Supporting Information

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SI Materials and Methods

Drugs and Chemicals. Icilin was purchased from Cayman Chemicals. Unless specified, 2,4,6-trinitrobenzenesulfonic acid (TNBS) and all other chemicals were obtained from Sigma.

Human Biopsies. Human intestinal biopsy samples were collected from participants consented through the Intestinal Inflammation Tissue Bank under an ethics protocol approved by the Conjoint Health Research Ethics Board at the University of Calgary.

Mice. C57BL/6 mice (6–8 wk old) were obtained from Charles River Laboratories. Transient Receptor Potential Melastatin–8 (TRPM8)–deficient mice were originally obtained from Dr. A. Patapoutian (The Scripps Research Institute, La Jolla, CA; generated as described in ref. 1) and were subsequently bred in the University of Calgary animal resource center (Dr. P. Whelan and Dr. S. Mandadi). *TRPM8^{GFP}* transgenic animals were generated as described in ref. 2 and were bred in the University of Calgary animal resource center (Dr. P. Whelan and Dr. S. Mandadi). All mice were genotyped before use in experiments. Mice were housed at room temperature under a 12 h light/dark cycle and had free access to food and water. All procedures were approved by the University of Calgary Animal Care Committee and were performed in accordance with the international guidelines for the ethical use of animals in research and guidelines of the Canadian Council on Animal Care.

Induction of Colitis. Colonic inflammation in mice was induced by TNBS or Dextran Sodium Sulfate (DSS) administration as described previously (3). TNBS [2 mg in 40% (vol/vol) ethanol] was administered as a single intrarectal injection on day 0, and animals were killed and assessed for disease parameters on day 7. DSS was dissolved in drinking water (2.5% wt/vol) and the animals were free to drink the DSS solution for 7 d, at which time they were killed and assessed for disease parameters. Water consumption was monitored daily and was equal in all groups of animals. Animal body weights were measured daily in all experiments. In some experiments designed to induce a low level of colonic inflammation, mice were administered 1% (wt/vol) DSS or 1 mg TNBS in 40% ethanol (EtOH). Following sacrifice, colons were excised and the bowel thickness was measured using an electronic caliper (Mitutoyo, resolution 0.01 mm). Disease severity was assessed through recording macroscopic damage scores, and tissue was harvested for histology, MPO assays, cytokine assays, and PCR analysis.

Study Design. Four groups of animals were studied. The first group (Vehicle) received daily icilin vehicle (1% tween-80/PBS) injections i.p and received intrarectal saline injections on day 0 (TNBS experiments) or received normal drinking water (DSS experiment). The second group (Icilin control) received daily i.p injections of Icilin (4 mg/kg) and received intrarectal saline injections on day 0 (TNBS experiments) or received normal drinking water (DSS experiment). The third group (Colitis control) received daily i.p. injections of vehicle and intrarectal TNBS injections on day 0 (TNBS experiment) or DSS in the drinking water (DSS experiments) for 7 d as described above. The fourth group (Treatment) received daily i.p injections of Icilin (4 mg/kg) and intrarectal TNBS injection on day 0 (TNBS experiment) or DSS administration in the drinking water (DSS experiments) for 7 d. In experiments designed to assess the effect of menthol on colitis, the treatment group consisted of menthol (40 mg/kg in 5% EtOH/PBS) and the vehicle group was administered 5% EtOH/PBS.

Macroscopic damage scores were assigned by observing and scoring the following parameters. Presence of hemorrhage, edema, stricture, ulceration, fecal blood, or diarrhea was each assigned a score of 1. Erythema was scored on a 0–2 scale depending on the length of the area affected (0, absent; 1, less than 1 cm; 2, more than 1 cm). Adhesions were scored based on severity (0, absent; 1, moderate; 2, severe) (4).

Histological assessment of microscopic damage in colonic tissue was done as described previously with some modifications (5). Excised colons were fixed in 10% neutral buffered formalin and embedded in paraffin. Section of 5 μM were cut and stained with H&E. Microscopic damage scores were assessed by a pathologist blinded to the treatment. The following features were graded on a 3-point scale (0, normal; 1, mild; 2, moderate; 3, severe): (*i*) destruction of normal mucosal architecture, (*ii*) presence of immune cell infiltration, and (*iii*) muscle thickening. Goblet cell depletion was also scored (0, absent; 1, present). The scores were summed up to give a maximum possible microscopic damage score of 10. Images were acquired on an Olympus BX51 microscope and the Q capture pro imaging software (Qimaging).

