PNAS
www.pnas.org

Supplementary Information for

The ATP-hydrolyzing ectoenzyme E-NTPD8 attenuates colitis through modulation of P2X4 receptor-dependent metabolism in myeloid cells

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SI Materials and Methods

Reagents

Adenosine 5′-[γ-thio] triphosphate tetralithium salt (ATPγS), FITC-conjugated dextran (average molecular weight of 3,000-5,000), *Pluronic® F-127*, the Ca^{2+} ionophore A23187, 2-Deoxy-D-glucose (2-DG), $^{13}C_6$ D-glucose, (1S) - (+)-10-Camphorsulfonic acid and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dextran sulfate sodium salt (DSS) was purchased from MP Biomedicals (Kaysersberg, France). Ampicillin sodium salt, neomycin sulfate, metronidazole, BAPTA-AM, RPMI1640 without D-(+)-Glucose, and 4',6-Diamidino-2-phenylindole Dihydrochloride (DAPI) were purchased from Nacalai Tesque (Kyoto, Japan). Vancomycin hydrochloride was purchased from Duchefa Biochemie B.V. (Haarlem, Netherlands). An ATP assay kit was purchased from Toyo Ink Co., Ltd (Tokyo, Japan). Rat IgG2b, κ isotype control, FITC-conjugated anti-mouse CD326 (EpCAM) (Clone: G8.8) antibody, Biotin-conjugated anti-Ly6G antibody (Clone: 1A8) and Purified anti-mouse CD4 antibody (Clone: GK1.5) were purchased from BioLegend (San Diego, CA, USA). Anti-cleaved Caspase 3 antibody (Asp175) was purchased from Cell signaling technology (Massachusetts, USA). Alexa 647-conjugated streptavidin and Alexa 488-conjugated anti-rabbit IgG were purchased from Invitrogen (Massachusetts, USA). MEBCYTO® Apoptosis Kit (Annexin V-FITC Kit) was purchased from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). Polyclonal anti-ENTPD8 antibody was purchased from Biorbyt (Cambridge, UK). Alexa 594-conjugated anti-rabbit IgG was purchased from Life Technologies (Carlsbad, CA, USA). Seahorse XF Cell Mito Stress Test Kit (Only ROT/AA was used), Seahorse XF RPMI Medium, Seahorse XF Glucose, Seahorse XF Pyruvate and Seahorse XF L-Glutamine were purchased from Agilent Technologies (Santa Clara, CA, USA). Corning® Cell-Tak™ Cell and Tissue Adhesive was purchased from Corning (Corning, NY, USA). Percoll was purchased from GE Healthcare (Chicago, IL, USA). Dormicum (Midazolam) was purchased from

Astellas (Tokyo, Japan). Butorphanol Tartrate. was purchased from Meiji Seika Pharma (Tokyo, Japan). Domitor (Medetomidine) was purchased from Zenoaq (Fukushima, Japan). Probenecid (water soluble) was purchased from Invitrogen (Carlsbad, CA, USA). P-EGFP N3 vector was purchased from Takara Bio (Shiga, Japan).

Mice

BALB/c and C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). *P2rx4*$^{-/-}$ mice were generated as described previously(1). All mice were maintained under specific pathogen-free conditions. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University (28-065). A cocktail of antibiotics (0.05% ampicillin, 0.05% neomycin, 0.05% metronidazole, and 0.025% vancomycin) was administered to 6- to 8-week-old mice in their drinking water for 12 weeks. For the analysis of epithelial permeability, mice were starved for 4 hours and then orally administered FITC-conjugated dextran (molecular weight of 3,000-5,000) at a dose of 50 mg/100 g of body weight. After 4 hours, serum was collected, and the concentration of FITC-conjugated dextran was measured. To generate BM-chimeric mice, 8- to 10-week-old mice were injected intravenously with 10^7 BM cells at 20 hours after 7.5 Gy irradiation. Seven weeks later, the mice were used for experiments. To deplete neutrophils and monocytes, wild-type and *Entpd8*$^{-/-}$ mice were injected intraperitoneally with either 250 μg of anti-Gr-1 antibody (RB6-8C5)(2) or control IgG on days 2 and 4 after DSS administration. To deplete $CD4^+$ T cells, wild-type and *Entpd8*$^{-/-}$ mice were intraperitoneally injected with 500 μg control IgG or anti-CD4 antibody on days -1, 2, and 5 during DSS administration.

DSS-induced colitis

Acute colitis was induced in 8- to 20-week-old mice by administering DSS (36–50 kDa) in their drinking water for 5-7 days. The DAI score, which incorporates bleeding score

and pasty stool score, was used to evaluate the severity of large intestinal inflammation, as reported previously(3). In addition, the mice were analyzed for changes in large intestinal histology. The colons collected from mice after the initiation of DSS administration were fixed in 4% paraformaldehyde. Paraffin-embedded sections mounted on glass slides were used for hematoxylin and eosin (H&E) staining, and images of H&E staining were taken using Biozero (BZ9000, Keyence, Osaka, Japan). Each section of the large intestine was evaluated using inflammation scores, as described previously(4, 5).

ATP measurement

Concentrations of fecal and luminal ATP were measured as described previously(6). In brief, to analyze the fecal ATP levels, feces were collected, weighted, and suspended in PBS to generate ten-fold dilutions of the homogenates. After the samples were centrifuged at 13,000 ×*g* for 10 minutes, the resulting supernatants were collected, and their ATP levels were measured by using a luciferin-luciferase assay performed with a Kinsiro ATP Luminescence kit (Toyo B-Net, Tokyo, Japan). To measure the luminal ATP levels, mice were first anesthetized via intraperitoneal injection with 250 μl of a mixture of three types of anesthetic agents (0.003% medetomidine, 0.04% midazolam, 0.05% butorphanol tartrate). The peritoneal cavity was opened, and the large and small intestines were ligated with nylon threads to make a closed intestinal loop. Then,300 μl of PBS was applied luminally with a 29G needle, and 5 minutes later, the luminal fluids were recovered using a 29G needle, and collected in microtubes. After these samples were centrifuged at 13,000 ×*g* for 10 minutes, the ATP levels in the resulting supernatants were measured.

Measurement of intracellular Ca^{2+} in Gr-1^+ $CD11b^+$ cells

Intracellular Ca^{2+} in Gr-1^+ $CD11b^+$ cells was measured as previously described, with modifications(7). Large intestinal Gr-1^+ $CD11b^+$ cells from mice administered 3.0% DSS

for 6 days were stained with 2 μM Ca^{2+} indicator fluo-8 AM in the presence of 0.02% *Pluronic® F-127*, 2.5 mM probenecid, 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 U/ml penicillin (Nacalai Tesque) at 37 °C for 30 minutes and washed with HBSS (Nacalai Tesque) containing 0.02% *Pluronic® F-127*, 2.5 mM probenecid and then suspended in HBSS containing 0.02% *Pluronic® F-127*, 2.5 mM probenecid. Intracellular Ca^{2+} level was measured with flow cytometry for 100 seconds prior to addition of $CaCl_2$ with ATPγS or vehicle to determine the baseline Ca^{2+} level in Gr-1^+ $CD11b^+$ cells and the cells were stimulated with or without 100 μM ATPγS. The fluorescence emission of Fluo-8 was assessed for 400 seconds and the average of the fluorescence was plotted (every 10 seconds). Changes in intracellular Ca^{2+} level were calculated as a fold change of the fluorescence of Fluo-8 after addition of $CaCl_2$ with vehicle (at 110 sec) in wild-type or *P2rx4*$^{-/-}$ cells.

Apoptosis assay

Gr-1^+ $CD11b^+$ cells, Ly6G^+ $CD11b^+$ neutrophils, or Ly6C^+ $CD11b^+$ monocytes isolated from the colons of mice that had been administered 3% DSS for 6 days were cultured at 37 °C with or without ATPγS in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 5 hours, the cells were washed with PBS and then stained with annexin V and PI. The frequencies of annexin V positive cells were analyzed by flow cytometry. In some experiments, either 10 μM BAPTA-AM or 100 μM 2-DG were added into the culture of Gr-1^+ $CD11b^+$ cells in the presence or absence of ATPγS.

