Supplemental Data

Intestinal proinflammatory macrophages induce a phenotypic switch in interstitial cells of Cajal

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Supplemental Methods

Colon histopathology and immunohistochemistry (IHC) staining

Since the dilated colon of 3-weeks old *Ednrb*$^{-/-}$ mice extended to nearly the proximal colon, we divided mouse colon into distal colon, middle colon and proximal colon, which was analogous to the human distal narrowed colon, transition zone and dilated colon. Human and mouse colon tissues were fixed in 4% paraformaldehyde (PFA) solution for 24 h, embedded in paraffin, sectioned, and stained with H&E. Immunohistochemistry was performed by incubating tissue sections with rabbit anti-C-KIT antibody (1:200 dilution, catalog. ab114992, Abcam; Cambridge, MA, USA) at 4℃ overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody (catalog WGZ-074-1506, Servicebio, Wuhan, China). Positive reactions were confirmed by staining with diaminobenzidine (catalog G1212-200, Servicebio). Cell nuclei were stained with hematoxylin (catalog G1004-100, Servicebio). The stained sections were examined with a Nikon E800 Microscope (Nikon, Shinagawa-Ku, Tokyo, Japan).

Immunofluorescence (IF) staining

Paraffin embedded colon sections were rehydrated through a graduated ethanol solution, 5% normal goat serum was used for non-specific blocking. Primary antibodies (Supplemental Table 1) were incubated at 4℃ overnight, and then slides were washed and incubated with goat anti-rabbit IgG Cy3-conjugated secondary

antibody (catalog WGZ5-715-165-150, Servicebio) or goat anti-rat IgG fluorescein-isothiocyanate-conjugated secondary antibody (catalog GB22403, Servicebio) for 1h at room temperature. 4,6-diamidino-2-phenylindole (DAPI, catalog MBD0020, Sigma-Aldrich, St. Louis, MO, USA) was used for nuclear staining. Cells were subsequently visualized under a Nikon E800 Microscope. Photoshop software (Adobe Photoshop CS5, Adobe Systems Inc, CA, USA) was used for image merging.

Whole mount colon specimens were prepared by longitudinally opening and stripping the mucosa, the remaining muscularis specimens were fixed by 4% PFA, primary antibodies (Supplemental Table 1) were incubated at 4℃ overnight, specimens were washed and secondarily stained with goat anti-rabbit IgG Cy3-conjugated secondary antibody (catalog WGZ5-715-165-150, Servicebio) or goat anti-rat IgG fluorescein-isothiocyanate-conjugated secondary antibody (catalog GB22403, Servicebio) for 1h at room temperature. The specimens were examined using confocal microscopy (Zeiss LSM 710) creating full thickness, z stack image sets.

Flow Cytometry

The 1, 2, and 3- weeks old *Ednrb*$^{-/-}$ and *Ednrb*$^{+/+}$ mice, along with the 3-weeks old mice treated with Clod, were sacrificed under anesthesia. Intestinal leukocytes were isolated as described previously (1). Briefly, colon was removed and cut into small pieces. To remove the epithelial layer, tissues were incubated in HBSS (Catalog

14025092, Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA) contained with 2 mM EDTA (Catalog 15040066, Gibco) at 37℃ for 15 min, then digested in complete RPMI 1640 medium (Catalog 11875093, Gibco) supplemented with 10% FBS (Catalog 16000044, Gibco), 2mM L-glutamine (Catalog 25030081, Gibco), 100 U/ml penicillin/streptomycin (Catalog 15140122, Gibco) and 50μM 2-mercaptoethanol (Catalog 21985023, Gibco) containing 0.5 mg/mL collagenase D (Catalog 11088858001, Roche, Mannheim, Germany), 3 mg/ml dispase (Catalog 17105-401, Gibco), 30 μg/ml DNase (Catalog 04716728001，Roche) for 40 min shaking at 37℃. Single cell suspensions were washed with FACS buffer (PBS with 1% FBS) and incubated with combinations of antibodies (Supplemental Table 2). Cells were analyzed on a FACS Calibur (BD Immunocytometry Systems, BD Biosciences, San Jose, CA, USA). Analysis was performed using FlowJo software (version 10, FlowJo, LLC, Ashland, OR, USA).

Quantitative real-time PCR (qRT-PCR)

Quantitative RT-PCR was performed to determine the expression levels of miRNA and mRNA. Total RNA was obtained from tissues or cells using TRIzol reagent as described by the manufacturer (Catalog 12183555, Invitrogen). For mRNA detection, real-time PCR was performed using an SYBR Premix Ex Taq kit (Code no. PCR-311, TOYOBO LIFE SCIENCE, Osaka, Japan) on a Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), using β-actin as the

endogenous control. For microRNA analysis, TaqMan® MicroRNA Assays (Catalog 4427975, Applied Biosystems, Carlsbad, CA, USA) were used as the probe for miR221 and U6 which act as a normalized control. PCR primers used in the study are listed in Supplemental Table 3 as human samples, Supplemental Table 4 as murine samples, and Supplemental Table 5 as miRNA detection.

Western blot

Total protein was extracted by using a commercially available kit (Catalog KGP250, Nanjing Keygen Biotech Co. Ltd., Nanjing, China). Protein concentrations were determined by the BCA method. Protein samples were separated by SDS-polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After being blocked, PVDF membranes were incubated with antibodies, the antibodies used are listed in the Supplemental Table 6. Protein bands were quantified by densitometry with Quantity One Software (BioRad, Hercules, CA, USA).

Measurement of colonic electrical activity

Mice were anaesthetized with an intraperitoneal injection of 80 mg/kg of 1% sodium pentobarbital (Catalog 1507002, Sigma-Aldrich). After opening the abdominal cavity, two bipolar electrodes were placed on the serosa circumferentially around the lumen at an interval of 2 cm. Mouse colon myoelectrical activity was

recorded and analyzed using a multiple-channel recorder (model BL-420E+xz; Chengdu Techman Software Co. LTD, Chengdu, China). The amplifier was set at a cutoff frequency of 52 Hz. Tracings were displayed on an on-line monitor and saved on a hard disk with a sampling frequency of 100 Hz.

Macrophage depletion by intraperitoneal injection of Clod in vivo

Two-week old mice were treated with 100 μL of Clod (5 mg/mL) (Catalog F70101C-N, FormuMax Scientific Inc., Sunnyvale, USA) via intraperitoneal injection at 1 day and 4 days prior to measuring endpoints. Mice were sacrificed at the end of the 3rd week after birth to harvest colons.

TNF-α neutralization in vivo

To neutralize TNF-α *in vivo,* 2-week old mice were treated with 300 μg of anti-TNF-α monoclonal antibody (clone XT3.11, catalog BE0058, Bioxcell, West Lebanon, NH, USA) via intraperitoneal injection at 1 and 4 days prior to measuring endpoints. Mice were sacrificed at the end of the 3rd week after birth, and colons were harvested for IHC staining and qRT-PCR.

ICCs isolation and culture

Primary ICCs were isolated from the colon of 3-week old *Ednrb*$^{+/+}$ mice as we have previously described (2). ICCs were cultured at 37°C in a 5% CO_2 incubator.

After 24 h of incubation, and cells in the experimental group were incubated with 5 ng/mL TNF-α (Catalog 654245, Sigma-Aldrich). One day later, cells were collected and used for IF staining, protein and mRNA assay, miRNA detection, and recording of pacemaker currents.

