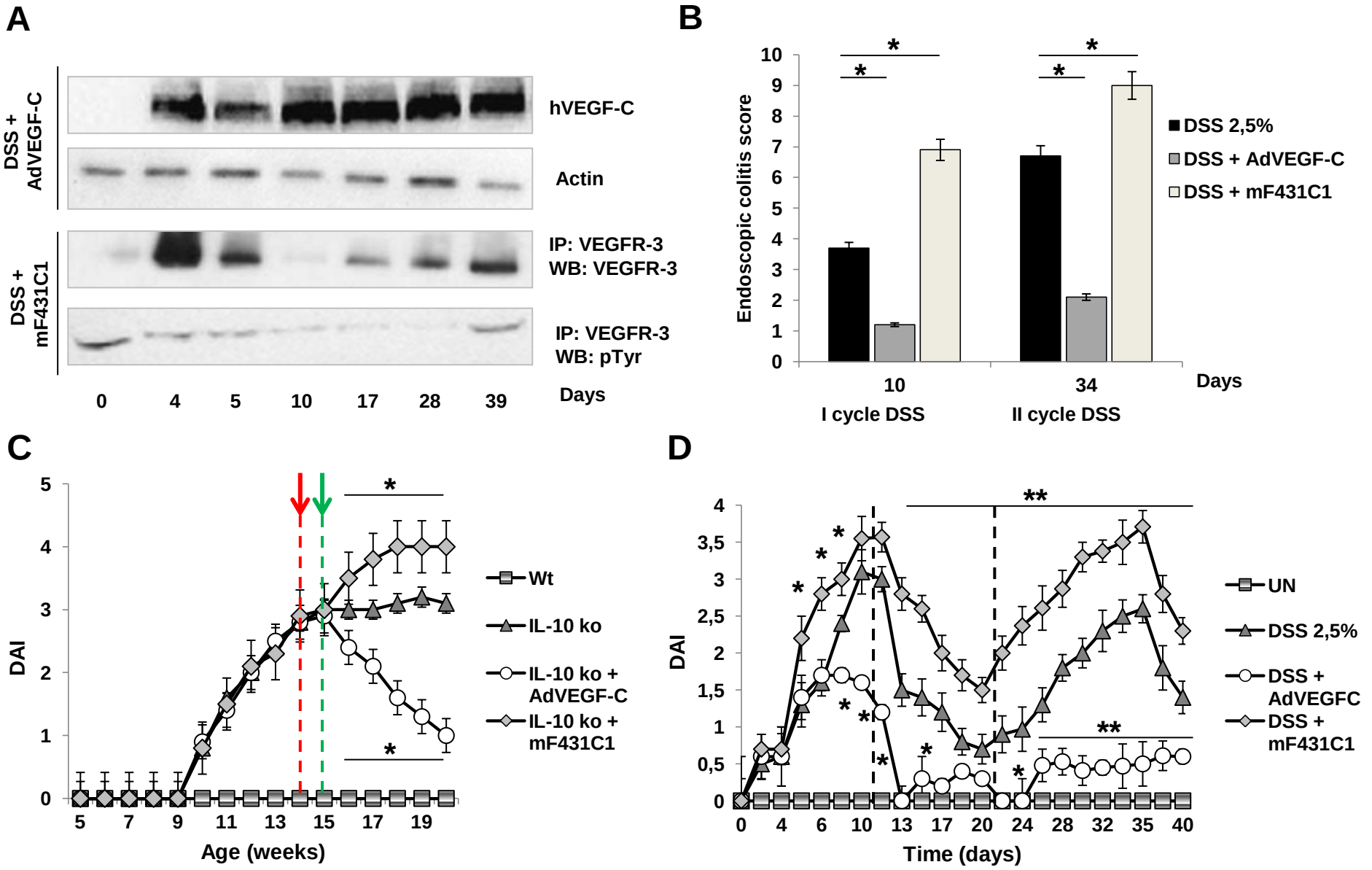

Figure S2 (Related to Figure 2). Systemic delivery of VEGF-C significantly improves colitis both clinically and endoscopically. Mice undergoing two cycles of DSS treatment and 15-week-old IL-10 ko mice with established colitis were injected with adenoviruses encoding hVEGF-C (n=8/time point), or anti-VEGFR3 antibody (mF431C1, n=8/time point). (**A**) Colonic samples from normal and colitic mice with the indicated treatments and at the indicated time points were mechanically homogenized in lysis buffer for protein extraction. Immunoprecipitation and immunoblotting were performed as described in methods, and representative blots are presented. Actin is used as loading control. (**B-D**) Systemic delivery of VEGF-C significantly reduced the severity of colitis, based on endoscopic colitis score (**B**) and Disease Activity Index (**C, D**), whereas mF431C1 worsened the colitis, compared to control mice (only DSS and Il-10 ko). UN: no DSS. Wt: IL-10 wild-type. Black dotted lines represent the two DSS cycles. Red and green dotted lines indicate the first VEGF-C and mF431C1 administration, respectively. Values and bars represent the mean per experimental group ± SEM The *P<0,05; **P<0,001.