Isolation of intestinal lamina propria cells

Adaptive lymphocytes and myeloid cells were isolated from the lamina propria of the large intestine using a previously described protocol(8). In brief, the large intestines were opened longitudinally and washed with PBS to remove the feces. The large intestines

were then placed in HBSS with 5 mM EDTA and incubated at 37 °C for 20 min in a shaking water bath. After being washed in PBS, the tissues were cut into small pieces and incubated in RPMI 1640 containing 4% FBS, 1 mg/ml collagenase D (Roche, Basel, Switzerland), 0.5 mg/ml dispase (Thermo Fisher Scientific, MA, USA), and 40μg/ml DNase I (Sigma Aldrich) for 35 minutes at 37 °C in a shaking water bath. The digested tissues were passed through a 40 μm cell strainer, and the isolated cells were washed in PBS containing 2% FBS. Finally, the Gr-1$^+$ CD11b$^+$ cells, CD64$^-$ DCs, and CD64$^+$ Mϕ were purified using a FACS Aria system. For the isolation of large intestinal lamina propria lymphocytes, the digested tissues were resuspended in 5ml of 40% Percoll (GE Healthcare) and overlaid on 2.5 ml of 80% Percoll in a 15 ml tube. Percoll gradient separation was performed via centrifugation at 780 ×*g* for 20 min at room temperature. The lamina propria lymphocytes at the interface of the Percoll gradient were collected and washed with PBS containing 2% FBS.

Intracellular cytokine staining

Intracellular expression of IFN-γ, IL-10, and IL-17A in intestinal CD4$^+$ T cells was analyzed by using a Cytofix/Cytoperm Plus Kit with GolgiStop (BD Biosciences; Franklin Lakes, NJ, USA), in accordance with the manufacturer's instructions. In brief, the cells were stimulated with 50 ng/ml PMA and 5 μM calcium ionophore A23187 in the presence of GolgiStop at 37 °C for 4 hours. After surface staining with anti-CD4 antibody, the cells were permeabilized with Cytofix/Cytoperm solution and stained with anti-IFN-γ, anti-IL-10, and anti-IL-17A antibodies.

Flow cytometry

The following antibodies were purchased from BD Biosciences: anti-mouse CD16/32 (2.4.G2), PE/Cy7-conjugated anti-CD11b (M1/70), PE-conjugated anti-mouse Ly-6G (1A8), PE-conjugated anti-mouse CD64 (X54-5/7.1.1), FITC-conjugated anti-mouse IgA

(C10-3), Biotinylated anti-mouse Ly-6G and Ly-6C (Gr-1) (RB6-8C5), and PE/Cy7-conjugated anti-mouse Ly-6C (AL-21). Anti-mouse CD16/32 (S17011E), Pacific Blue-conjugated anti-mouse CD45 (30-F11), PE-conjugated anti-mouse CD45 (30-F11), FITC-/APC-conjugated anti-mouse/human CD11b (M1/70), FITC-conjugated anti-mouse Ly-6G (1A8), FITC-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) (RB6-8C5), Pacific Blue-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) (RB6-8C5), PerCP/Cy5.5-conjugated anti-mouse CD4 (GK1.5), FITC-conjugated anti-mouse IFN-γ (XMG1.2), APC-conjugated anti-mouse IL-17A (TC11-18H10.1), APC/Cy7-conjugated anti-mouse B220 (RA3-6B2), and PE-conjugated anti-mouse IL-10 (JES5-16E3) were purchased from BioLegend. FITC-conjugated anti mouse MHC class II (M5/114.15.2) was purchased from eBioscience (San Diego, CA, USA). Dead cells were stained with 7-AAD Viability Staining Solution (BioLegend). Flow cytometric analysis was performed with a FACSCanto II flow cytometer (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR, USA). $7AAD^{-}$ $CD45^{+}$ $CD11b^{+}$ $Gr\text{-}1^{+}$ cells, $7AAD^{-}$ $CD45^{+}$ $CD11b^{+}$ $Ly6G^{+}$ cells, $7AAD^{-}$ $CD45^{+}$ $CD11b^{+}$ $Ly6C^{+}$ cells, $7AAD^{-}$ $CD45^{+}$ $CD11b^{+}$ MHC II^{+} $CD64^{+}$ Mϕ, and $7AAD^{-}$ $CD45^{+}$ $CD11b^{+}$ MHC II^{+} $CD64^{-}$ DCs were isolated with a FACSAria flow cytometer (BD Biosciences). The instrumental compensation was set in each experiment using single-color, two-color, or four-color stained samples.

Real-time RT-PCR

Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and the RNA was reverse transcribed with ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo; Osaka, Japan). Real-time RT-PCR was performed on a Step One Plus™ Real-Time PCR System (Applied Biosystems; Waltham, MA, USA) using GoTaq qPCR Master Mix (Promega; Madison, Wisconsin, United States). All values were normalized to the expression of *Gapdh*, encoding glyceraldhyde-3-phosphate dehydrogenase, and the fold difference in expression relative to that of

Gapdh is shown. The amplification conditions were 50 °C (2 min), 95 °C (10 min), and 40 cycles of 95 °C (15 s) and 60 °C (60 s). The following primer sets were used: *Gapdh,* 5′-cctcgtcccgtagacaaaatg-3′ and 5′-tctccactttgccactgcaa-3′, *Il17,* 5′ -ggactctccaccgcaatga-3′ and 5′ -ggcactgagcttcccagatc-3′, *Mpo,* 5′-atcacggcctcccaggatacaatg-3′ and 5′-accgcccatccagatgtcaat-3′, *ENTPD1*, 5′-ggttgctcaggatggaaagt-3′ and 5′-atgggactatgctgaaccac-3′, *ENTPD2*, 5′-tgctcaacctgaccaatcca-3′ and 5′-acccagccgtacttgatgaa-3′, *ENTPD7,* 5′-tcaagccaggaatctctgca-3′ and 5′-atagccaactgcttcctctc-3′, *ENTPD8,* 5′-gctggtcaagtactccttca-3′ and 5′-tgtgagtgtagacgctgtag-3′, *ENPP1*, 5′-cagcattgtcaccggattgt-3′ and 5′-gctgtgacccaaattggttc-3′, *ENPP2,* 5′-ctttggccactgggctatat-3′ and 5′-ggctgtaatccatagcggtt-3′, *ENPP3*, 5′-cttacaacggaagtgtccca-3′ and 5′-attactctggcactgactgg-3′, *GAPDH,* 5′-gggagtcaacggatttggtc-3′ and 5′-gaatttgccatgggtggaat-3′.

Extraction of bacterial DNA from feces

Feces were collected in tubes, and RNAlater (Ambion; Austin, TX, USA) was added to make ten-fold dilutions of homogenates. Fecal homogenates (200 μl) were washed twice with 1 ml of PBS, after which 0.3 g of glass beads (diameter, 0.1 mm) (BioSpec Products; Bartlesville, OK, USA), 300 μl of Tris-SDS solution, and 500 μl of TE-saturated phenol were added to the suspension, and the mixture was vortexed using a FastPrep-24 (MP Biomedicals; Kaysersberg, France) at power level 5.0 for 30 s. Following centrifugation of the samples at 20,000 ×*g* for 5 minutes at 4 °C, phenol-chloroform extraction was performed on 400 μl of the resulting supernatants. Isopropanol precipitation was then performed 250 μl of supernatants generated form phenol chloroform extraction. Finally, DNA from the fecal samples was suspended in 200 μl of TE buffer.

Determination of fecal commensal bacteria composition by deep sequencing

Libraries were prepared in accordance with “Illumina 16S Metagenomic Sequencing

Library Preparation Guide" by using a primer set (27Fmod, 5′-AGRGTTTGATCMTGGCTCAG-3′ and 338R, 5′-TGCTGCCTCCCGTAGGAGT-3′) targeting the V1–V2 region of the 16S rRNA gene. Afterwards, 251 bp paired end sequencing of the amplicon was performed on a MiSeq (Illumina, San Diego, CA, USA) using a MiSeq v2 500 cycle kit. Raw paired end sequences were merged using PEAR (http://sco.h-its.org/exelixis/web/software/pear/), and 30,000 reads per sample were randomly selected for further analysis using seqtk (https://github.com/lh3/seqtk). The processed sequences were clustered into OTUs defined at a 97% similarity cutoff using UCLUST version 1.2.22q. Representative sequences for each OTU were then classified taxonomically by using RDP Classifier version 2.2, with the Greengenes 13_8 database. The bioinformatics pipeline QIIME, version 1.9.1, was used as the informatics environment for all relevant processing of raw sequencing data and the calculation of relative bacterial abundances.

LC-MS analysis

BALB/c mice were administered 3% DSS for 6 days and isolated $Ly6G^+$ $CD11b^+$ neutrophils from the colon. Neutrophils were cultured in the presence or absence of 100 μM ATPγS for 3 hours in RPMI 1640 medium supplemented with 5.5 mM $^{13}C_6$ D-glucose (Sigma-Aldrich). These cells were washed with PBS and resuspended in 200 μL ultrapure water (Wako, Tokyo) and then incubated in a thermomixer (eppendorf, Hamburg, Germany) (1,200 rpm) at 4 ℃. After 10 minutes, these samples were added 800 μL methanol and incubated in a thermomixer (1,200 rpm) at 4 ℃ for 10 minutes and then added (1S) - (+)-10-Camphorsulfonic acid (Sigma-Aldrich) as an internal standard. Following centrifugation at 16,000 × g for 3 minutes, 800 μl of the supernatants were concentrated by evaporating with an Spin Dryer Standard VC-96R (Taitec, Saitama, Japan) to dryness and the dried samples were dissolved in 50 μl of ultrapure water and then filtered through 0.2 μm filter (Merk).