For treatment groups, primary ICCs were cultured for 24 h, then 20 μM PDTC (Catalog S1808, Beyotime, Shanghai, China) or 100 nM micrOFF mmu-miR-221-3p inhibitor (Catalog miR30000669-4-5, Ribobio, Guangzhou, China) was added to the cell culture medium, 1 h later, cells in the experimental group were incubated with 5 ng/mL TNF-α. One day later, cells were harvested for protein, mRNA and miRNA assays. PCR primers used in the study are listed in Supplemental Table 3 as human samples, Supplemental Table 4 as murine samples, and Supplemental Table 5 as miRNA detection.

Patch clamp studies of ICCs

A conventional dialyzed whole cell patch-clamp configuration was used to record membrane currents (voltage clamp) and potentials (current clamp, I = 0) from cells. Membrane currents or transmembrane potentials were amplified with an Axopatch 200B patch-clamp amplifier (Molecular Devices, San Jose, CA, USA) and digitized with a 16-bit analog-to-digital converter (Digidata 1322A, Molecular Devices). The currents and potentials were stored directly on-line using pCLAMP software (version 9.2, Molecular Devices). Data were sampled at 4 kHz and filtered at

2 kHz for whole cell experiments. Mini-Digi with Axoscope (version 9.2, Molecular Devices) was used to monitor changes in holding currents (basal currents) throughout each experiment. All data were analyzed using Clampfit (version 9.2, Molecular Devices) and GraphPad Prism (version 6.0, GraphPad Software, San Diego, CA, USA) software. The pipette tip resistance ranged between 3 and 6 MΩ for whole cell recordings, and experiments on ICCs were conducted at 30°C with the use of a Thermoclamp-1 temperature control system (Automate Scientific; Berkeley, CA, USA).

Immunofluorescence staining of ICCs

Cells were collected and fixed in 4% PFA. After blocking, the cells were incubated with anti-C-KIT (1:200 dilution, catalog. ab171227, Abcam) or anti-CD34 (1:200 dilution, Catalog EP373Y, Abcam) antibodies overnight at 4°C; after which, they were incubated with goat anti-rat IgG Cy3-conjugated secondary antibody (Catalog GB21302, Servicebio) or goat anti-rabbit IgG fluorescein-isothiocyanate-conjugated secondary antibody (Catalog GB22403, Servicebio) for 1 h. DAPI (catalog MBD0020, Sigma-Aldrich, St. Louis, MO, USA) was used for nuclear staining. Cells were subsequently visualized under a laser scanning confocal microscope (Olympus, FV3000, Tokyo, Japan). Photoshop software (Adobe Photoshop CS5, Adobe Systems Inc, CA, USA) was used for image merging.

References

1. Zigmond E, Varol C, Farache J, Elmaliah E, Satpathy AT, Friedlander G, et al. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. Immunity. 2012;37(6):1076-90.

2. Wei J, et al. Effects of lipopolysaccharide-induced inflammation on the interstitial cells of Cajal. Cell Tissue Res. 2014;356(1):29-37.

Supplemental Table 1. Primary antibodies used for IF

Antibody (# Catalog)	Dilution	Company
CD68 (#ab31630)	1:200	Abcam
TNF-α (# ab6671)	1:200	Abcam
iNOS (# 18985-1-AP)	1:200	Proteintech
C-KIT (# ab65525)	1:200	Abcam
TMEM16A (#ab53212)	1:200	Abcam

IF: Immunofluorescence staining.

Supplemental Table 2. Antibodies used for flow cytometry analysis

Antibody (#Catalog)	Dilution	Company
V500 Rat Anti-Mouse CD45 (#561487)	1:200	BD Biosciences
PE Rat Anti-Mouse F4/80 (#565410)	1:200	BD Biosciences
BV605 Rat Anti-CD11b (#563015)	1:200	BD Biosciences
PE-Cy™7 Hamster Anti-Mouse CD11c (#561002)	1:200	BD Biosciences
PerCP-Cy™5 .5 Rat Anti-Mouse TNF (#MP6-XT22)	1:200	BD Biosciences
iNOS (D6B6S) Rabbit mAb (Alexa Fluor® 647 Conjugate) (#48866)	1:200	CST
Fixable Viability Stain 780 (#565388)	1:200	BD Biosciences

Supplemental Table 3. Primer sequences used for real-time PCR of human specimens (5′-3′)

Primers for real time PCR		
β-Actin	forward	CTGAGAGGGAAATCGTGCGT
	reverse	CCACAGGATTCCATACCCAAGA
iNOS	forward	AATTGAATGAGGAGCAGGTCG
	reverse	CTGTCCTTCTTCGCCTCGTAA
TNFA	forward	TCTACTCCCAGGTCCTCTTCAAG
	reverse	GGAAGACCCCTCCCAGATAGA
C-KIT	forward	GGCACGGTTGAATGTAAGGC
	reverse	ACGAAACCAATCAGCAAAGGAG
CD34	forward	TTGCCCAGTCTGAGGTGAGG
	reverse	CAGGAAATAGCCAGTGATGCC

Supplemental Table 4. Primer sequences used for real-time PCR of murine specimens (5′-3′)

Primers for real-time PCR		
β-Actin	forward	GTGACGTTGACATCCGTAAAGA
	reverse	GTAACAGTCCGCCTAGAAGCAC
iNos	forward	CGGAGCCTTTAGACCTCAACAGA
	reverse	TAGGACAATCCACAACTCGCTCC
Tnfa	forward	ACCCTCACACTCACAAACCA
	reverse	ATAGCAAATCGGCTGACGGT
c-Kit	forward	GACCCGACGCAACTTCCTTA
	reverse	GAGCATCTTCACGGCAACTGT
Cd34	forward	TTTCACAACCACAGACTTCCCC
	reverse	GCCAACCTCACTTCTCGGATTC
Scf	forward	GAATCTCCGAAGAGGCCAGAA
	reverse	GCTGCAACAGGGGGTAACAT

Supplemental Table 5. Primer sequences used for miR221 of isolated murine interstitial cells of Cajal (5′-3′)

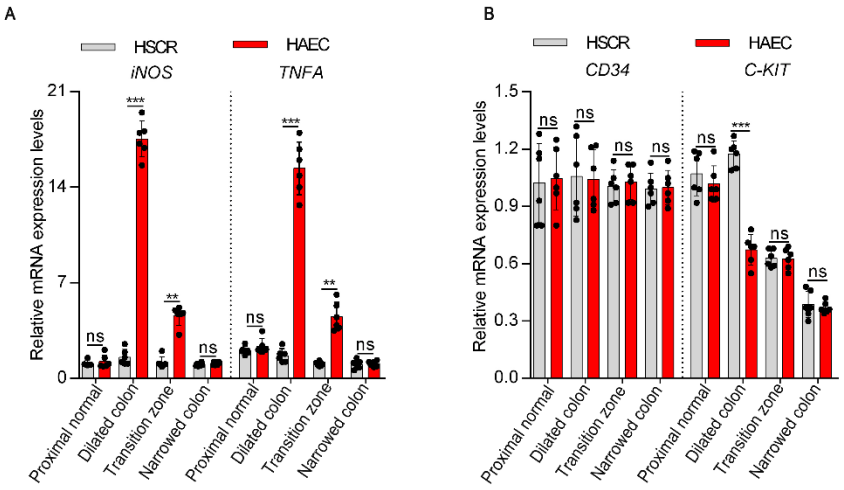
Primer for miRNA		
mmu-miR-221	forward	GCGAGCTACATTGTCTGCTGG
	reverse	CAGTGCAGGGTCCGAGGTAT
U6	forward	CTCGCTTCGGCAGCACATA
	reverse	CGAATTTGCGTGTCATCCT