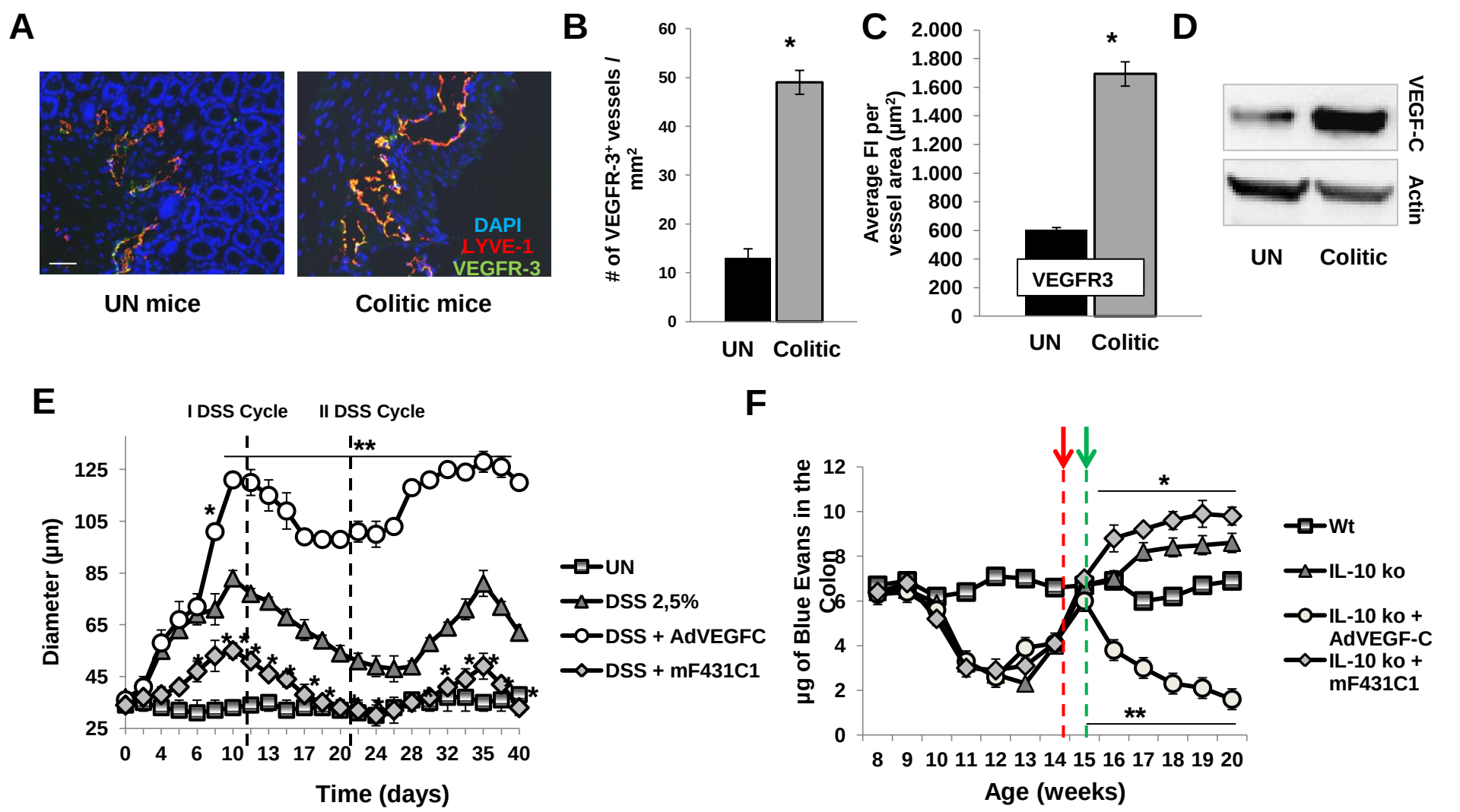

Figure S3 (Related to Figure 3 and 5). Systemic delivery of VEGF-C increases LV dimension and lymphatic drainage in colitic mice. Mice undergoing two cycles of DSS treatment, were sacrificed at the end of the experiment (day 40) and colons were collected for the evaluation of the expression of VEGF-C and VEGFR-3. (**A**) Representative frozen sections from the colons of control (UN, n=5) or DSS-treated (colitic, n=5) mice double-stained with anti-Lyve-1 and anti-VEGFR-3 antibodies. (**B**) VEGFR-3+ lymphatic vessels were quantified by stereological point counting of 10 regions per section and the numbers were normalized per total section area expressed in square millimeters. The results are presented as the mean vessel density per group ± SEM. *P<0,05. (**C**) The average fluorescence intensity (FI) per vessel was analyzed on VEGFR-3+ LVs (10-15 vessels per section). FI is expressed as relative units normalized per vascular area expressed in square micrometers. *P<0,05. (**D**) Representative blots showing VEGF-C protein levels in control (UN) and DSS-treated (colitic) mice, with Actin as loading control. (**E**) Whole mounts of proximal, distal and rectal colonic segments from DSS-treated mice with acute and chronic colitis were stained with antibodies against LYVE-1 and CD31 to measure LV dimension. LV diameter was quantified at the indicated time points and reported as mean values ± SEM, for each group (n=8 mice/group with 5 fields/colonic segment). *P<0,05; **P<0,001 vs DSS alone. (**F**) Ten micrograms of Evans blue dye was injected into the colonic mucosa of healthy (IL-10 Wt, n=5/time point), and colitic (IL-10 ko) AdVEGF-C- (n=5/time point) and mF431C1-treated mice (n=5/time point). IL-10 ko animals (n=5/time point) were used as a control group. Evans blue was then extracted 16 hours after the dye injection from distal colons of comparable weight. The graph shows the total dye remaining in the colon expressed in µg. Black dotted lines represent the two DSS cycles. Red and green dotted lines indicate the first VEGF-C and mF431C1 administration, respectively. Data represent the mean per group ± SEM. *P<0,05; **P<0,001 vs IL-10 ko.