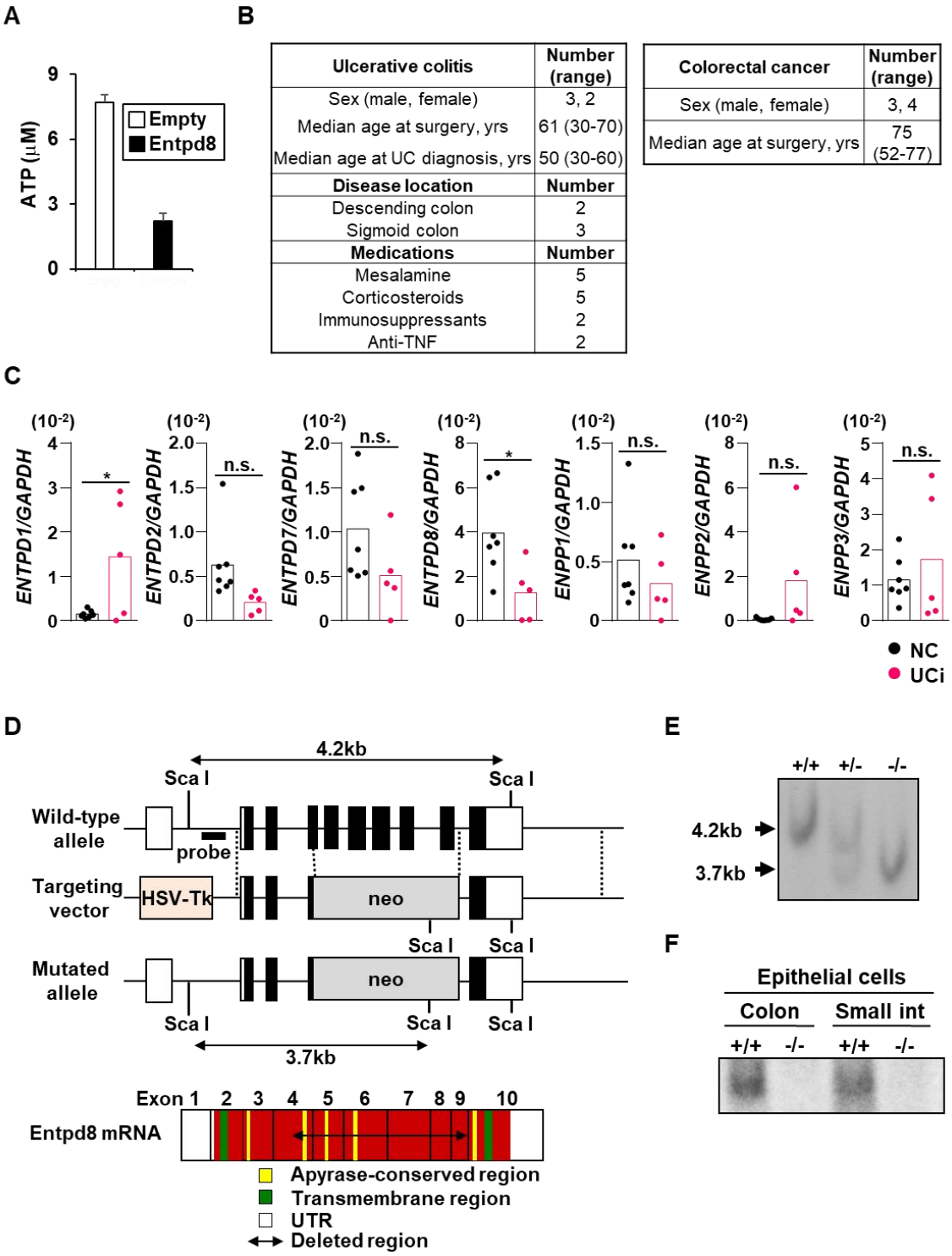
Relative levels of analyte abundances and ^{13}C incorporation into glycolytic intermediates were measured with LCMS-8030Plus (Shimadzu, Kyoto, Japan) operated in negative electrospray ionization mode, as previously described, with slight modifications(9). Three μL of sample was injected on a L-Column2 that was kept at 45 ℃ (2.1 mm i.d. × 150 mm, 3 μm, metal-free) (CERI, Tokyo, Japan). The gradient started with 0% solvent B (methanol) and 100% solvent A (10 mM TBA, 15 mM acetic acid in water) until 1 min after injection. Then, a linear gradient to 15% solvent B was performed until 1.5 min and the gradient at 15% B was kept in 1.5-3.0 minutes. A linear gradient to 50% solvent B was carried out until 8 minutes and next a linear gradient to 100% solvent B was carried out until 10 minutes. The gradient at 100% solvent B was hold on between 10 to 11 minutes. The gradient returned to 0% solvent B at 11.5 minutes and it was kept until 17 minutes. The flow was constantly kept at 0.2 ml/min. The MS operated by using an interface voltage of 4 kV, nebulizer gas at 2 L/min, heating gas at 10 L/min, drying gas at 10 L/min, interface temperature at 300 ℃, desolvation line temperature at 250 ℃, and heat block temperature at 400 ℃. Data collection and analysis were performed by using LabSolutions (Shimadzu, Kyoto, Japan). Total abundance of Glu-6-P is a measure of all mass isotopologues of Glu-6-P m+0 (only ^{12}C carbons) to Glu-6-P m+6 (all 6 carbons are ^{13}C-labeled). Isotype collection was performed by using an in-house software tool, as previously described, with modifications(10).

Immunostaining

Frozen sections (6 μm) of the colon fixed with 4% PFA (Nacalai Tesque) were prepared using a cryostat (Leica; Wetzlar, Germany), mounted on glass slides, and air dried. The sections were subsequently permeabilized with 1% Triton X-100 (Nacalai Tesque) and 1% bovine serum albumin (BSA) in PBS for 5 minutes at room temperature, after which they were performed blocking with 10% BSA in PBS for 30 minutes at room temperature and stained with anti-E-NTPD8 antibody for 18 hours at 4°C. The samples were then

washed with PBS three times and incubated with Alexa 488-conjugated anti-rabbit IgG and 4′,6-diamidino-2-phenylindole (DAPI) (Nacalai Tesuque) for 1 hour at room temperature. After being washed with PBS three times, the samples were then embedded in Antifade mount (Invitrogen) and examined by using a IX 71 fluorescence microscope (Olympus, Tokyo, Japan). Frozen sections of the colon from wild-type and *Entpd8*$^{-/-}$ mice administered 3% DSS for 7 days were prepared using cryostat (Leica; Wetzlar, Germany) and fixed with 4 % PFA for 20 minutes, after which they were performed blocking with 3 % BSA in PBS for 1 hour at room temperature and stained with biotin-conjugated anti-Ly6G (1A8) antibody for 18 hours at 4°C. Then, the samples were washed with PBS three times and incubated with Alexa 647-conjugated streptavidin for 1 hour at room temperature and subsequently treated with 0.1 % triton for 10 minutes at room temperature and stained with anti-cleaved-Caspase 3 (Asp175) antibody for 18 hours at 4°C. Then, they were washed with PBS three times and stained with Alexa 488-conjugated anti-rabbit IgG for 1 hour at room temperature and DAPI. Images were taken by confocal laser scanning microscope (FV3000, Olympus, Tokyo, Japan).

Figure S1



Ulcerative colitis	Number (range)
Sex (male, female)	3, 2
Median age at surgery, yrs	61 (30-70)
Median age at UC diagnosis, yrs	50 (30-60)
Disease location	**Number**
Descending colon	2
Sigmoid colon	3
Medications	**Number**
Mesalamine	5
Corticosteroids	5
Immunosuppressants	2
Anti-TNF	2

Colorectal cancer	Number (range)
Sex (male, female)	3, 4
Median age at surgery, yrs	75 (52-77)

Fig. S1: Generation of *Entpd8*$^{-/-}$ mice.