Supplemental Table 6. Primary antibodies used for WB

Antibody (# Catalog)	Dilution	Company
iNOS (#18985-1-AP)	1:1000	Proteintech
TNF-α (# sc-12744)	1:1000	Santa
C-KIT (# ab171227)	1:1000	Abcam
CD34 (# EP373Y)	1:1000	Abcam
SCF (#bs-0545R)	1:1000	Bioss
p-p65(Ser536) (#3033)	1:1000	CST
P65 (#8242)	1:1000	CST
β-actin (#GB11001)	1:2000	Servicebio

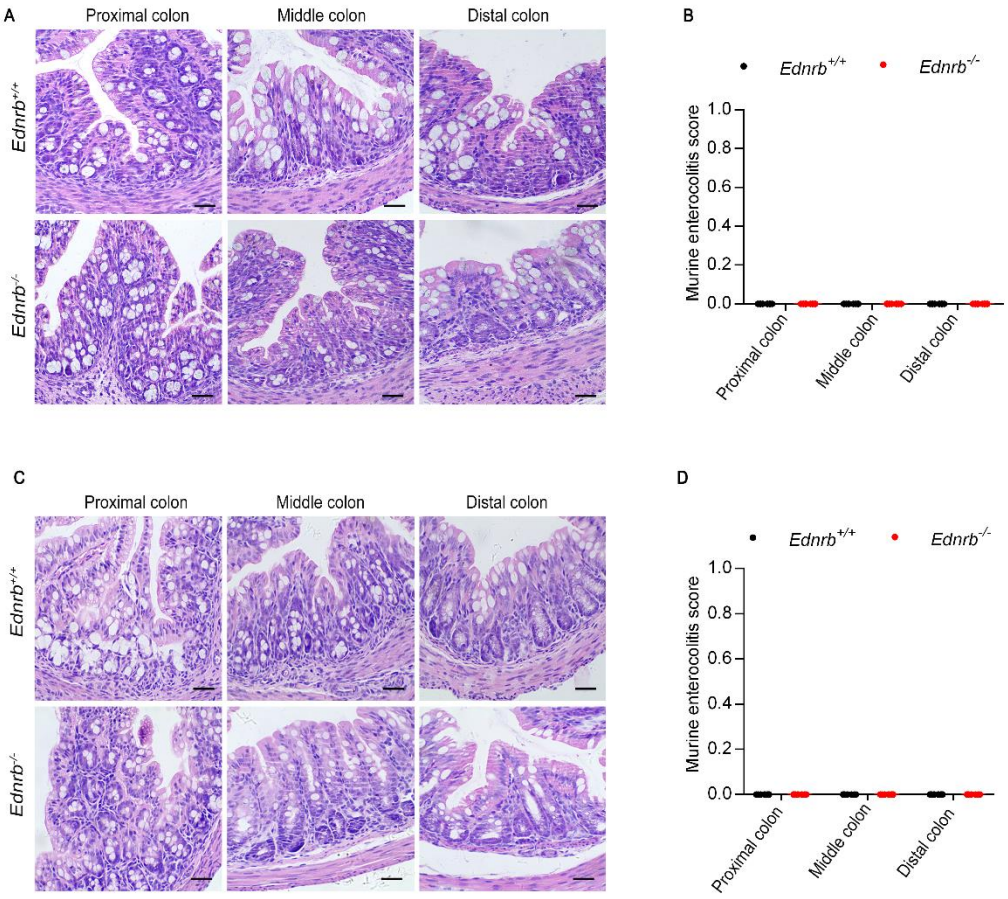
WB: western blot.

Supplemental figures and figure legends

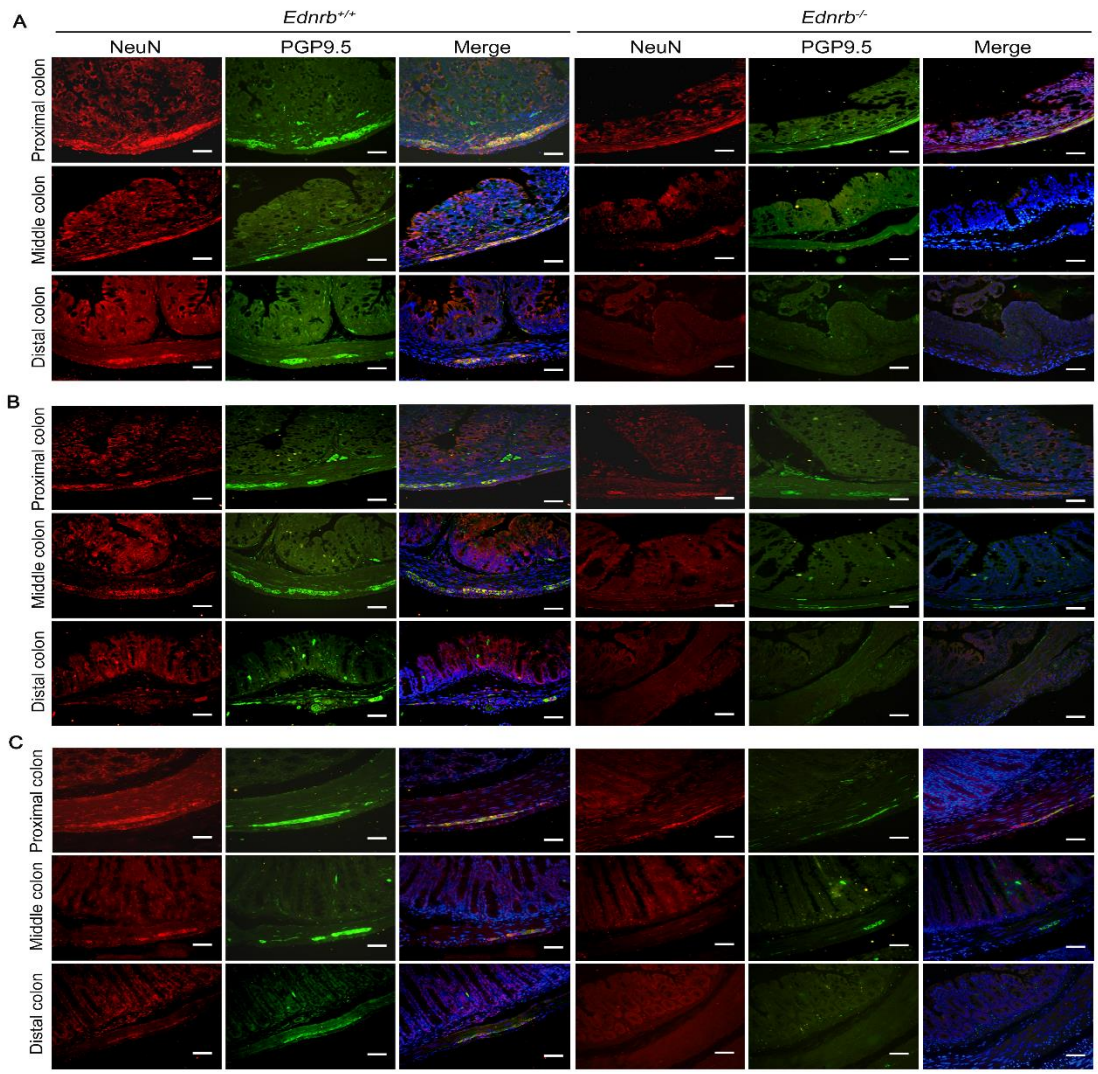


Supplemental Figure 1: mRNA expression levels in HSCR and HAEC human samples.