mRNA Extraction and Real-Time PCR Detection of TRPM8. ***mRNA extraction and qPCR from human biopsies.*** Colonic biopsies were placed directly into RNA later and kept at 4 °C for a minimum of 16 h. Biopsies were kept at –80 °C for long-term storage. For RNA extraction, biopsies were transferred into TRIzol reagent (Life Technologies) and homogenized either by a hand-held rotor homogenizer or by stainless steel-bead–based lysis using a Bullet Blender. Phase separation was achieved by adding chloroform (20% of original TRIzol volume), mixing, and then centrifugation at 12,000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to a new tube to which isopropanol was added (50% of original TRIzol volume). Precipitated RNA was pelleted by centrifugation at 12 000 × *g* for 10 min at 4 °C and then washed in 75% ethanol. The remaining RNA pellet was resuspended in water and purified through an RNeasy spin column (Qiagen) according to the RNeasy Mini kit instructions (including on-column DNase digestion step). Quantity and purity of RNA was determined on a Nanodrop spectrophotometer. Between 800 and 1,000 ng RNA was used to synthesize cDNA according to the RT2 First Strand Kit (SaBiosciences/Qiagen) instructions.

mRNA extraction and qPCR from mouse colon. Mouse colon tissue was homogenized using a polytron homogenizer, and RNA was extracted using the RNeasy spin column extraction kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of 1 μg of RNA using the Quantitect reverse transcription kit (Qiagen).

TRPM8 in human and mouse cDNA was amplified with the human (Cat. No./ID QT00038906) and mouse (Cat. No./ID QT00137256) Qauntitect primer assay (Qiagen) on an ABI 7900 HT Fast real-time PCR cycler (Applied Biosystems) using SYBR green (Qiagen) as the reporter dye and GAPDH as the reference gene.

Immunocytochemical Detection of TRPM8, CGRP, and ZO-1 Expression in the Mouse Colon. Colonic expression of TRPM8 was detected using *TRPM8^{GFP}* transgenic mice essentially as described (2, 6). Mice were anesthesized with ketamine/xylazine, and tissue was fixed by transcardial perfusion with 10% buffered formalin. Colons were excised, rinsed with PBS, and cryoprotected in 10% sucrose solution overnight and snap frozen in Optimal Cutting Temperature Compound (OCT)(Tissue-Tek). OCT embedded tissue was sectioned with a cryostat at 7 μm and mounted on

Superfrost Plus slides. Slides were stored at –80 °C. Frozen slides were dried at 4 °C for 30 min, then at room temperature for 30 min, and washed with deionized water for 30 s. Slide-mounted sections were washed three times with PBS and once with PBS containing 0.1% Triton X-100 for 30 min. Following another three washes with PBS, slides were blocked (1 h in PBS/0.1% Tween 20/20% goat serum). Rabbit anti-GFP antibody (Torrey Pines biolabs) was diluted 1:5,000 in PBS/10% goat serum and incubated at 4 °C overnight. Sections were washed three times with PBS containing 0.1% Triton X-100 and incubated for 2 h at room temperature with secondary antibodies conjugated to Alexa-488 (Molecular Probes) diluted 1:500 in blocking solution. Sections were washed five times with PBS containing 0.1% Tween 20 and coverslipped with FluorSave mounting medium (Calbiochem-EMD/Millipore). Double-staining experiments were performed with a chicken anti-GFP antibody (Aves Labs) (diluted 1:5,000 in PBS/0.3% triton-X and 3% FBS) and calcitonin-gene–related peptide (CGRP)–targeted (Calbiochem-EMD/Millipore) or Zona Occludens protein-1 (ZO-1)–targeted (Invitrogen) rabbit polyclonal antibodies. Digital images were acquired using an Olympus FV1000 confocal system on an Olympus IX70 microscope with the Fluoview system software or on a Zeiss LSM 510 meta inverted microscope.

Measurements of Myeloperoxidase Activity. MPO activity was measured as an index of granulocyte infiltration into the gut. Briefly, tissue samples were homogenized in a solution of 0.5% hexadecyltrimethylammonium bromide dissolved in phosphate buffer solution (pH 6.0). The homogenized tissues were centrifuged at 13,000 × *g* at 4 °C for 5 min. Supernatants were added to a buffer supplemented with 1% hydrogen peroxide and O-dianisidine dihydrocholoride solution. Optical density readings at 450 nm were taken for 1 min at 30 s intervals on a microplate reader (Molecular Devices).