(A) ATP levels in the culture supernatants of HEK293 cells transfected with an empty or mouse *Entpd8* expression vector at 5 minutes after the addition of 50 μM ATP. Data are representative of two independent experiments (mean values with ± SD). **(B)** Characteristics of patients with ulcerative colitis (UC) (left) or colorectal cancer (right). **(C)** Expression of ATP-hydrolyzing enzymes belonging to E-NTPD or E-NPP family in epithelial cells from normal sites of the colon from patients with colorectal cancer (n = 7) (NC) and inflamed sites of colon from patients with ulcerative colitis (n = 5) (UCi). *$p <$ 0.05. n.s., not significant. **(D)** Map of the E-NTPD8 wild-type genome, targeting vector, and predicted targeted gene. Open boxes: non-coding exons; closed boxes: coding exons. **(E)** Southern blot analysis of offspring from heterozygote intercrosses. Genomic DNA from the mouse tails was digested with *Sca*I, separated electrophoretically, and then hybridized with the indicated probe in (D). The approximate sizes of the wild-type and mutated bands are 4.2 kbp and 3.7 kbp, respectively. **(F)** Northern blot analysis of mRNA isolated from the colon or small intestine of wild-type or *Entpd8*$^{-/-}$ mice.

Figure S2

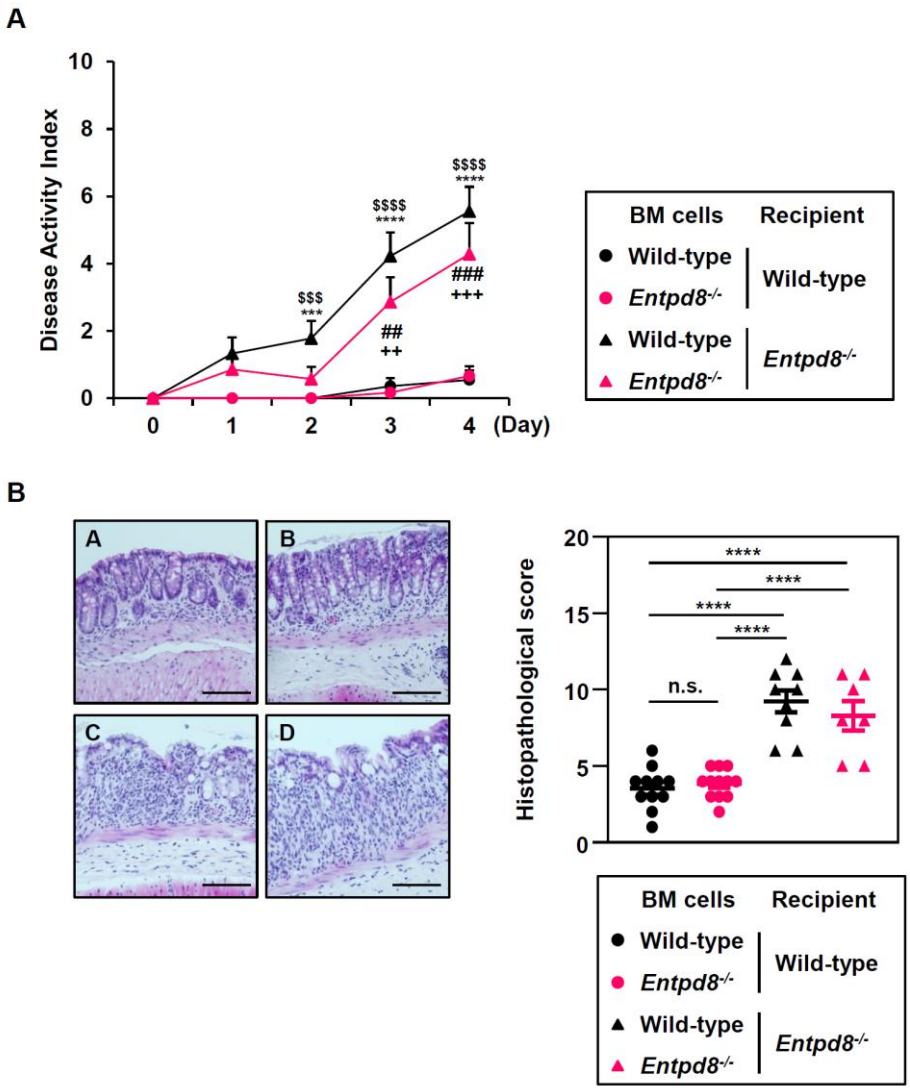


Fig. S2: E-NTPD8 on non-hematopoietic cells is required for the inhibition of colitis. Wild-type mice were transplanted with wild-type (WT/WT; n = 11) or *Entpd8*$^{-/-}$ (WT/KO; n= 12) BM cells, and *Entpd8*$^{-/-}$ mice were transplanted with wild-type (KO/WT; n = 9) or *Entpd8*$^{-/-}$ (KO/KO; n = 7) BM cells. After 7 weeks, the mice were administered 3% DSS for 7 days. **(A)** The DAI scores (mean values ± SEM). ***p < 0.005, ****p < 0.001. $^{\$\$\$}p$ < 0.005, $^{\$\$\$\$}p$ < 0.001. $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.005. ^{++}p < 0.01, ^{+++}p < 0.005. *p denotes significance between WT/WT and KO/WT mice. $^{\$}p$ denotes significance between WT/KO and KO/WT mice. $^{\#}p$ denotes significance between WT/WT and KO/KO mice. ^{+}p denotes significance between WT/KO and KO/KO mice. All data are from two independent experiments. **(B)** Representative sections (left) and histopathological scores (right) of colons from WT/WT (n = 11), WT/KO (n = 12), KO/WT (n = 9), and KO/KO (n = 7). ****p < 0.001. n.s., not significant. Scale bars, 100 μm.

Figure S3

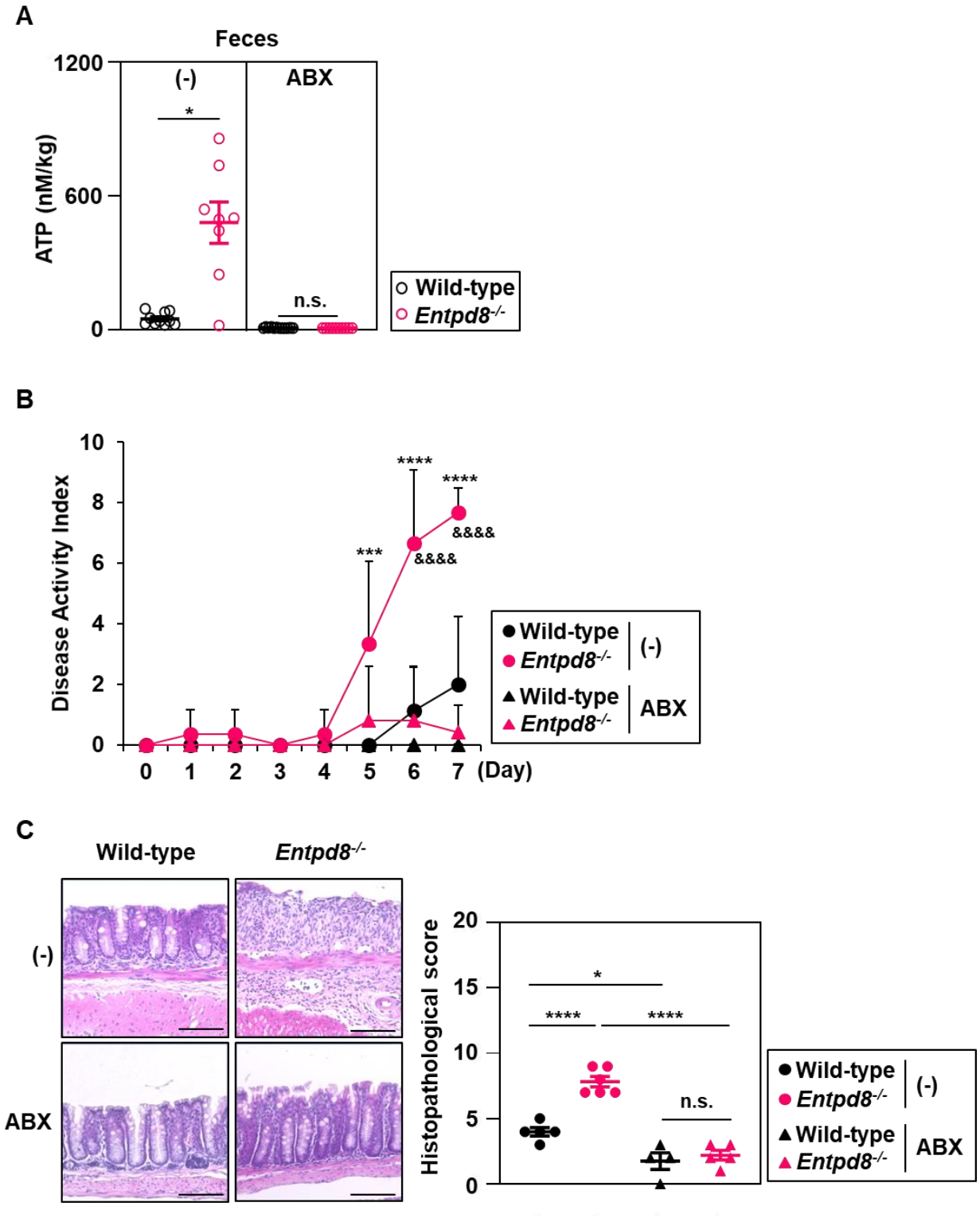


Fig. S3: Intestinal bacteria contribute to exacerbation of DSS-induced colitis in *Entpd8*$^{-/-}$ mice. (A) Concentrations of fecal ATP in wild-type or *Entpd8*$^{-/-}$ mice treated