mRNA expression levels of (**A**) *iNOS* and *TNFA*, and (**B**) *CD34* and *C-KIT* in proximal normal colon, dilated colon, transition zone and narrowed colon from HSCR and HAEC patients. Data are representative from 6 independent experiments. One-way ANOVA: ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

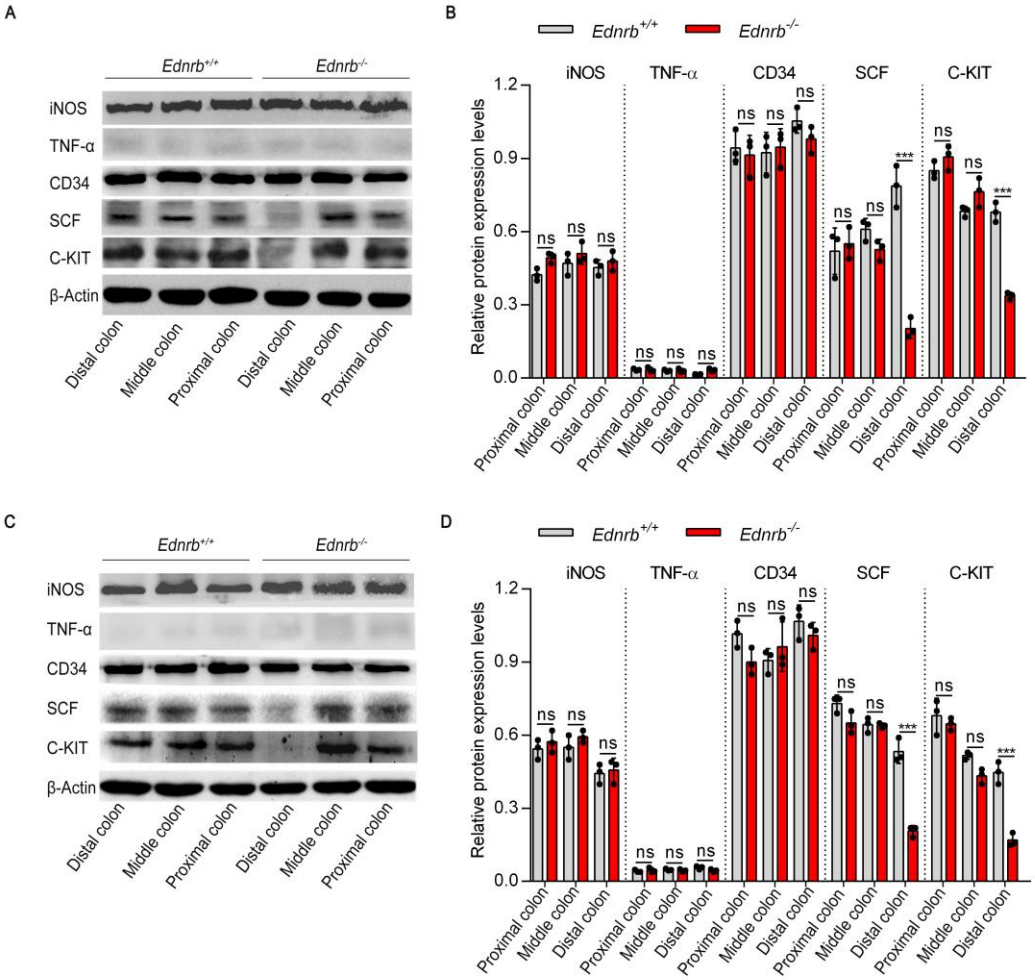


Supplemental Figure 2: Enterocolitis scores in 1- and 2- week old *Ednrb*$^{-/-}$ mice. (**A**) H&E staining of proximal, middle, and distal colon sections from 1-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Scale bar: 100µm. (**B**) An enterocolitis grading system was used to evaluate inflammation scores in 1- week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**C**) H&E staining of proximal, middle, and distal colon sections from 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Scale bar: 100µm. (**D**) An enterocolitis grading system was used to evaluate inflammation scores in 2-weeksold *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Data shown represent results from 6 mice per group. One-way ANOVA analysis.



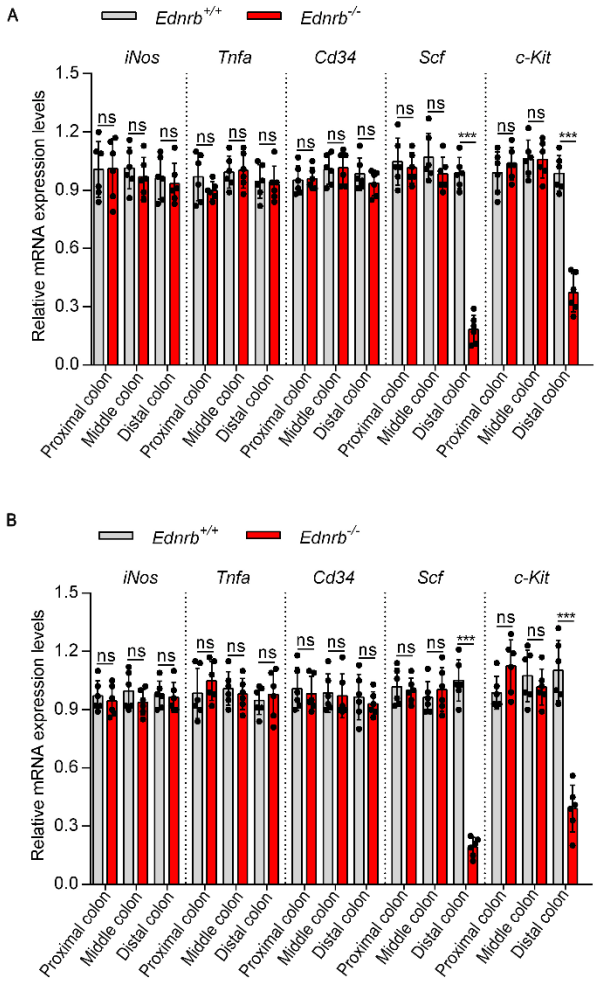
Supplemental Figure 3: NeuN and PGP9.5 immunofluorescence double staining of enteric neurons in *Ednrb*$^{-/-}$ mice.

The enteric neurons are revealed by immunofluorescence double staining of NeuN and PGP9.5 in murine colon. (**A**) NeuN and PGP9.5 staining in 1-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**B**) NeuN and PGP9.5 staining in 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**C**) NeuN and PGP9.5 staining in 3-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Red, NeuN; Green, PGP9.5; Blue, DAPI. Scale bar: 100µm. Experiments were repeat 3 times.