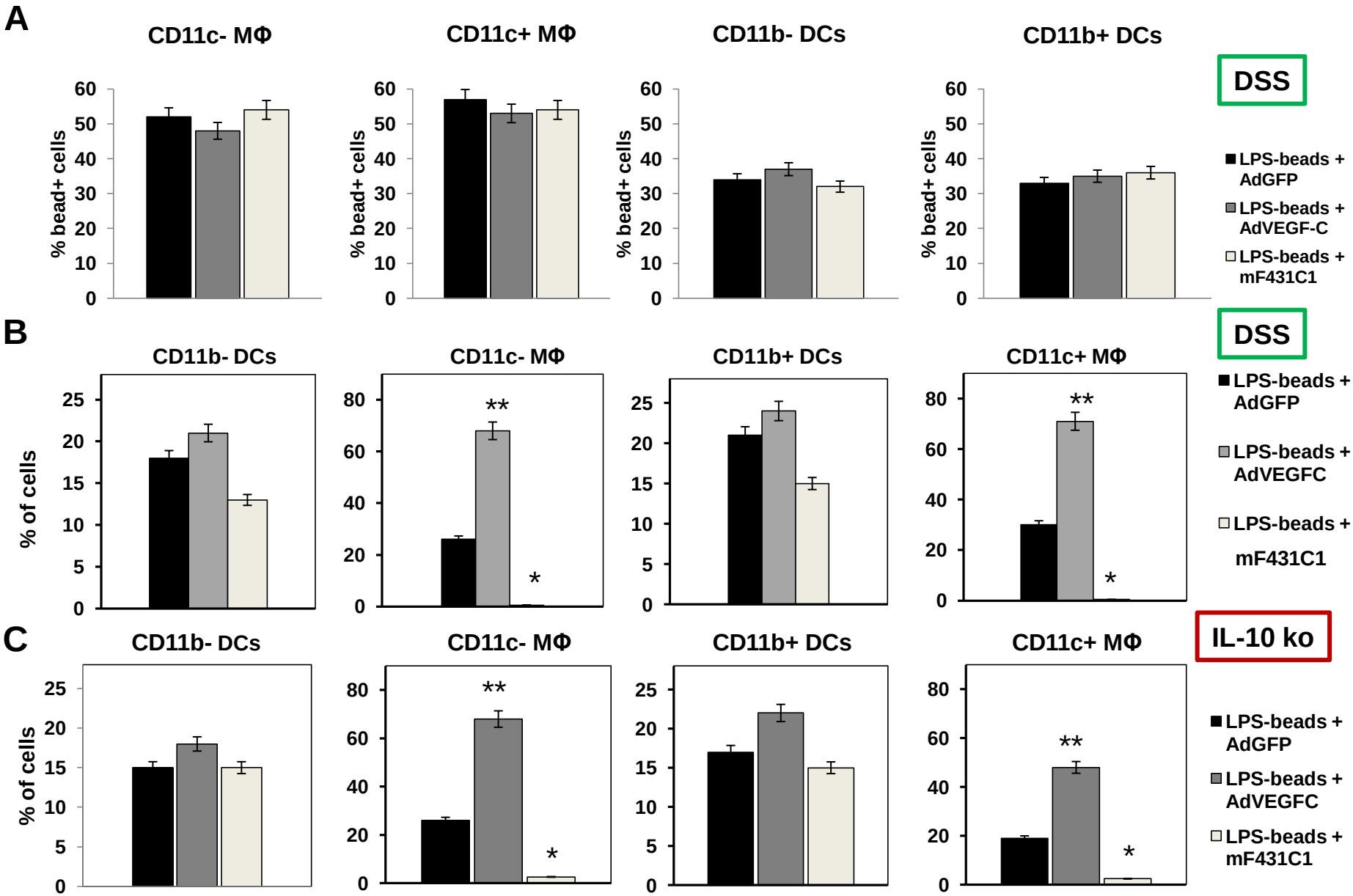

Figure S4 (Related to Figure 6). Systemic delivery of VEGF-C accelerates migration of both CD11c- and CD11c+ macrophages. A single intramucosal injection of carboxylated crimson fluorescent LPS-coated beads was performed at day 5 after the second DSS cycle (chronic inflammation) and at day 21 after the first AdVEGF-C/mF431C1 administration in DSS-treated and IL-10 ko mice. Colitic animals treated with an adenovirus overexpressing GFP (AdGFP) and injected with LPS-coated beads were used as control. (**A**) Uptake of coated beads by each subpopulation was quantified in the colon of DSS-treated animals 12 h after the injection and the percentage of LPS-bead+ cells is reported. Data represent the mean per group ± SEM. (**B,C**) The phenotype of cleared LPS-coated beads^{+} cells in the DLNs 3 days after LPS injection was analyzed. The percentage of the indicated subpopulations, according to their CD11b and CD11c expression levels, is reported in the graphs for both the DSS (**B**) and the IL-10 ko model of chronic colitis (**C**). Values are expressed as mean per group ± SEM. n=5 mice per group. *P<0,05; **P<0,001.