Measurement of Cytokine Levels. The inflammatory cytokine profile was studied in the TNBS colitis model. Tissue sections were obtained from four groups of animals treated as described above. Tissues were homogenized in HBSS buffer containing a protease inhibitor mixture (Roche), and protein levels in the homogenate were measured with the BCA protein assay kit (Pierce) and normalized. The inflammatory cytokine/chemokine profile in each sample was analyzed using a multiplex assay with the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (EMD Millipore) on a Luminex xMAP multiplexing technology (Eve Technologies Corporation). The analytes profiled included Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, keratinocyte-derived chemokine (KC), Leukemia inhibitory factor (LIF), LPS-induced CXC chemokine (LIX), M-CSF, monocyte chemoattractant protein-1 (MCP-1), monokine induced by IFN-γ (MIG), macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2, Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES), TNF-α and VEGF.

Intravital Microscopy. Intravital microscopy was done as described earlier (7,8). Mice received i.p. injections of either TNF-α (20 μg/kg), icilin (4 mg/kg), or vehicle solutions (all 100 μL volume), and intravital microscopy was initiated 3 h later. Control animals received saline along with icilin vehicle (10% DMSO in 1% tween-80/PBS). Another group of animals was administered icilin along with i.p saline (TNF-α vehicle). The study also included a TNF-α control group with the animals receiving TNF-α with icilin vehicle and the test group in which mice received TNF-α coadministered with icilin. Mice were anesthetized 3 h later with a mixture of xylazine (10 mg/kg; MTC Pharmaceuticals) and ketamine (200 mg/kg; Rogar/STB) by i.p. injection. Anesthetized mice were administered 100 μL rhodamine 6G (Sigma-Aldrich) in NaCl 0.9% (0.3 mg/kg, administered in 0.1 mL) via tail vein injections. The dosage of rhodamine 6G used in this study has been shown to have no effect on leukocyte kinetics while effectively labeling leukocytes and platelets. A midline abdominal incision was then made, and a segment of colon was exteriorized and placed on top of a viewing pedestal and superfused with bicarbonate-buffered saline (pH 7.4). After 10 min of equilibration, single venules (20–40 μm in diameter) were selected and visualized using an inverted fluorescent microscope (Olympus) with a 203 objective lens by epi-illumination at 510–560 nm, using a 590 nm emission filter. A 5 min recording of the selected field was made and the end of this recording was designated as 0 min. Additional 5 min recordings of the selected field were then made at 20, 35, 50, 65, and 80 min. The recorded images were assessed by video playback in which leukocyte rolling, adherence, and vessel diameter were measured. Leukocyte flux was defined as the number of leukocytes per minute moving at a velocity less than that of the erythrocytes that passed a reference point in the venule. A leukocyte was considered adherent to the vessel wall if it remained stationary for 30 s or longer. The change in vessel diameter and leukocyte flux was evaluated as the differences between the values at each interval and the basal value observed at 0 min. The parameters measured were plotted over the 80 min time of observation, and the area under the curve was calculated.

CGRP Enzyme Immunometric Assay. For basal CGRP-level assessment in WT and TRPM8-KO animals, colonic samples were collected from control and DSS-treated animals. Samples were homogenized in 30 μL of enzyme immunometric assay (EIA) buffer (1 M phosphate solution containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide) per milligram of tissue using a bullet blender and 0.9–2.0 mm stainless steel beads (both from Next Advance). Samples were centrifuged at 13,000 × *g* for 10 min and supernatants collected and assayed immediately.

For ex vivo stimulation experiments, distal colonic tissues (0.5 cm) from WT control animals were collected in HBSS on ice. Samples were equilibrated in extracellular bath solution (NaCl 135 mM, KCl 5 mM, CaCl2 3 mM, MgCl2 2 mM, D-Glucose 10 mM, Hepes 10 mM, pH 7.3) (30 μL per mg of tissue) for 10 min at 37 °C, then exposed to icilin (30 μM) or capsaicin (1 μM) either alone or sequentially for 5 min, after which supernatants were snap frozen and stored at –80 °C until use. The CGRP assay (SPI Bio–Cederlane) was performed according to the manufacturer's instructions, and assay plates were read at 405 nm using a microplate reader (Molecular Devices).

