**Supplementary Material**

**Cell Culture**

All cell lines (SW480, SW620, RKO, HCT116, HT29) derived from human colon cancer were used between 18-24 passages, and cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum, 1 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L non-essential amino acids, and 100 U/mL) antibiotics (penicillin and streptomycin) at 37 °C in 5% CO2.

**Animals**

Male and female C57BL/6 S1PR2-/- mice aged 5-12 weeks old (generated by the team of Dr. Richard L. Proia, NIH, Bethesda, MD) were maintained according to national (D.Lgs 26/2014) and international animal care criteria and used to generate S1PR2-/- and S1PR2+/+ littermates in the facility of Humanitas Clinical and Research Center. Female and male C57BL/6 Apcmin/+ aged 5-12mice were purchased from the Jackson Laboratory (Stock No: 002020). The double transgenic mouse S1PR2-/- Apcmin/+ was generated in the facility of the Humanitas Clinical and Research Center by crossbreeding S1PR2-/- and Apcmin/+ mice. Athymic female CD-1 nude mice (Strain code: 086) were purchased from the Charles River Laboratories. Charles River Laboratories All animals were fed a standard diet and housed in standard polypropylene mouse cages on sawdust bedding. Rooms were kept at 23°C and maintained on a 12-hr light and 12-hr dark cycle. All experiments were performed accordingly with the approval from the ethics committees of the Humanitas Clinical and Research Center, in agreement with national (D.Lgs 26/2014) and international animal care criteria. The genotypes were determined by PCR analysis of genomic DNA extracted from tail biopsies by NaOH 60 Mm tail lysis. The primer sequences used for the genotyping of the S1PR2 gene were: 5’- GCA GTG ACA AAA GCT GCC GAA TGC TG-3’, 5’- AGA TGG TGA CCA CGC AGA GCA CGT AG -3’ and 5’- TGA CCG CTT CTT CGT GCT TTA CGG TAT -3. The sequences of primers used for the genotyping of the Apcgene were 5’- GCC ATC CCT TCA CGT TAG -3’, 5’- TTC CAC TTT GGC ATA AGG C -3’ and 5’- GTG CAA TCC ATC TTG TTC AAT -3’.

**Immunohistochemistry staining**

Murine and human samples were fixed in 4% formalin, processed, paraffin-embedded, and sectioned in 2 μm slides. The colonic and intestinal paraffin-embedded tissue slides were deparaffinized, dehydrated with ethanol, and stained with hematoxylin (Dako) and eosin (Diapath) for the single-blinded histological evaluation of the inflammation (Rachmilewitz score) and tumor count or immune-stained. The staining for S1PR2 on murine and human tissue samples was carried out by heating the sections in 10 mM citrate buffer (pH 6.0). The endogenous peroxidase activity was inactivated in hydrogen peroxide 3% for 20 minutes, and the endogenous mouse IgG and non-specific background were blocked by incubating 30 minutes in Rodent Block M (Biocare Medical) or Background Sniper (Biocare Medical) respectively murine and human sections. The primary antibody used for the S1PR2 staining was the anti-mouse/human rabbit S1PR2 antibody (Acris;1:200) for 1 hour at RT. For the primary antibody detection, the MACH 1 Universal HRP-Polymer (Biocare Medical) and the Betazoid DAB Chromogen Kit (Biocare Medical) have been used. Primary antibodies against Lgr5-GPR49 (Abcam;1:20) for 2 hours at RT in Da Vinci Diluent (Biocare Medical), anti-GFP, biotin-conjugated (Invitrogen;1:500) 1 hour at RT, Olfm4 (Cell Signaling; 1:400) 1 hour at RT, Ki67 (Abcam;1:800) 1 hour at RT, anti-BrdU (Serotec; 1:600) 40 minutes in Da Vinci Green Diluent (Biocare Medical), β Catenin (Abcam; 1:800)1 hour at RT.

**Immunoreactive Scoring system.**

The final score of immunoreaction was calculated as described by Klein et al 1(**Supplemetary** **Table 1**). Briefly, the percentage of labelled was assessed as 0 = no labeled cells; 1 = < 30%; 2= 30-60%; and 3= > 60%. The intensity was calculated from 0to 3, where 0 was no reaction; 1 weak, 2 mild, and 3 strong intensity.