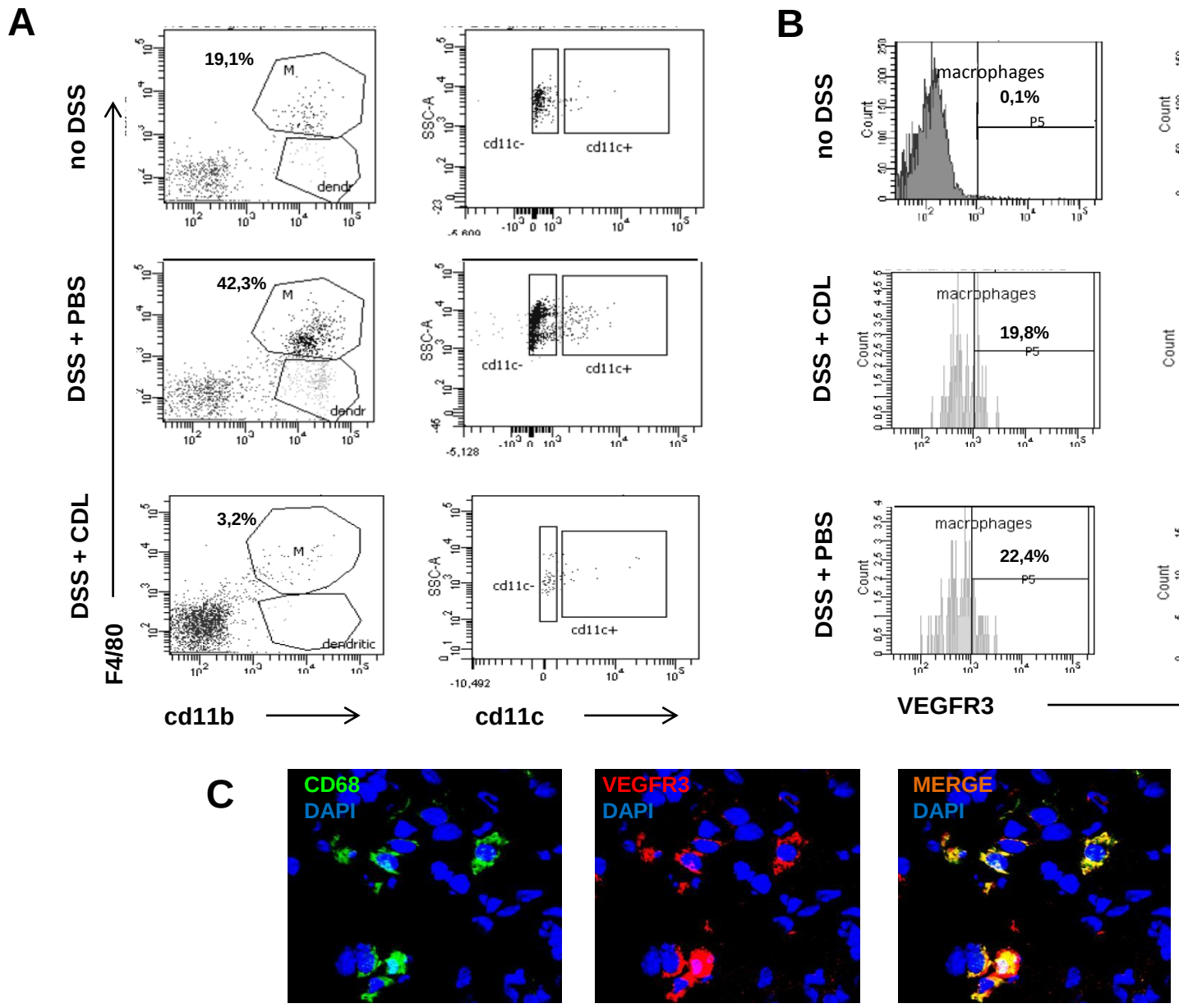

Figure S5 (Related to Figure 7). Effect of rectal administration of CDL on local MΦ in the intestine. Healthy (no DSS) and DSS-treated mice received intramucosal injections of CDL or PBS alone starting at Day 0 and every two days for the entire experiment of chronic inflammation. (**A**) Representative FACS plots showing macrophages (MΦ) and dendritic cells (DCs) (left panels), and cd11c+ and cd11c- cells (right panel) within the MΦ cell population at day 21. (**B**) Representative FACS plots of VEGFR3 expression in MΦ and DCs at the indicated experimental conditions. (**C**) Representative immunofluorescence images of VEGFR-3-expressing MΦ in the colon of colitic mice at day 21. n=5 mice per group.