Calcium Signaling. Calcium signaling was monitored in transfected HEK cell monolayers using an Olympus IX51 microscope with the cellSens digital imaging software (Olympus). Briefly, cells were transiently transfected with the pcDNA3 vector encoding rat TRPM8 (kind gift from Dr. David Julius, University of California, San Francisco, CA), rat Transient Receptor Potential Vanilloid-1 (TRPV1) (kind gift from Dr. Ardem Patapoutian, The Scripps Research Insititute, La Jolla, CA), and mCherry pcDNA3.1 vector to identify transfected cells. Cells were loaded with the calcium sensitive dye Fluo3-AM (acetoxymethyl ester of Fluo3) (0.5 μM for 30 min, Invitrogen), then perfused (~2 mL/min) with capsaicin (1 μM) (Sigma), or icilin (30 μM) (Cayman Chemicals), and calcium signaling was monitored. Band-limited excitation (420–495 nm) was provided by a mercury arc lamp and filter. Cells were imaged using a 20× 0.5NA objective. Images were acquired using a CCD camera (Olympus) at an effective sampling rate of 1 Hz, and digitized. Regions of interest (ROIs) were fitted around the perimeter of cells using ImageJ software, and intensity variations for each ROI were corrected for background levels and expressed in relation to a baseline fluorescence level preceding capsaicin or icilin responses to obtain ΔF/F fluorescent intensity values.

Statistical Tests. Statistical tests were performed with Graphpad Prism software. Comparisons among groups were made with

ANOVA followed by a Tukey's posttest. The data are expressed as mean ± SEM. $P < 0.05$ was considered to be significant. Nonparametric data were analyzed using the Mann–Whitney test.

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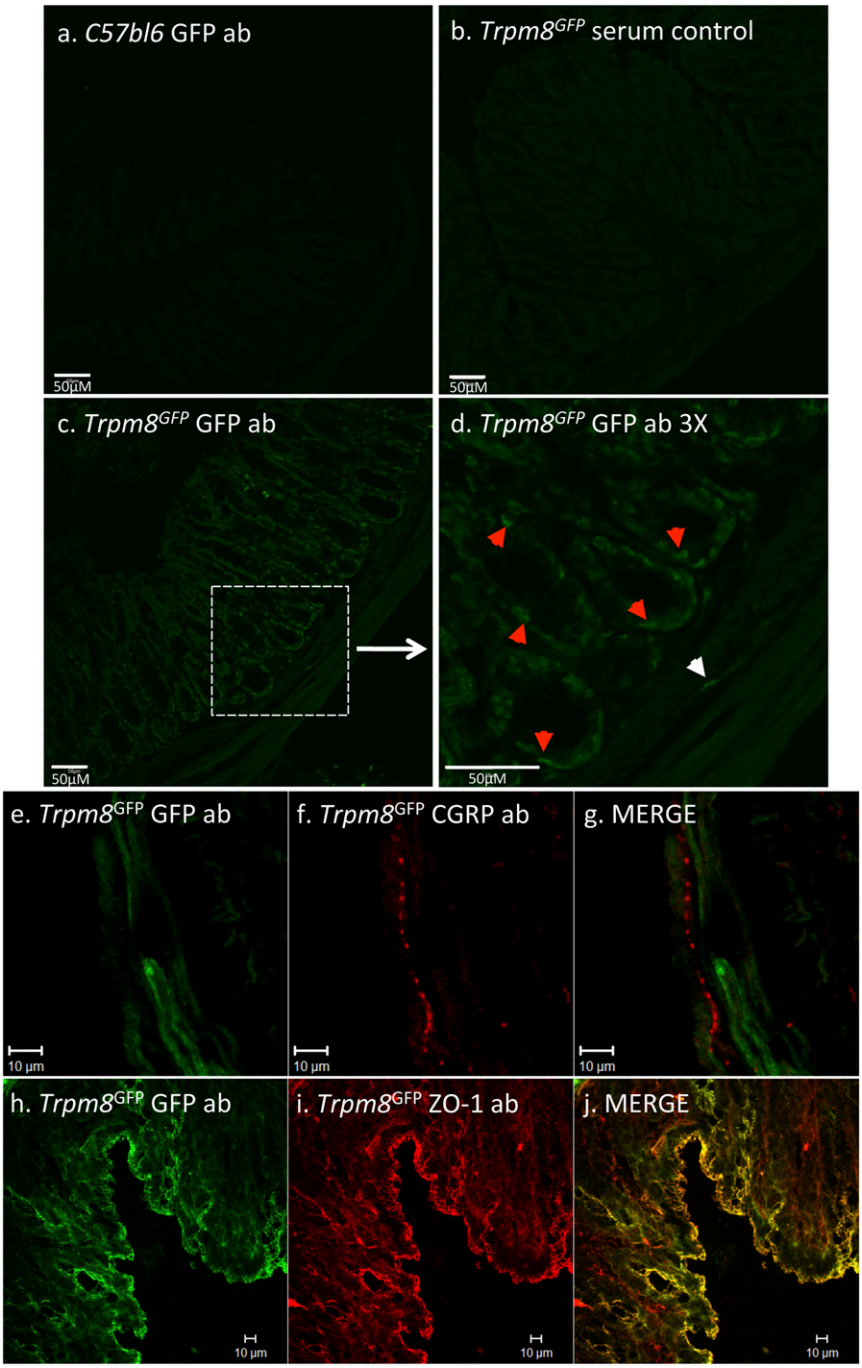