**Supplementary Table 1. Immunoreaction scoring system**

|  |  |  |
| --- | --- | --- |
| A % of IHC + labeled cells | B intensity of IHC reaction | Final score (A + B) |
| 0 = 0 | 0 = no reaction | No reaction = 0 |
| 1 = <30 | 1 = weak | Low = 2 |
| 2 = 30-60 | 2 = mild | Medium = 3-4 |
| 3 = >60 | 3 = strong | High = 5-6 |

**Intestinal Epithelial Cell isolation**

*Human Intestinal Epithelial Cells*. Mucosal samples were first incubated in 2 Mm DTT at 37°C for 15 minutes to remove the residual mucus and then digested mechanically into smaller pieces and incubated in a chelating compound ethylenediaminetetraacetic acid (EDTA) 1Mm at 37°C for 30 minutes. After this time, the mucosal pieces resuspended in a solution supplemented with 2.5% fetal calf serum (FCS) (Sigma-Aldrich) Hepes (Sigma-Aldrich) 20mM and Penicillin/Streptomycin (Sigma-Aldrich) 100 units/ml were agitated for 1-2 min on the stirrer at maximum speed. Whole crypts were collected from following through with a 100-µm cell strainer, and their viability was determined with a 0,4% Trypan blue solution. Final centrifugation at 1300rpm for 5 minutes at 4° C allowed the disgregation of the crypts; single epithelial cells were subsequently counted, pellet, and finally stored at -80°C.

*Murine intestinal stem cells*. Lgr5-expressing stem cells were isolated from Lgr5-EGFP-IRES-creERT2 heterozygous mice aged from 6 to 8 weeks, that express EGFP protein under the control of Lgr5 promoter following the procedure described by Mahe et al2. The intestines were extracted from the abdominal cavity, washed to remove the feces and opened lengthwise. The tissue was cut in small pieces and incubated in Phosphate-Buffered Saline (PBS) 2mM EDTA at 37°C for 30 minutes. Then, the crypts were collected by shaking the tissue and filtering with a 70-µm cell strainer. They were further dissociated in single cells after 90 minutes of incubating in TryPLE Express supplemented with 10uM Y27632 (Invitrogen, Carlsbad, CA, USA) and filtering with a 40um cell strainer. To specifically isolate epithelial cells, the single-cell suspension was stained with the anti-mouse CD326 (EpCAM) eFluor 450 Monoclonal Antibody, clone G8.8 (eBioscience) and sorted by FACS. Dead cells were stained by using a LIVE/DEAD cell viability assay kit (Invitrogen, Carlsbad, CA, USA). Live EpCAM+Lgr5-GFP+ and EpCAM+Lgr5-GFP- cells were sorted into RNAlysis buffer provided by the RNAeasy micro kit (QIAGEN) supplemented with 1% β-mercaptoethanol to directly lysate the cells for the mRNA extraction.

**Matrigel invasion assay**

Scrumble and overexpressed S1PR2 RKO cells (3x104 cells per well) were allowed after serum starvation to migrate across the precoated inserts (Corning Matrigel Invasion Chamber, BioCoat) at 37°C for 24 hours. Then unmigrated cells from the top of the chamber were removed, and the migrated cells were viewed using an inverted microscope with x10 objective (Olympus IX51).

**Proliferation Assay**

The cell cycle was evaluated by the use of the BD Pharmigen TM 647 EdU Click Proliferation Kit (cat.n°: 565456). According to the protocol, 1x106 cells were co-stained with a DNA dye 7-AAD, and cell populations were segmented by flow cytometry into the G0/G1-phases (2N DNA content, EdU-negative), S-phase (2N-4N DNA content, EdU-positive), or G2/M-phases (4N DNA content, EdU-negative).