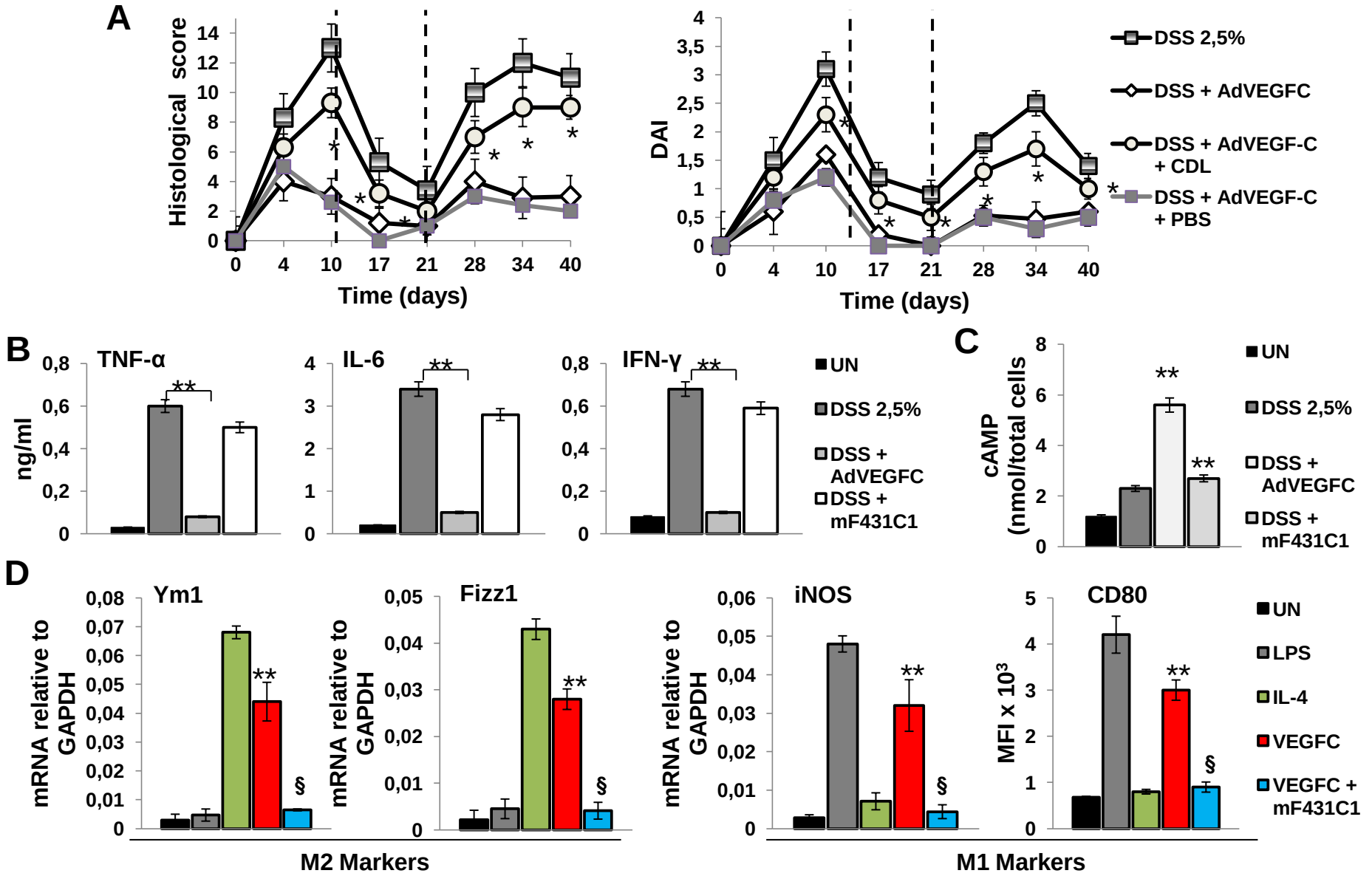

Figure S6 (Related to Figure 7 and 8). MΦ depletion using clodronate liposomes (CDL) and role of the VEGFC/VEGFR-3 signaling on MΦ plasticity and activation during DSS-induced colitis. (**A**) Mice undergoing two cycles of DSS treatment were injected with adenoviruses encoding hVEGF-C (n=4/time point), and, were indicated, with intrarectal CDL or PBS (n=4/time point), as described in methods. CDL administration significantly reduced the protective effect of VEGFC, based on histological score (**A, left panel**), and DAI score (**A, right panel**), compared to control mice (only DSS + VEGFC). Black dotted lines represent the two DSS cycles. *P<0,05. (**B, C**) Increased production of pro-inflammatory cytokines (**B**) and cAMP (**C**) in colonic MΦ isolated from healthy (UN), and DSS-treated mice in the presence of AdVEGFC or mF431C1 and kept in culture for 24 h. Concentration of cytokines in the supernatant of colon MΦ were measured by ELISA. Data are mean values per group (n=4-6/group). **P<0,01 vs. DSS alone. **(D)** Bone marrow-derived MΦ were cultured and stimulated *in vitro* with LPS (M1), IL-4 (M2) , VEGFC and VEGFC + mF431C1 for 24 h. The expression of the indicated genes was then examined with Real-Time PCR or FACS. The data shown are the means ± SEM of three independent experiments. **P<0,01 vs. untreated (UN). §P<0,001 vs. VEGFC alone.