with (n = 12 or n = 10, respectively) or without (n = 10 or n = 8, respectively) antibiotics (ABX) for 12 weeks (mean values ± SEM). *$p < 0.05$. n.s., not significant. All data are from two independent experiments. **(B)** The DAI scores of mice that were administered 3% DSS for 7 days following treatment with (wild-type, n = 4; and *Entpd8*$^{-/-}$, n = 5) or without (wild-type, n = 9; and *Entpd8*$^{-/-}$, n = 6) ABX for 12 weeks (mean values ± SD). ***$p < 0.005$, ****$p < 0.001$. $^{\&\&\&\&}p < 0.001$. *p denotes significance between ABX-untreated wild-type and ABX-untreated *Entpd8*$^{-/-}$ mice. $^{\&}p$ denotes significance between ABX-untreated *Entpd8*$^{-/-}$ and ABX-treated *Entpd8*$^{-/-}$ mice. All data are from two independent experiments. **(C)** Representative sections (left) and histopathological scores (right) of colons from ABX-untreated or treated wild-type (n = 5 or n = 4, respectively) or *Entpd8*$^{-/-}$ (n = 6 or n = 5, respectively) mice (mean values ± SEM). Scale bars, 100 μm. *$p < 0.05$, ****$p < 0.001$. n.s., not significant.

Figure S4

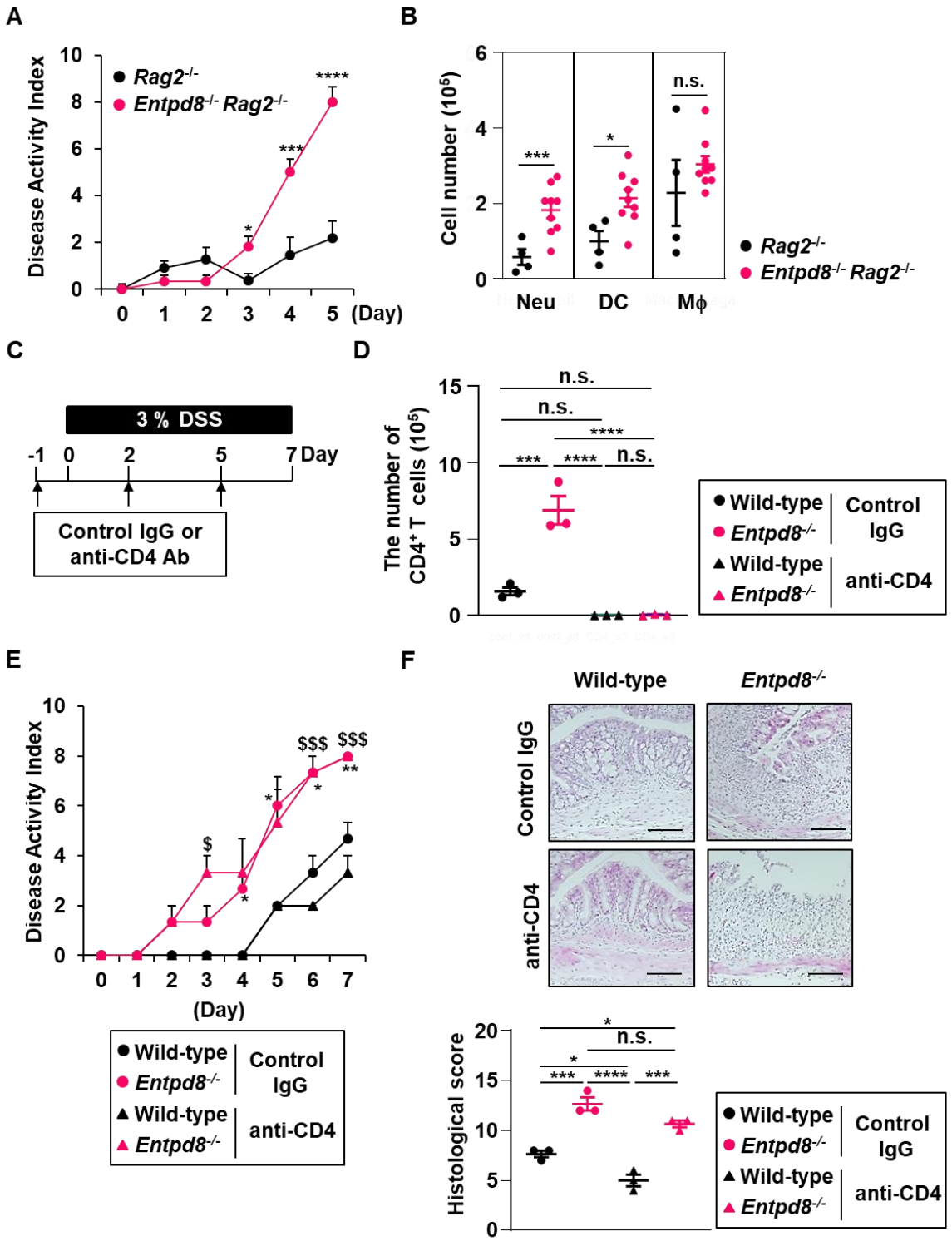


Fig. S4: Adaptive lymphocytes are not involved in exaggeration of intestinal inflammation in *Entpd8*$^{-/-}$ mice. (A) *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ *Entpd8*$^{-/-}$ mice were administered

2.5% DSS for 5 days. The DAI scores of *Rag2*$^{-/-}$ (n = 11) and *Rag2*$^{-/-}$ *Entpd8*$^{-/-}$ (n = 12) mice (mean values ± SEM). *$p < 0.05$, ***$p < 0.005$, ****$p < 0.001$. **(B)** Cell numbers of the indicated myeloid cell types in the large intestinal lamina propria of *Rag2*$^{-/-}$ (n = 4) and *Rag2*$^{-/-}$ *Entpd8*$^{-/-}$ (n = 9) mice (mean values ± SEM). *$p < 0.05$, ***$p < 0.005$. n.s., not significant. All data are pooled from two independent experiments. **(C)** The scheme of the experiment. **(D)** The number of $CD4^+$ T cell in the colonic lamina propria. All data are mean values ± SEM from three mice in each group. ***$p < 0.005$, ****$p < 0.001$. n.s., not significant. **(E)** The DAI scores of wild-type or *Entpd8*$^{-/-}$ mice injected with control IgG or anti-CD4 antibody. All graphs show mean values ± SEM from three mice. *$p < 0.05$, **$p < 0.01$. $^{\$}p < 0.05$, $^{\$\$\$}p < 0.005$. *$p$ denotes significance between control IgG-injected wild-type and *Entpd8*$^{-/-}$ mice. $^{\$}p$ denotes significance between anti-CD4 antibody-injected wild-type and anti-CD4 antibody-injected *Entpd8*$^{-/-}$ mice. **(F)** Representative sections of the colon (left) and histopathological scores (right). All data are mean values ± SEM from three mice. *$p < 0.05$, ***$p < 0.05$, ****$p < 0.001$. n.s., not significant. Scale bars, 100 μm.