**Animal experiments**

The acute colitis model was induced in C57BL/6 S1PR2 -/- and S1PR2 +/+ mice by administration of dextran sodium sulfate (DSS) (MP Biomedicals) 3% *ad libitum* in their drinking water for nine days. The inflammation-driven colon carcinogenesis model was induced by a single intraperitoneal injection with the Azoxymethane (AOM Sigma) 10 mg per kg body weight, followed by four cycles of DSS 2.5% in drinking water *ad libitum* for four days spaced out by ten days. The mice were monitored during the entire experiment for body weight changes, bleeding, and consistency of stool three times for a week. The disease activity index (DAI) was evaluated according to the criteria as reported previously3. The number of tumors was recorded by endoscopy (Coloview system, STORZ) under general anesthesia with 100 mg/kg intraperitoneal ketamine and 10mg/kg xylazine, at the last DSS cycle and by histology on fixed colon and small intestine paraffin-embedded. Histological analysis of inflammatory status was performed by a single-blinded pathologist accordingly to Rachmilewitz score3. S1PR2-/- Apcmin/+ and S1PR2+/+Apcmin/+ littermates have been sacrificed at 14 or 21-weeks old, and the colon and intestine tissues were collected.

S1PR2+/+Apcmin/+mice received every two other day oral gavage intake of a specific S1PR2 inhibitor, JTE013 (10009458, Cayman Chemical) 10 mg/kg, or vehicle for five weeks after weaning. C57BL/6 S1PR2 +/+ and S1PR2 -/- mice were irradiated with X-ray at 9 Gy (RADGIL X-ray generator) and monitored for body weight changes daily for seven days. On the day of sacrifice, the intestines were recovered and fixed by 10% formaldehyde for histological examination. Mice received an intraperitoneal injection of BrdU (BD PharmigenTM; 5 mg/kg/body weight) 24 hours before sacrifice.

**In vivo xenograft model**

Athymic female CD-1 nude mice received via subcutaneous injection 3x106 of scramble or overexpressing GFP-RKO cells. After injection, the tumor formation was monitored daily over 23 days and measured by the use of a vernier caliper. After this time, mice were sacrificed, and the tumors enzymatically digested in RPMI medium supplemented with Collagenase/Dyspase (1mg/ml) (Roche#1109711300; Roche Diagnostics GmbH) and DNAase I (20μg/ml) (Roche#10104159001); Roche Diagnostics GmbH) at 37°C for 30 minutes.

**Organoid culture**

The culture of small intestinal organoids was performed as previously described2. The intestine was opened lengthwise, cut into 2 cm pieces, and washed in ice-cold PBS. The tissue was further cut in smaller pieces and incubated in PBS 2mM EDTA at 37°C for 30 minutes. Then the crypts were collected by shaking the tissue and filtering the solution through a 70-µm filter to remove the villus fraction. The crypts isolated were counted and plated within the Matrigel®, cultured in complete DMEM/F12 medium (B27 supplement, N2 supplement, PenStrep, HEPES, Glutamine, 500 ng/ml R-spondin1 (Peprotec), 100 µg/ml Noggin (Peprotec) and 50 ng/ml murine EGF (Peprotec)) and the budding neurospheres completely developed after seven days.

**Immunoblotting analysis**

Proteins were extracted from human and murine samples by mechanical homogenization in the Lysis Buffer (Tris HCl ph 7.4 50 Mm, EDTA ph 8 1 mM, NaCl 150 mM, 1% Triton, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate) through the bead mill Tissue Lyzer II (QIAGEN). Human epithelial cells have been homogenized by disrupting the pellet mechanically by an 18G-needle syringe in the Lysis Buffer. The protein was quantified by using DC protein assay kit (Biorad) and separated on 10% SDS-polyacrylamide gel. The following primary antibodies were used: anti-mouse/human S1PR2 rabbit (1:200 in TBS-T 1X 5% milk, Acris AP01198PU-N), anti Phospho-AKT1 (Ser473) Clone (D7F10) (1:1000 in TBTS-T 3% BSA, MAB-94125 Immunological Science). The levels of proteins were normalized on total AKT (1:1000 in TBS-T 1X 3% BSA; 9272 Cell Signaling Technology) and anti-Actin C-11 (1:1000 in TBS-T 1X 5% milk, Santa Cruz sc-1615) expression. Finally, the immunoreactivity was detected by an enhanced chemiluminescence reaction (ECL - Millipore) and developed by the ChemiDoc Imaging System.