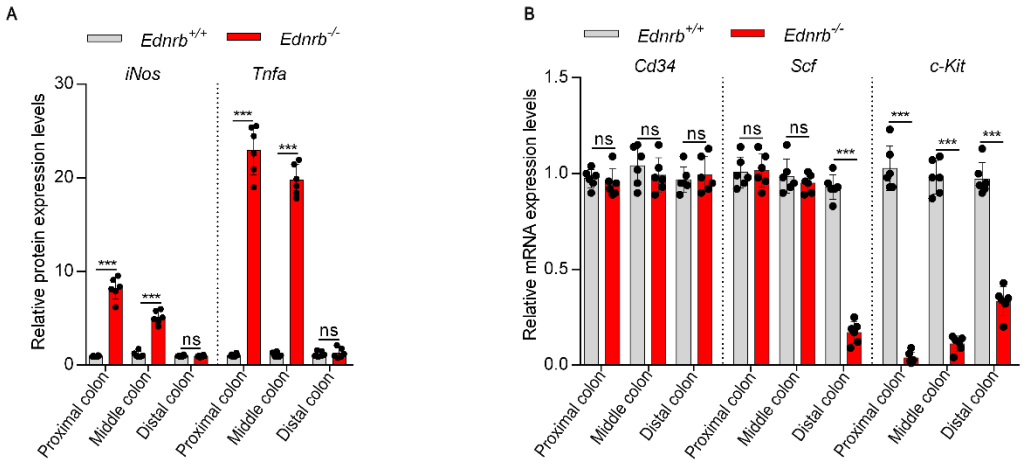
Supplemental Figure 4: Pro-inflammatory cytokines and ICCs phenotype in the 1- and 2-week old *Ednrb*$^{-/-}$ mice.

(**A**) Western blot analysis of colon tissues in 1-week old mice; (**B**) Semi-quantitative analysis of protein expression levels in 1-week old mice, with each protein being normalized to β-actin; (**C**) Western blot analysis of colon tissues in 2-weeks old mice; (**D**) Semi-quantitative analysis of protein expression levels in 2-week old mice, with each protein being normalized to β-actin. Data are representative from 6 independent experiments. One-way ANOVA: ns, non-significant; ***, $P < 0.001$.

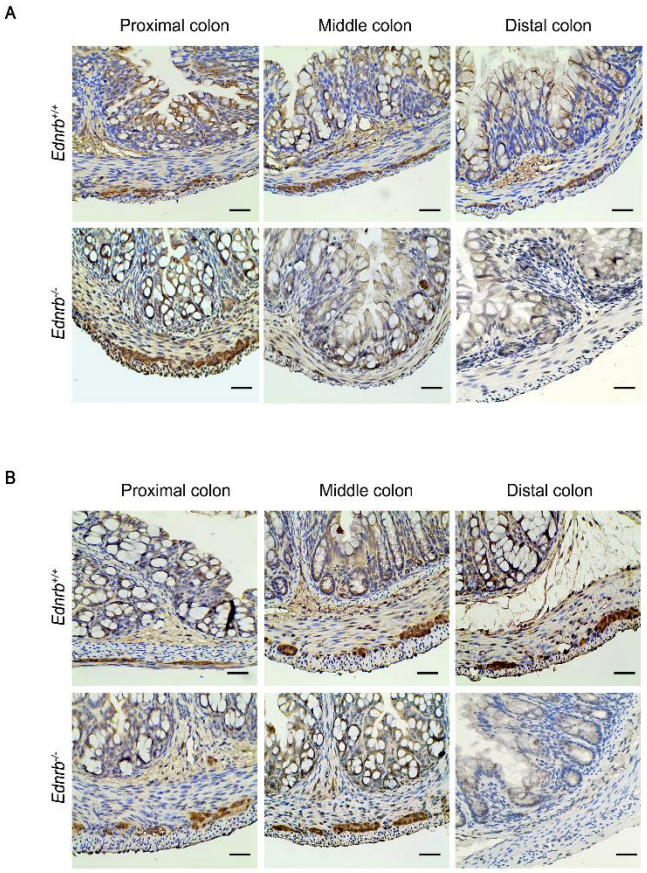


Supplemental Figure 5: mRNA expression levels in 1- and 2-week old *Ednrb*$^{-/-}$ mice.

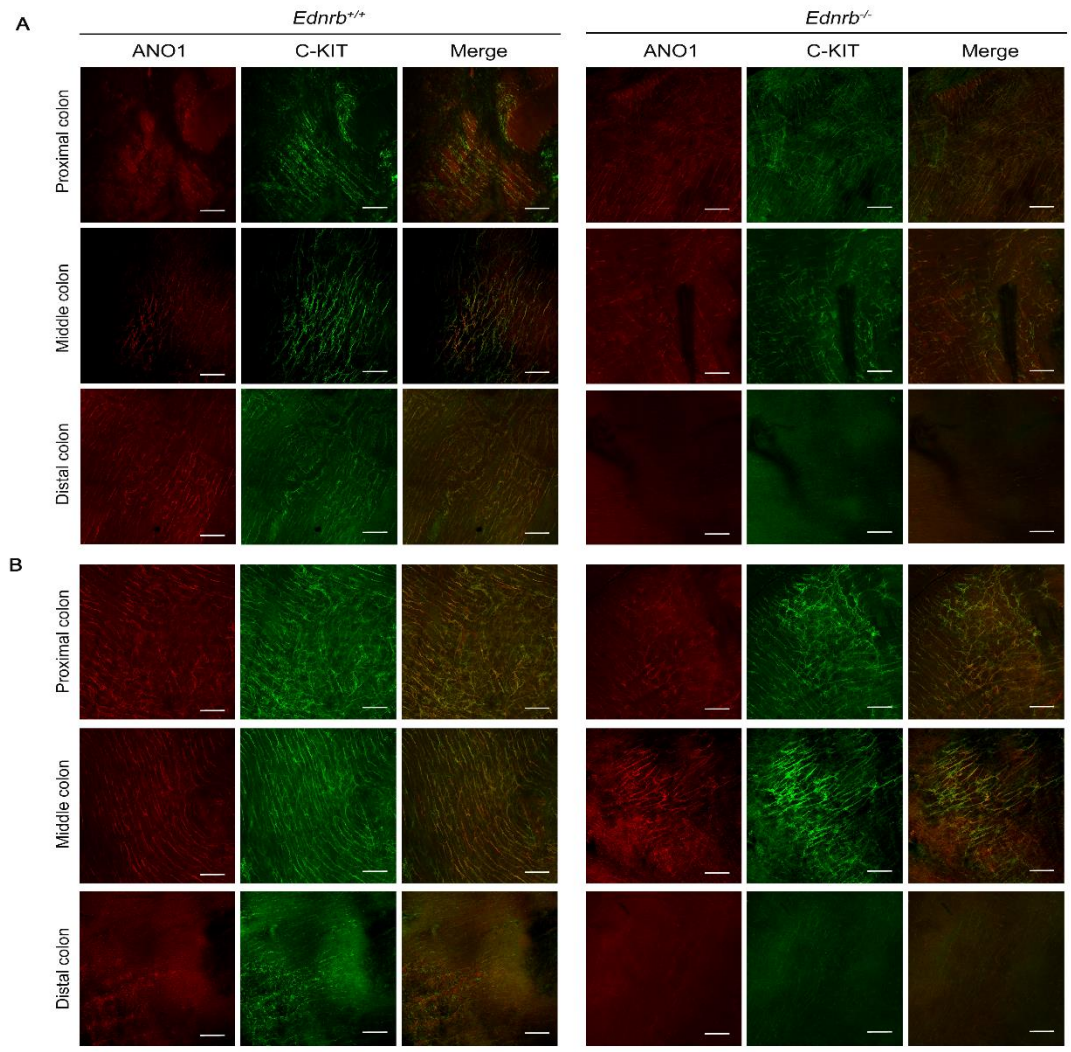
(**A**) mRNA expression levels of *iNos*, *Tnfa*, *Cd34*, *Scf*, *c-Kit* in the colon of the 1-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice; (**B**) mRNA expression levels of *iNos*, *Tnfa*, *Cd34*, *Scf*, *c-Kit* in the colon of the 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Data are representative from 6 independent experiments. One-way ANOVA: ns, non-significant; ***, $P < 0.001$.