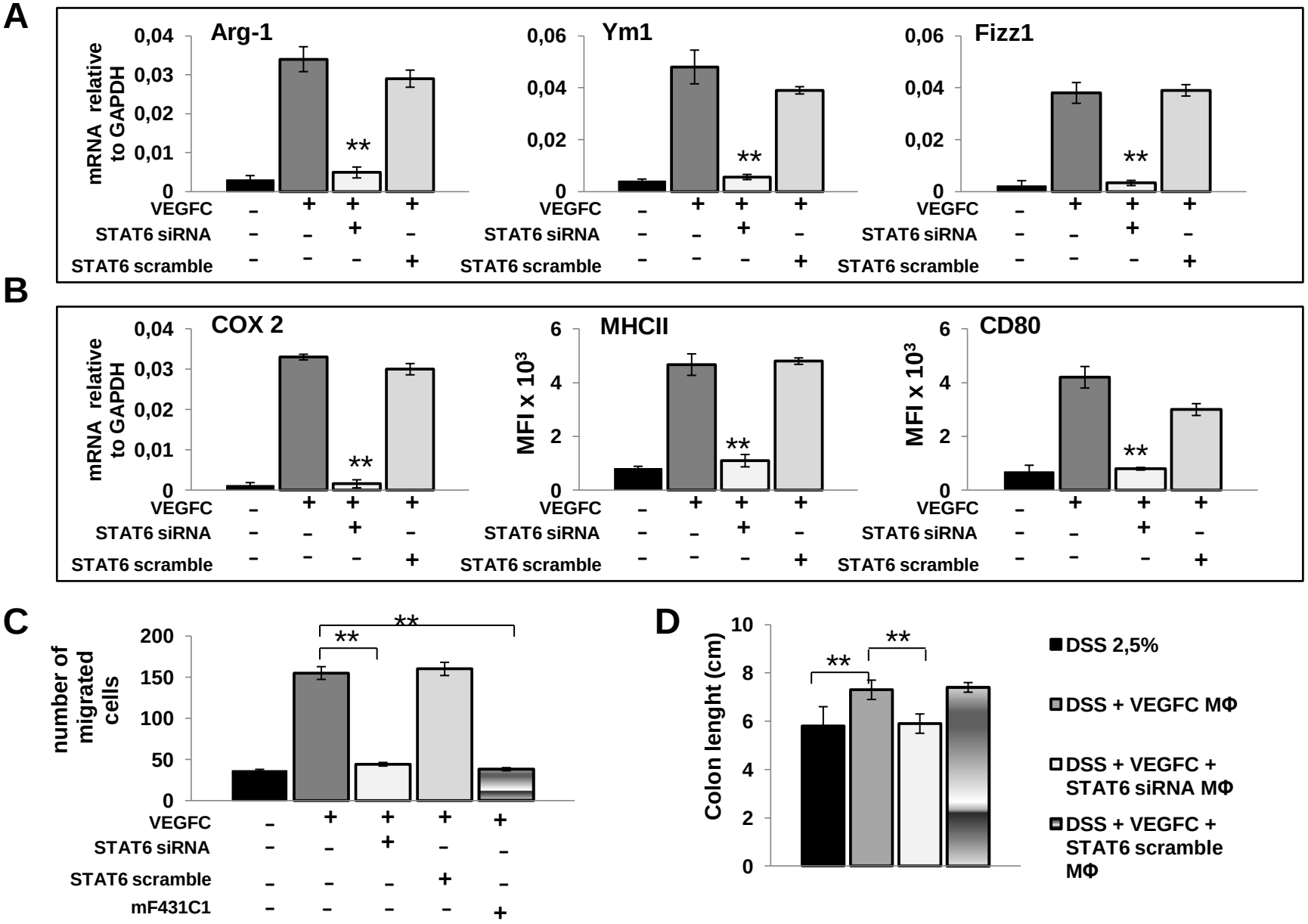

Figure S7 (Related to Figure 9). VEGFC modulate resolving MΦ plasticity and migration in a STAT-6 dependent manner. (A, B) Bone marrow-derived MΦ were cultured, transfected with a STAT6 specific siRNA or a STAT6 scramble, and stimulated *in vitro* with VEGFC for 24 h. The expression of the M2 (**A**) and M1 (**B**) MΦ-associated genes was then examined with Real-Time PCR or ELISA. The data shown are the means ± SEM of three independent experiments. **P<0,01 vs VEGFC alone. (C) Bone marrow-derived MΦ described in (**A**) were subjected to migration through matrigel-coated filters for 8 h and the number of migrated cells per filter is reported. Values are means ± SEM of triplicates of three independent experiments. . **P<0,01. (D) Cells described in Figure 9D were injected in the rectal mucosa of DSS-treated mice (n=4/group), and colon length expressed in cm was evaluated. Results are presented as the mean value per group ± SEM . **P<0,01.