**Overexpression constructs**

For the transfection of the RKO cell line, GFP-tagged lentiviral vectors (cat. no RG-2101663) harboring human sphingosine-1 phosphate receptor 2 and empty GFP-tagged as control vectors, both obtained from OriGene, were used. The production of the lentiviral particle was performed by transient transfection of 293T cells following standard protocol4. Briefly, 293T cells at 70% confluency were co-trasfected with 11.7 μg of the transfer plasmid, 3.5 μg of the packaging plasmid, and the same amount of envelope plasmid and 5 μg of rev-expressing plasmid by Lipofectamine. After 24 hours, the recombinant lentiviral vectors were collected, filtered, and used as a medium for the infection of the RKO cell line. The transfected cells were selected via flow cytometry sorting GFP positive cells, and infection efficiency were checked by RT-PCR. The transfected cells were used within 1-2 passages.

**RNA extraction and quantitative RT-PCR analysis**

Total RNA was extracted from human and murine samples using the RNeasy® Mini kit (Quiagen) according to the manufacturer’s protocol. The mRNA has been retrotranscribed in cDNA by using the High Capacity cDNA Reverse Transcription Kits(Applied Biosystem) and quantitative real-time PCR has been performed using the Fast SYBR® Green Master Mix (Applied Biosystems) and detected with 7900HT Sequence Detection System (Applied Biosystems). The primer sequences used are reported in the **supplementary** **Table 2**. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene has been used as housekeeping. The relative mRNA expression was determined by the 2-ΔCt method.

**Supplementary Table 2. List of primers**

|  |  |
| --- | --- |
| **Gene** | **Primer sequences** |
| *hS1PR1* | Forward: 5’- ATGGTGTCCACTAGCATCCC-3’  Reverse: 5’-CGATGTTCAACTTGCCTGTGTAG-3’ |
| *hS1PR2* | Forward: 5’- ATGGGCGGCTTATACTCAGAG -3’  Reverse: 5’- GCGCAGCACAAGATGATGAT -3’ |
| *hS1PR3* | Forward: 5’- CCATTGCCATTGAGCGGACAC -3’  Reverse: 5’- TTAGCCAGCACATCCCAATCA -3’ |
| *hMMP2* | Forward: 5’- GATACCCCTTTGACGGTAAGGA-3’  Reverse: 5’- CCTTCTCCCAAGGTCCATAGC-3’ |
| *hMMP1* | Forward 5’-AGCTAGCTCAGGATGACATTGATG-3′  Reverse: 5′-GCCGATGGGCTGGACAG-3 |
| *hSOX9* | Forward: 5’-GAGGAAGTCGGTGAAGAACG-3’  Reverse: 5’-ATCGAAGGTCTCGATGTTGG-3’ |
| *hAXIN2* | Forward: 5’-CAACACCAGGCGGAACGAA -3’  Reverse: 5’-GCCCAATAAGGAGTGTAAGGACT -3’ |
| *hGAPDH* | Forward: 5’- TGTGTCCGTCGTGGATCTGA -3’  Reverse: 5’- CCTGCTTCACCACCTTCTTGA -3’ |
| *hPTEN* | Forward: 5’-GAGCGTGCAGATAATGACAAG-3’  Reverse: 5’-GATTTGACGGCTCCTCTAACTG-3’ |
| *mOLFM4* | Forward: 5’-GGAGCGCTTAGAGTACACAG-3’  Reverse: 5’- GGAGCCTCTTCTCATACACAC-3’ |
| *mLGR5* | Forward: 5’- ATGAACAACATCAGTCAGCTAC-3’  Reverse: 5’- CTCCCTTGGGAATGTGTGTC-3’ |
| *mGAPDH* | Forward: 5’- CCATGTTCGTCATGGGTGTG -3’  Reverse: 5’- CAGGGGTGCTAAGCAGTTGG -3’ |

H=human; m=murine

**Measurement of S1P**

Approximately 20–60 mg of the tumor and normal mucosa sample was homogenized using a lysis buffer containing the internal standard solution (10.0 ng/mL, sphingosine17:1, and S1P 17:1) and analyzed by LC-MS/MS. The ratio of the peak area of the analyte to the internal standard was used to quantify the calibration curves. The results are reported as pmol/mg.

as a tumor suppressor. Its loss may represent a risk factor for the development of colorectal cancer. **References**

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3. Vetrano S, Rescigno M, Cera MR, et al. Unique role of junctional adhesion molecule-a in maintaining mucosal homeostasis in inflammatory bowel disease. Gastroenterology 2008;135:173-84.

4. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998;72:8463-71.