Fig. S1. *TRPM8* localization in the colon of *TRPM8*^{GFP} mice. (*A*) GFP staining in c57bl6 mice. No detectable signal could be observed. (*B*) Staining with rabbit serum instead of a GFP-specific antiserum. No detectable signal could be seen. (*C*) GFP-targeted polyclonal antibody staining in the colon of *TRPM8*^{GFP} mice. Expression of GFP is seen in the mucosal epithelial cells (red arrows) and in neuronal-like structures in the myenteric plexus (white arrow). (*D*) Enlarged (3× zoom) view of a section of the *TRPM8*^{GFP} mouse colon depicting GFP positive cells. (*E*) GFP staining in the muscularis of *TRPM8*^{GFP} mice. (*F*) CGRP staining in the muscularis of *TRPM8*^{GFP} mice. (*G*) Merged image showing TRPM8- and CGRP-expressing cells in the muscularis of *TRPM8*^{GFP} mice. (*H*) GFP staining in the mucosa of *TRPM8*^{GFP} mice. (*I*) ZO-1 staining in the mucosa of *TRPM8*^{GFP} mice. (*G*) Merged image showing TRPM8 and ZO-1 colocalization (yellow) in mucosal cells of *TRPM8*^{GFP} mice.

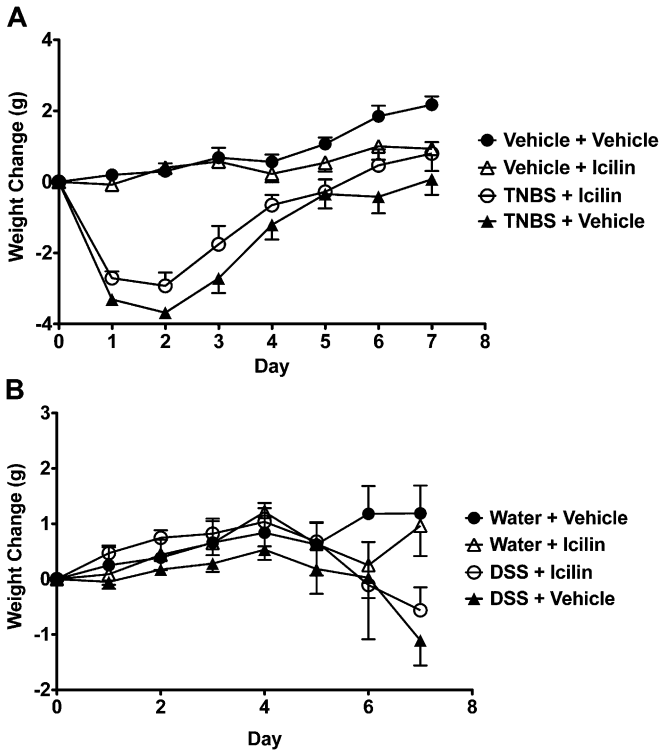

Fig. S2. Changes in body weight of mice following development of TNBS- or DSS-induced colitis. Mice were weighed before treatment with vehicle, icilin, TNBS or DSS, and TNBS or DSS plus icilin and subsequently every 24 h for 7 d. Change in weight for each mouse was calculated, and the change for each group is shown. $n = 8$ animals per group.