Figure S5

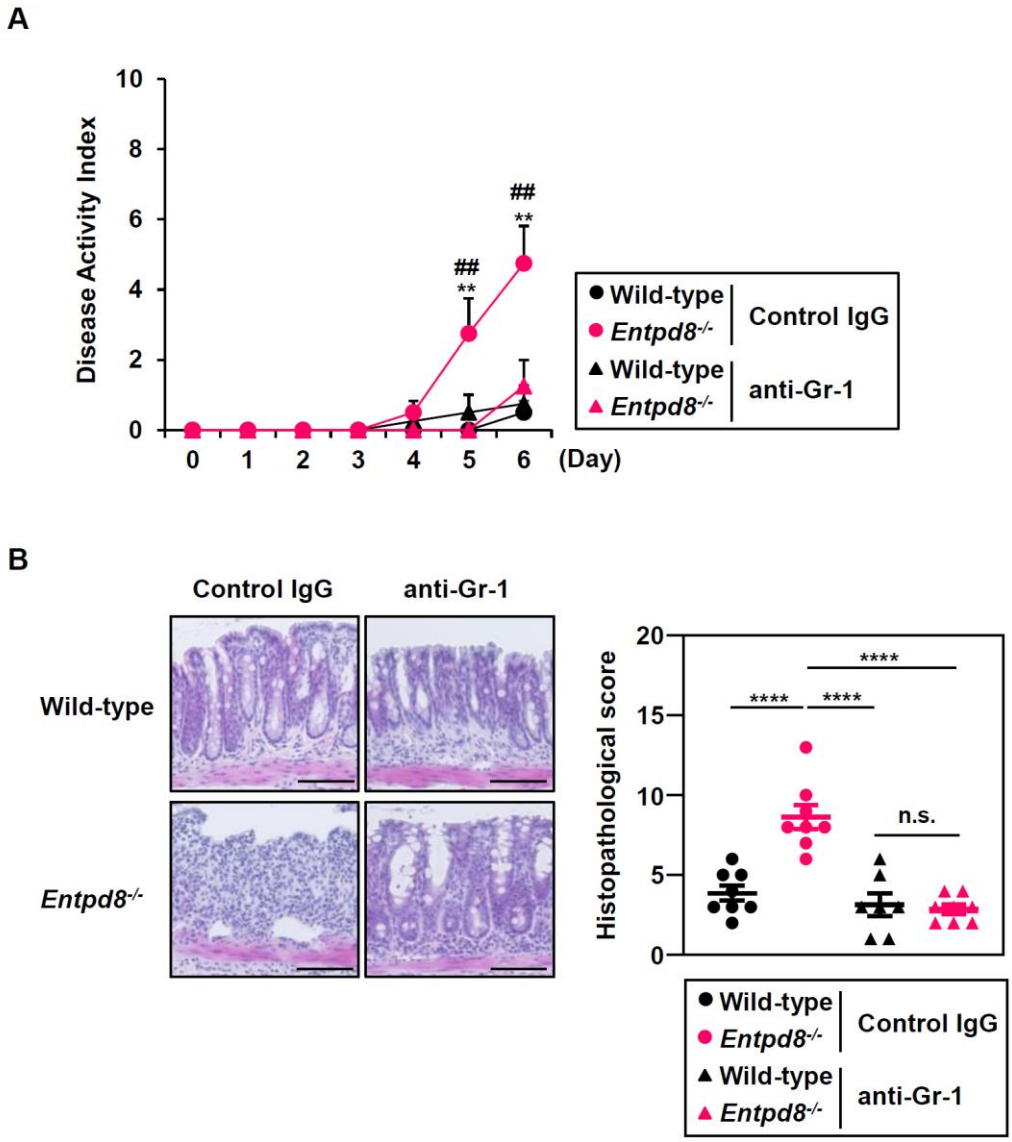


Fig. S5: *Entpd8* deficiency leads to severe innate intestinal pathology during DSS-induced colitis. Wild-type or *Entpd8*$^{-/-}$ mice were administered 3% DSS for 6 days and were intraperitoneally injected with control IgG or anti-Gr-1 antibody at 2 and 4 days after DSS administration was begun. **(A)** The DAI scores of wild-type or *Entpd8*$^{-/-}$ mice injected with control IgG or anti-Gr-1 antibody. All graphs shown mean values ± SEM from eight mice per group. $^{**}p < 0.01$. $^{\#\#}p < 0.01$. $^{*}p$ denotes significance between control IgG-injected wild-type and *Entpd8*$^{-/-}$ mice. $^{\#}p$ denotes significance between control IgG-injected *Entpd8*$^{-/-}$ and anti-Gr-1 antibody-injected *Entpd8*$^{-/-}$ mice. All data are from two independent experiments. **(B)** Representative sections (left) and histopathological scores (right) of murine colons. All data are mean values ± SEM from eight mice per group. $^{****}p < 0.001$. n.s., not significant. Scale bars, 100 μm.

Figure S6

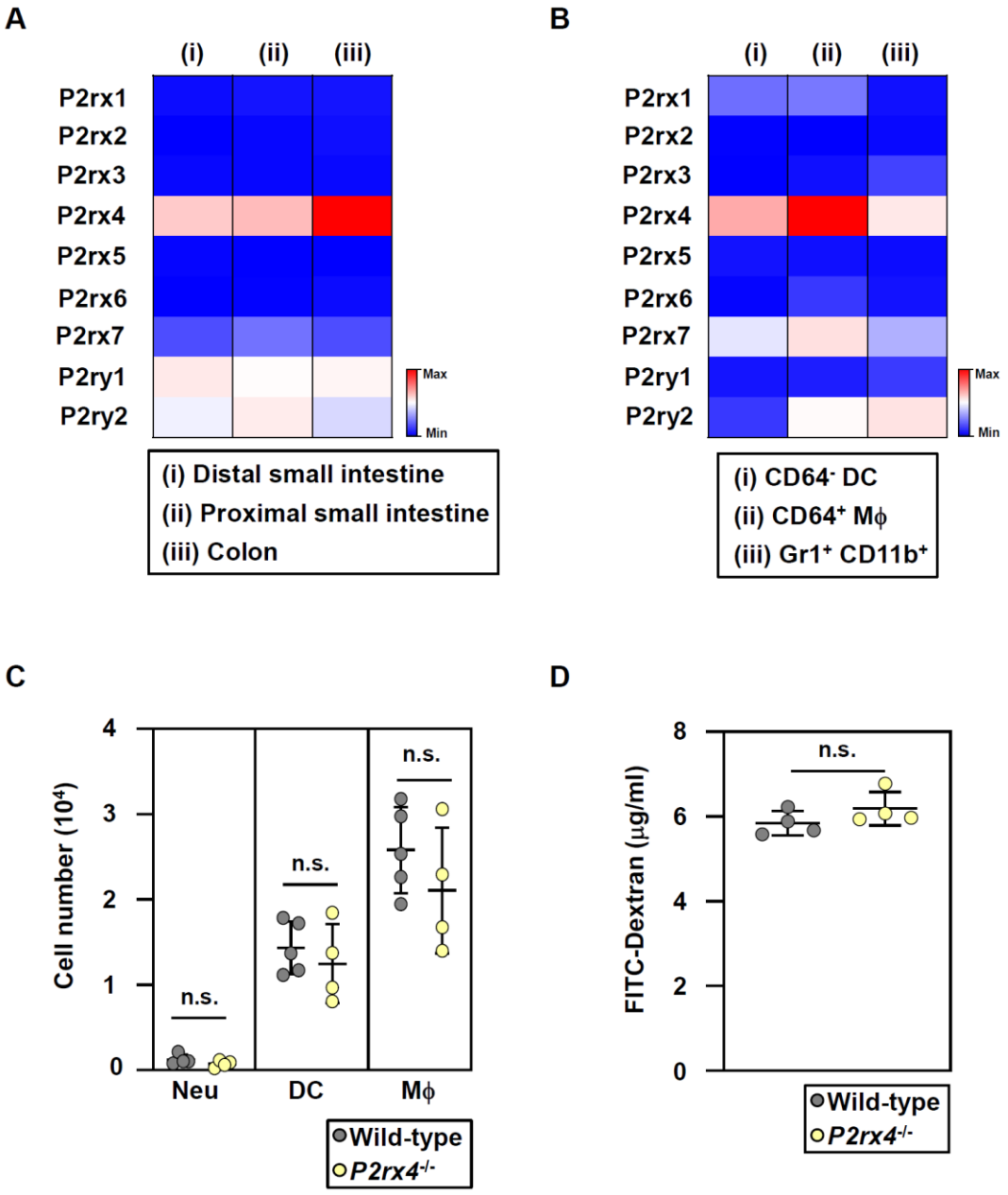


Fig. S6: Expression patterns of ATP receptors in colonic epithelial cells and myeloid cells. (A and B) Expression levels of ATP receptors according to FPKM values in large and small intestinal epithelia cells (A) and colonic myeloid cells (B). **(C)** The numbers of indicated myeloid cell types in the colonic lamina propria from wild-type (n = 5) or *P2rx4*$^{-/-}$ mice (n = 4) under steady state conditions (mean values ± SD). n.s., not significant. **(D)** Serum concentrations of FITC-conjugated dextran in wild-type (n = 4) or *P2rx4*$^{-/-}$ (n = 4) mice (mean values ± SD). n.s., not significant.