Supplemental Figure 6: mRNA expression levels in 3-week old *Ednrb*$^{-/-}$ mice.
mRNA expression levels of (**A**) *iNos* and *Tnfa* and (**B**) *Cd34*, *Scf* and *c-Kit* in proximal, middle and distal colon from 3-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Data are representative from 6 independent experiments. One-way ANOVA: ns, non-significant; ***, $P < 0.001$.

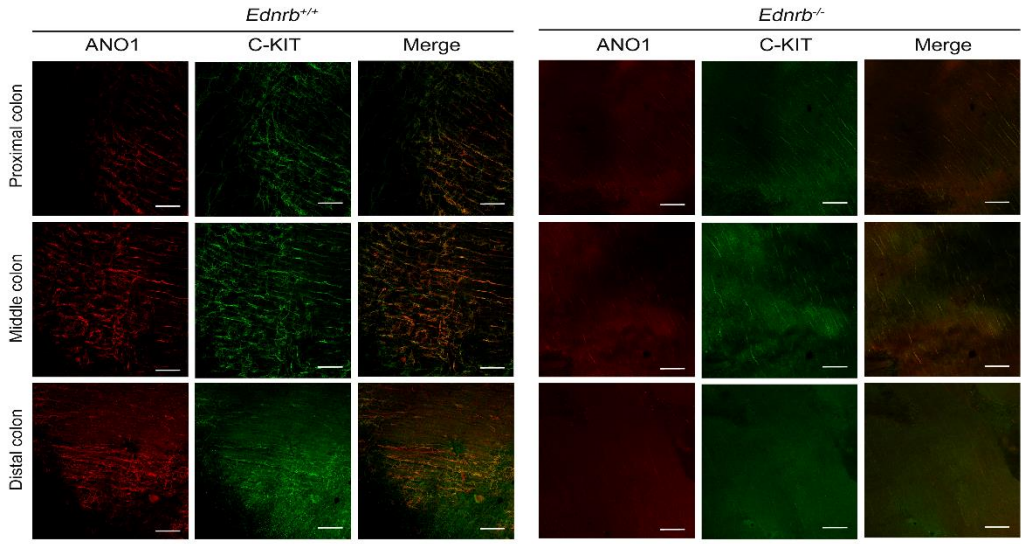


Supplemental Figure 7: C-KIT^{+} ICCs in the 1- and 2-week old *Ednrb*$^{-/-}$ mice. (A) Immunohistochemistry staining of C-KIT for ICCs from 1-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (B) Immunohistochemistry staining of C-KIT for ICCs from 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Scale bar: 100μm. Experiments were repeat 3 times.



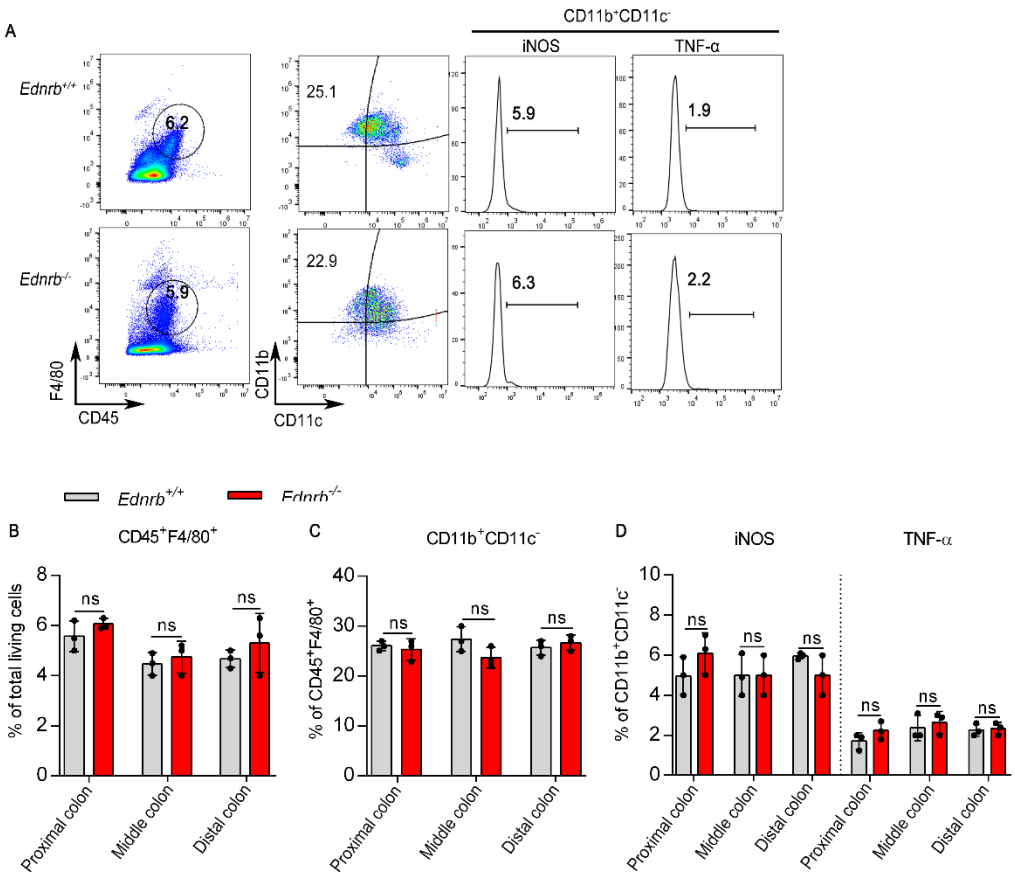
Supplemental Figure 8: Expressions of C-KIT and ANO1 in ICCs of 1- and 2-weeksold *Ednrb*$^{-/-}$ and *Ednrb*$^{+/+}$ mice.

(A) C-KIT and ANO1 were studied by wholemount staining of the proximal, middle and distal colon of 1- week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (B) C-KIT and ANO1 were studied by wholemount staining of the proximal, middle and distal colon of 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Red, ANO1; Green, C-KIT. Scale bar: 100µm. Experiments were repeat 3 times.