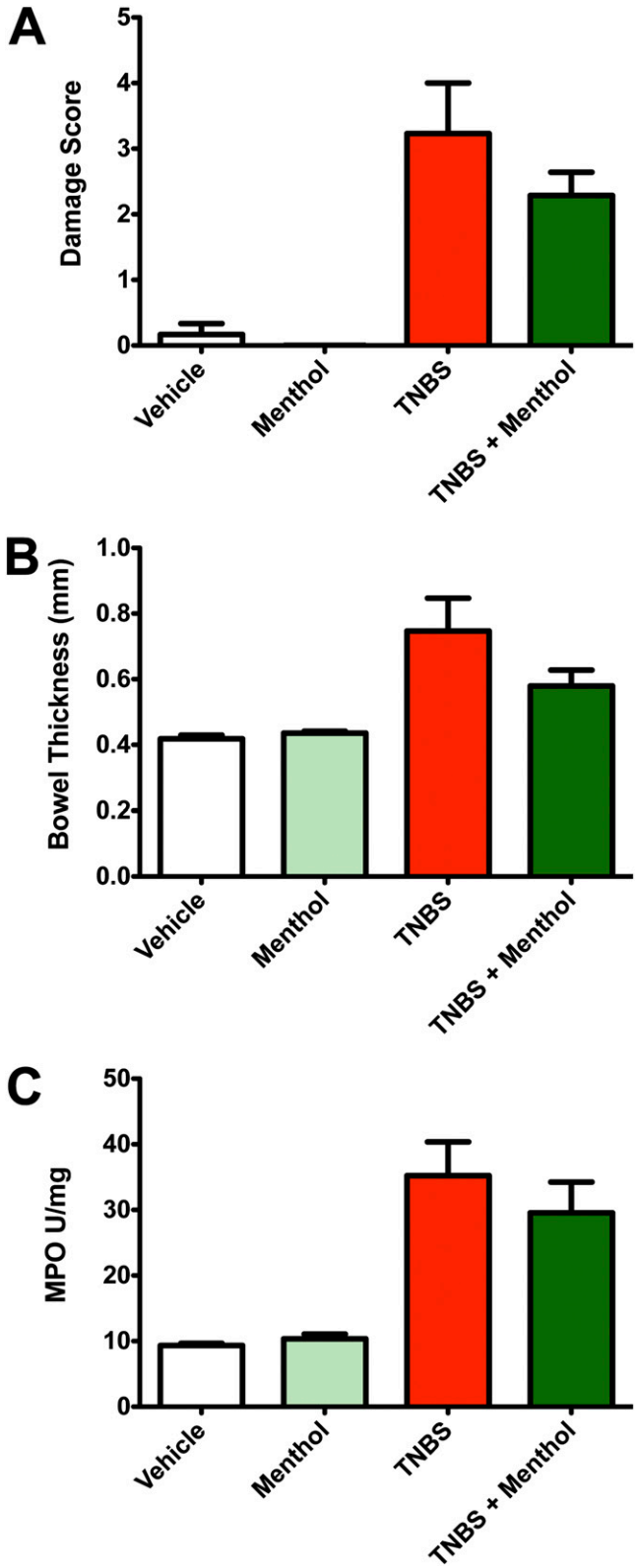

Fig. S3. Effect of menthol on modifying disease parameters in TNBS-induced colitis. (*A*) Assessment of intestinal damage scores, (*B*) bowel thickness, and (*C*) colonic MPO levels in mice treated with vehicle (5% EtOH/PBS), menthol (40 mg/kg in 5% EtOH/PBS), TNBS, or TNBS plus menthol. $n = 8$ animals per group.