Figure S7

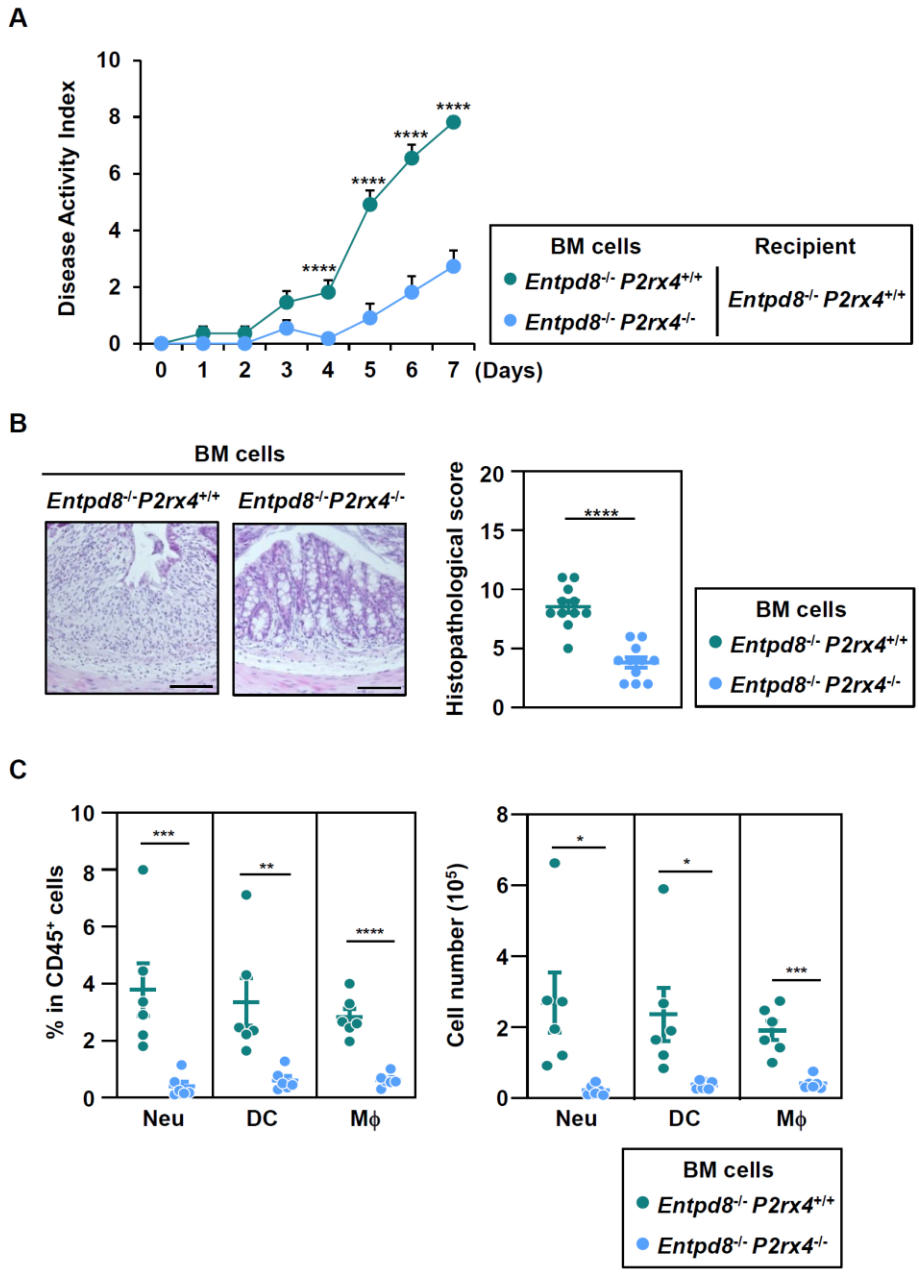


Fig. S7: P2X4 receptor in hematopoietic cell is implicated in the exacerbation of colitis in *Entpd8*$^{-/-}$ mice. *Entpd8*$^{-/-}$ *P2rx4*$^{+/+}$ recipients were reconstituted with BM cells from *Entpd8*$^{-/-}$ *P2rx4*$^{+/+}$ (n = 11) or *Entpd8*$^{-/-}$ *P2rx4*$^{-/-}$ (n = 11) donors. After 7 weeks, the mice were administered 3% DSS for 7 days. **(A)** The DAI scores (mean values ± SEM). ****$p < 0.001$. All data are from two independent experiments. **(B)** Representative sections (left) and histopathological scores (right) of the colons (mean values ± SEM). ****$p < 0.001$. Scale bars, 100 μm. **(C)** The frequencies (left) and numbers (right) of the indicated myeloid cell types in the large intestinal lamina propria (mean values ± SEM) (n = 6 per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****p < 0.001.

Figure S8

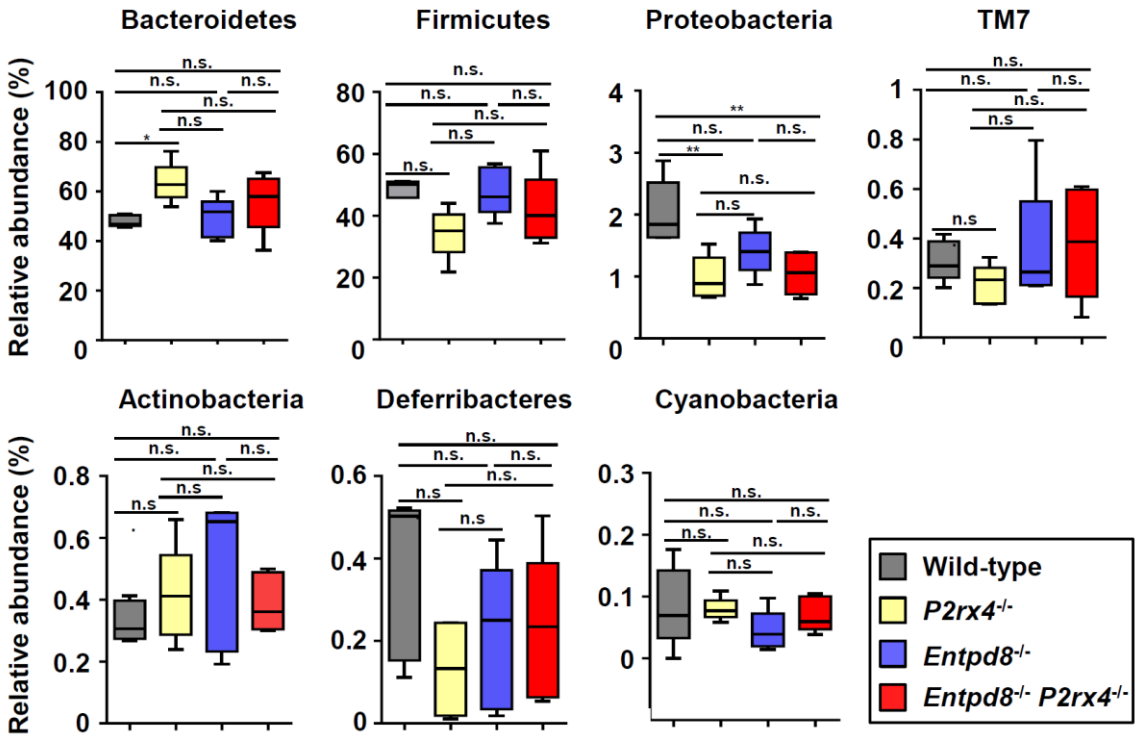


Fig. S8: Fecal microbiota composition in wild-type, *Entpd8*$^{-/-}$, *P2rx4*$^{-/-}$, and *Entpd8*$^{-/-}$ *P2rx4*$^{-/-}$ mice. Relative abundance of fecal bacteria at the phylum level in wild-type (n = 5), *Entpd8*$^{-/-}$ (n = 5), *P2rx4*$^{-/-}$ (n = 5), and *Entpd8*$^{-/-}$ *P2rx4*$^{-/-}$ (n = 5) mice (mean values ± SD). *$p < 0.05$, **$p < 0.01$, n.s., not significant.