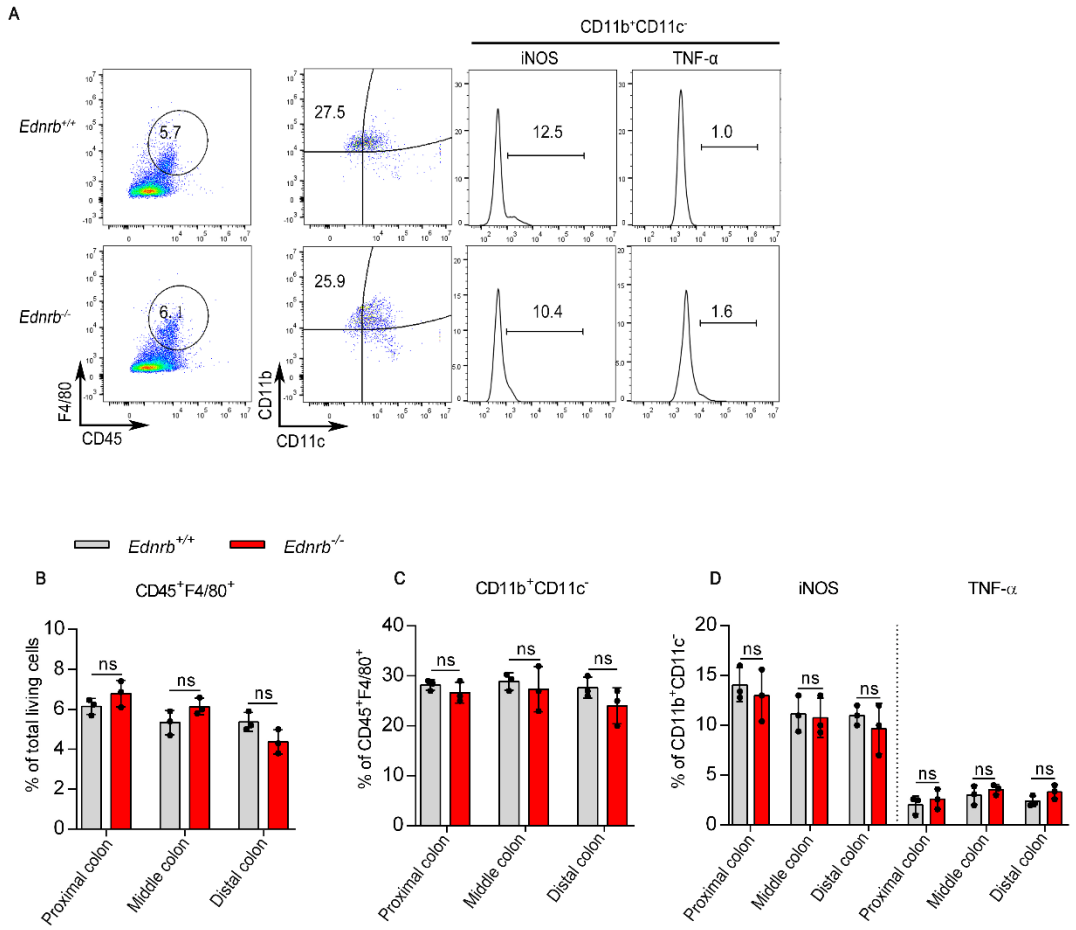
Supplemental Figure 9: Expressions of C-KIT and ANO1 in ICCs of 3-week old *Ednrb*$^{-/-}$ and *Ednrb*$^{+/+}$ mice.

C-KIT and ANO1 were studied by wholemount staining of the proximal, middle and distal colon of 3-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Red, ANO1; Green, C-KIT. Scale bar: 100μm. Experiments were repeat 3 times.



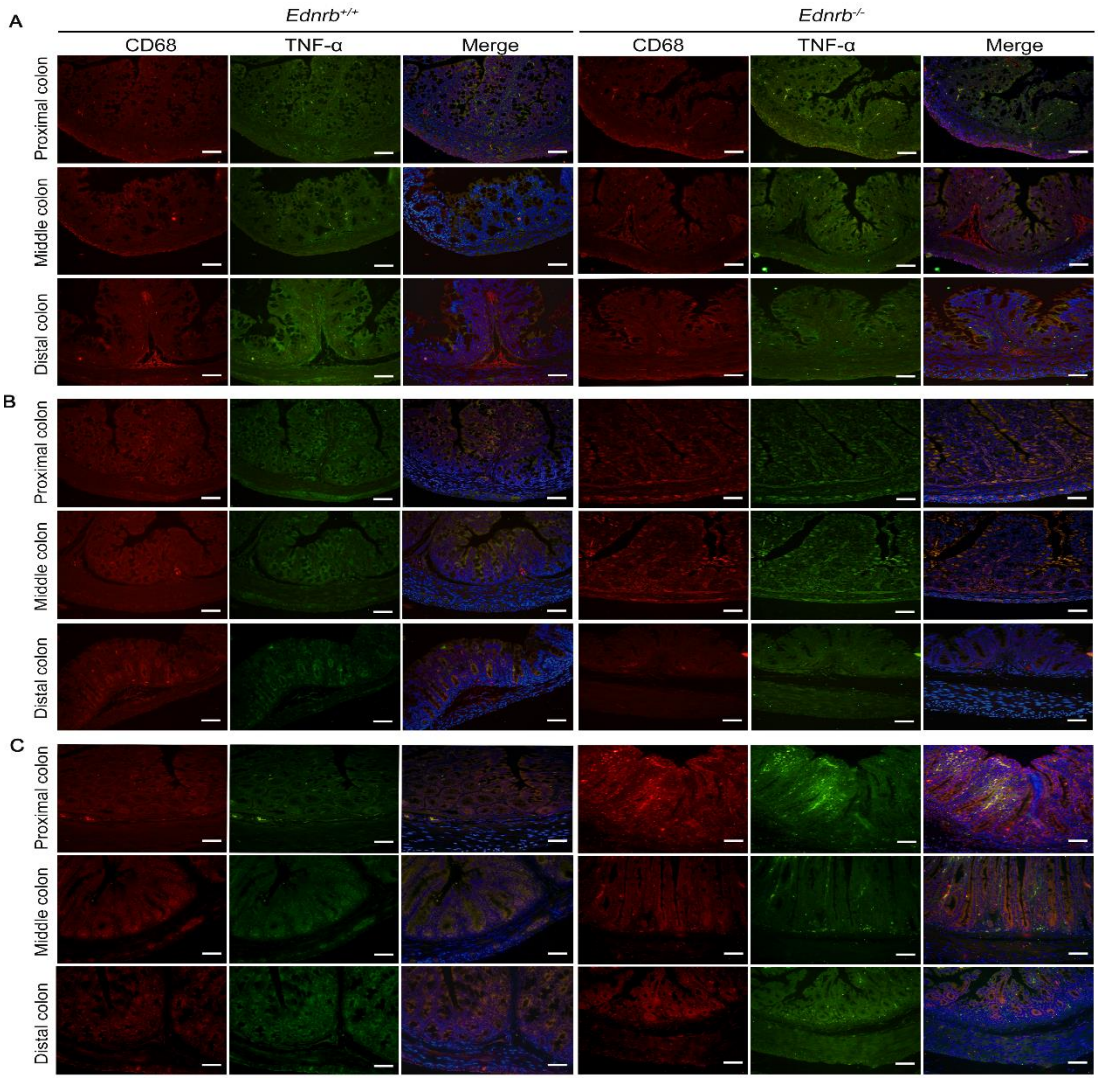
Supplemental Figure 10: Different subsets of monocytes in the 1-week old *Ednrb*$^{-/-}$ mice.

(**A**) Representative analysis of CD45^{+}F4/80^{+}, CD11b^{+}CD11c^{-}, iNOS^{+}, and TNF-α^{+} cells in the proximal colon from 1-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**B**) Percentage of CD45^{+}F4/80^{+} cells among total viable cells in colonic tissue. (**C**) Percentage of CD11b^{+}CD11c^{-} cells among CD45^{+}F4/80^{+} cells. (**D**) Percentage of iNOS^{+} cells and TNF-α^{+} cells among CD11b^{+}CD11c^{-}cells. Data are representative from 3 independent experiments. One-way ANOVA analysis: ns, non-significant.