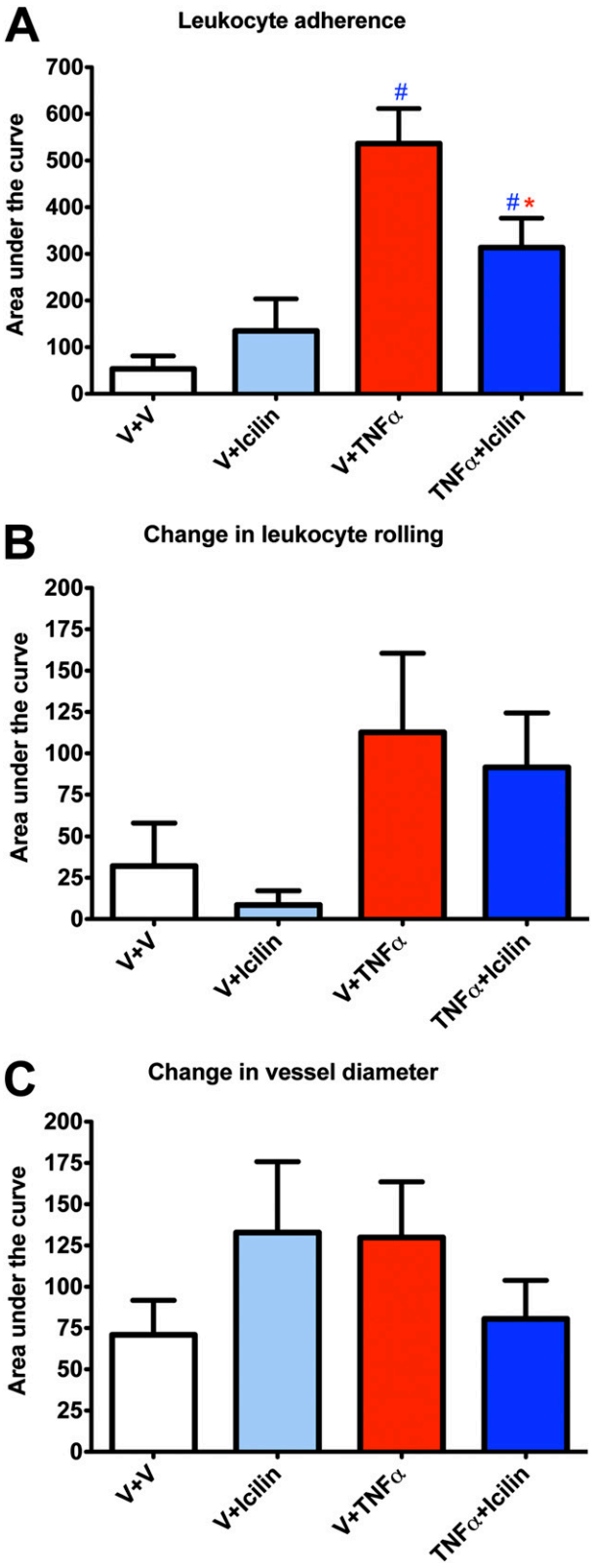

Fig. S4. Icilin reduces TNF-α–stimulated leukocyte adherence in mouse colonic venules. Changes in the flux of adherent (*A*) or rolling (*B*) leukocytes and the vessel diameter in mice treated with TNF-α for 3 h in the presence or absence of icilin treatment. Mice treated with icilin showed a significant reduction in TNF-α–stimulated leukocyte adherence. * indicates significant reduction from TNF-α–treated group, $P < 0.05$. # indicates significant increase compared with the vehicle-treated group, $P < 0.05$. $n = 6$–8 animals per group. AUC, area under the curve.

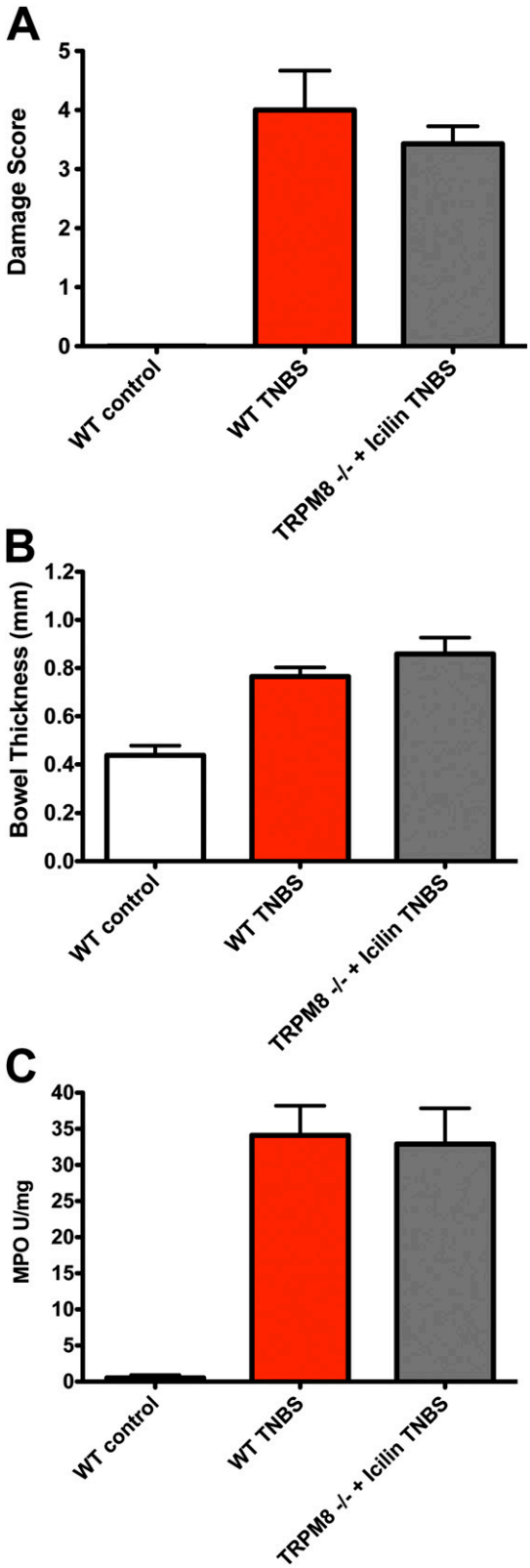

Fig. S5. Icilin does not attenuate TNBS-induced colonic inflammation in *TRPM8*$^{-/-}$ mice. Assessment of (*A*) intestinal damage scores, (*B*) bowel thickness, and (*C*) colonic MPO levels in WT and *TRPM8*$^{-/-}$ mice treated with TNBS or TNBS with icilin. Icilin treatment in *TRPM8*$^{-/-}$ mice failed to attenuate any of the assessed parameters. $n = 8$ animals per group.

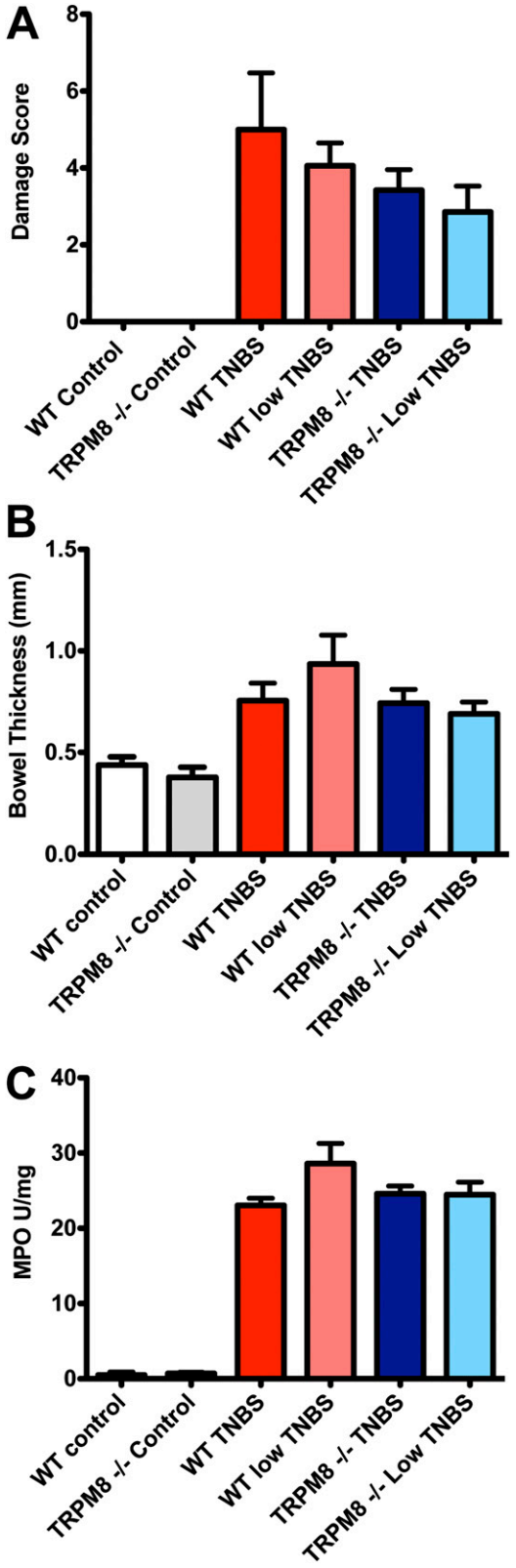

Fig. S6. *TRPM8*$^{-/-}$ mice do not show enhanced TNBS-induced colonic inflammation. Assessment of intestinal damage scores, colonic MPO levels, and bowel thickness in WT and *TRPM8*$^{-/-}$ mice treated with vehicle, TNBS (2 mg/40% EtOH), or low TNBS (1 mg/40% EtOH). *TRPM8*$^{-/-}$ mice do not show significantly different (*A*) damage scores, (*B*) bowel thickness, or (*C*) MPO levels compared with WT mice. $n = 8$ animals per group.