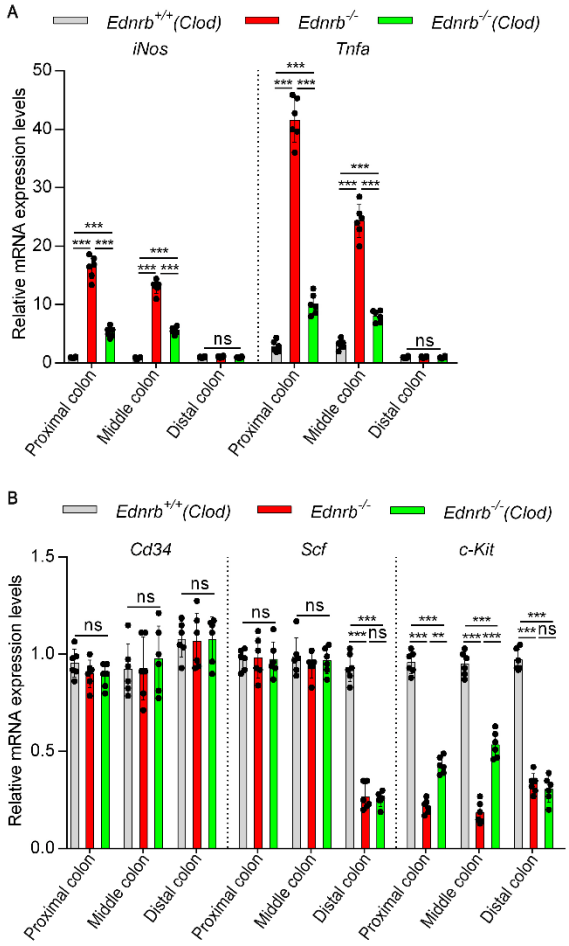
Supplemental Figure 11: Different subsets of monocytes in the 2-week old *Ednrb*$^{-/-}$ mice.

(**A**) Representative analysis of CD45^{+}F4/80^{+}, CD11b^{+}CD11c^{-}, iNOS^{+}, and TNF-α^{+} cells in the proximal colon from 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**B**) Percentage of CD45^{+}F4/80^{+} cells among total viable cells in colonic tissue. (**C**) Percentage of CD11b^{+}CD11c^{-} cells among CD45^{+}F4/80^{+} cells. (**D**) Percentage of iNOS^{+} cells and TNF-α^{+} cells among CD11b^{+}CD11c^{-} cells. Data are representative from 3 independent experiments. One-way ANOVA analysis: ns, non-significant.



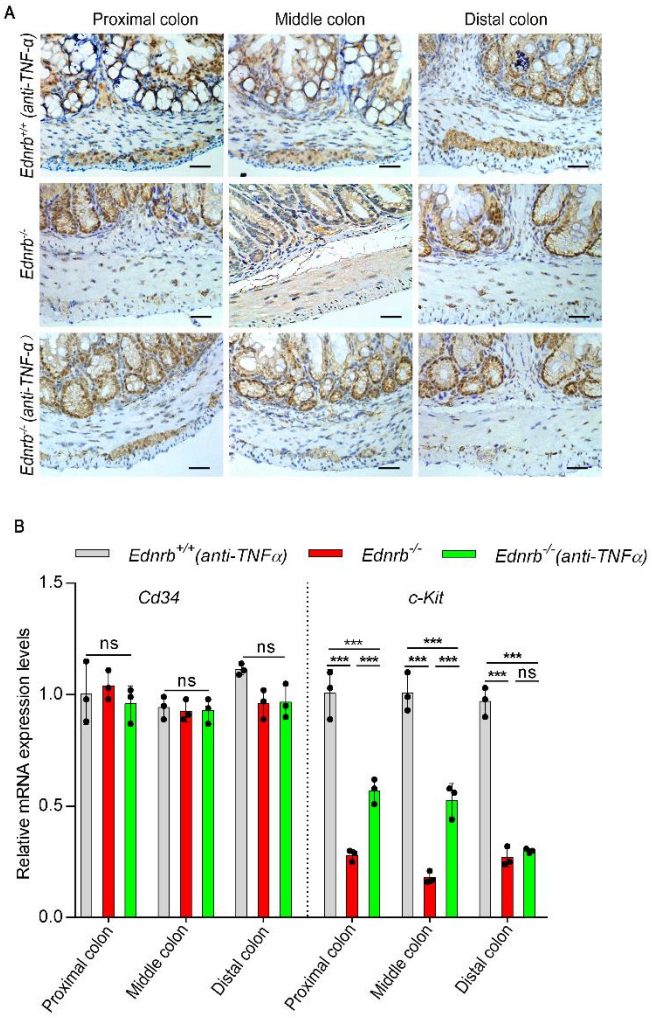
Supplemental Figure 12: Immunofluorescence double staining of CD68 and TNF-α in macrophages of *Ednrb*$^{-/-}$ mice.

(**A**) CD68 and TNF-α staining in 1- week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**B**) CD68 and TNF-α staining in 2-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. (**C**) CD68 and TNF-α staining in 3-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Red, CD68; Green, TNF-α; Blue, DAPI. Scale bar: 100μm. Experiments were repeat 3 times.



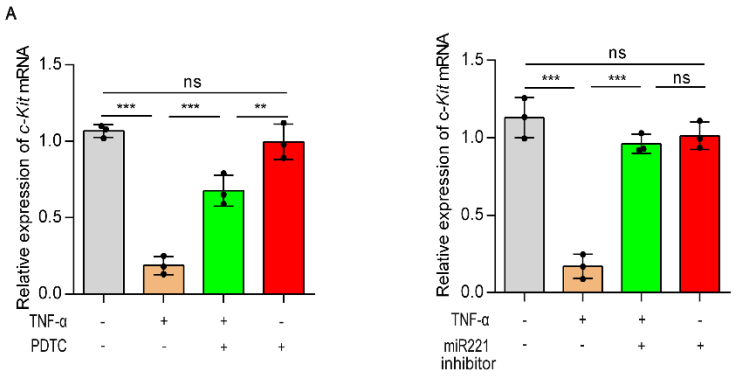
Supplemental Figure 13: mRNA expression levels in 3-week old *Ednrb*$^{-/-}$ mice treated with Clod.

mRNA expression levels of (**A**) *iNos* and *Tnfa* and (**B**) *Cd34*, *Scf* and *c-Kit* in proximal, middle and distal colon from 3-week old and 3-weeks old Clod-treated *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice. Data are representative from 6 independent experiments. One-way ANOVA: ns, non-significant; ***, $P < 0.001$.



Supplemental Figure 14: Phenotype of ICCs in 3-week old *Ednrb*$^{-/-}$ mice after TNF-α neutralization.

(**A**) Immunohistochemistry staining of C-KIT for ICCs from 3-week old *Ednrb*$^{+/+}$ and *Ednrb*$^{-/-}$ mice treated with TNF-α neutralization antibody. Scale bar: 100μm. (**B**) mRNA expression levels of *Cd34* and *c-Kit* in proximal, middle and distal colon. Data are representative from 3 independent experiments. One-way ANOVA: ns, non-significant; ***, $P < 0.001$.



Supplemental Figure 15: mRNA expression levels in the isolated ICCs treated with TNF-α, PDTC and miR221 inhibitor.

(A) *c-Kit* mRNA expression in isolated ICCs with or without TNF-α and PDTC treatment. (B) *c-Kit* mRNA expression in isolated ICCs with or without TNF-α and miR221 inhibitor treatment. Data shown represent results from 3 independent experiments. One-way ANOVA: ns, non-significant; **, $P < 0.01$; ***, $P < 0.001$.