Figure S9

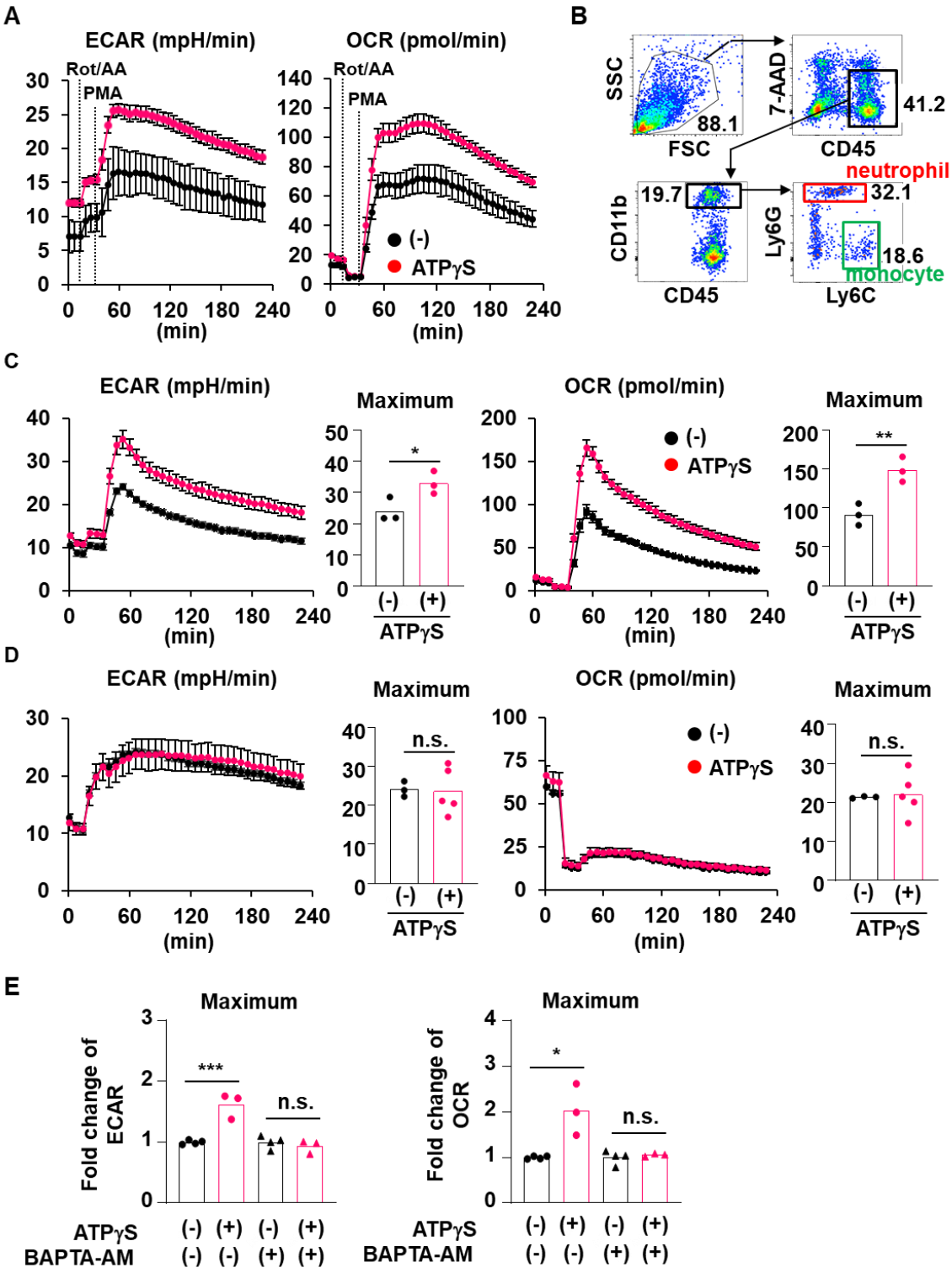


Fig. S9: Extracellular ATP promotes glycolysis through activation of the Ca^{2+} signaling in colonic neutrophils, but not monocytes. BALB/c mice were administered

3% DSS for 6 days and isolated Gr-1$^+$ CD11b$^+$, Ly6G$^+$ CD11b$^+$ neutrophils, or Ly6C$^+$ CD11b$^+$ monocytes from the colon. These cells were treated with 100 μM ATPγS for 3 hours and analyzed ECAR and OCR. **(A)** The values of ECAR (left) and OCR (right) in Gr-1$^+$ CD11b$^+$ cells. Data are representative of three independent experiments. **(B)** Gating strategy for neutrophils and monocytes in the colon of BALB/c mice administered 3% DSS for 6 days. **(C and D)** The values of ECAR and OCR in neutrophils (C) and monocytes (D) (mean values ± SEM). The graphs show averages of maximum OCR and ECAR from at least three wells. All data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$. n.s., not significant. **(E)** Neutrophils were stimulated with or without 100 μM ATPγS in the presence or absence of 10 μM BAPTA-AM. The bars show fold changes of the maximum ECAR (left) and OCR (right). All data are representative of three independent experiments. *$p < 0.05$, ***$p < 0.005$. n.s., not significant.

Figure S10

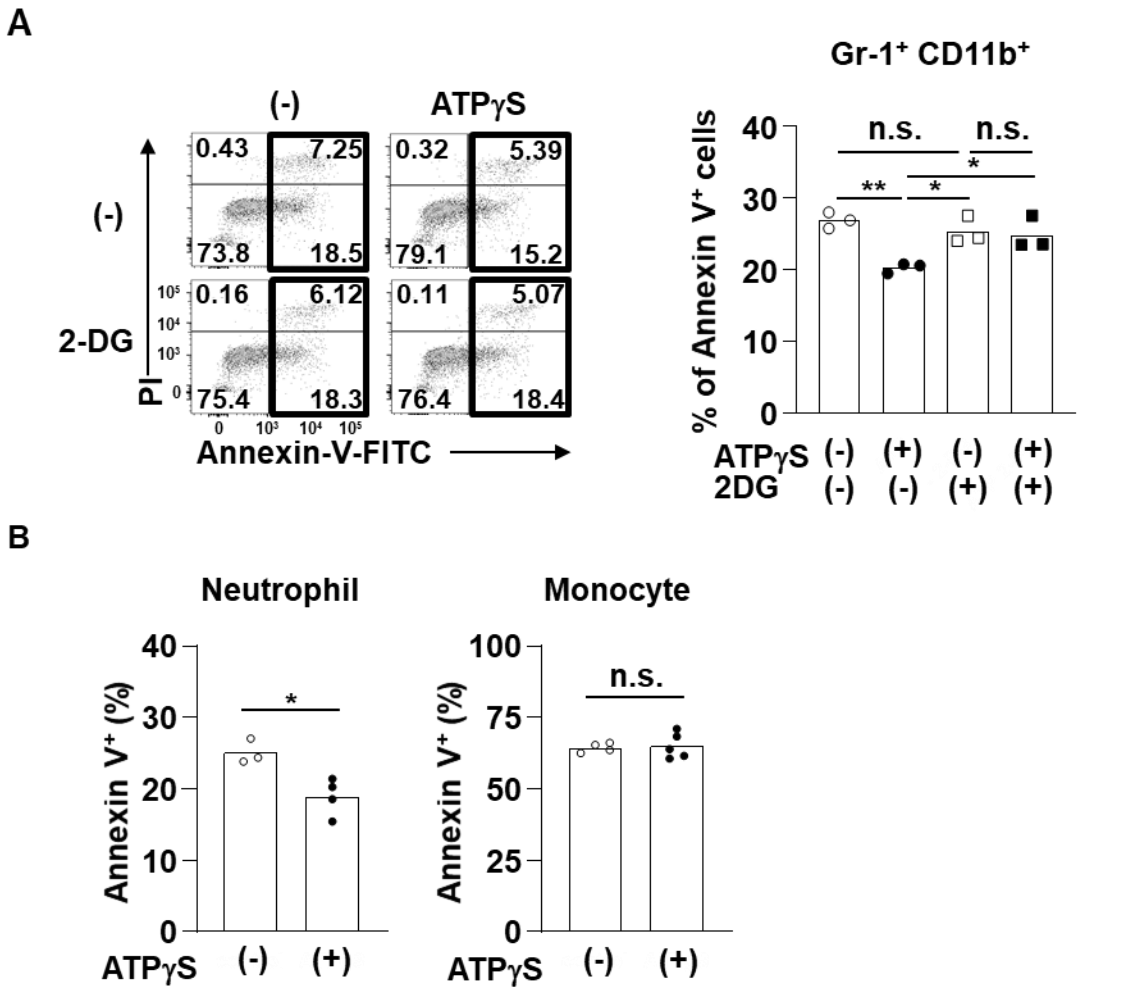


Fig. S10: ATPγS-mediated promotion of glycolysis suppresses neutrophil apoptosis. BALB/c mice were administered 3% DSS for 6 days and isolated Gr-1$^+$ CD11b$^+$ cells, Ly6G$^+$ CD11b$^+$ neutrophils, or Ly6C$^+$ CD11b$^+$ monocytes from the colon. These cells were treated with 100 μM ATPγS for 5 hours. **(A)** Flow cytometric dot plots (left) and frequencies of annexin V$^+$ cells (right) among Gr-1$^+$ CD11b$^+$ cells stimulated with or without ATPγS in the presence or absence of 2-DG. All data are from three independent experiments (mean values). *p < 0.05, **p < 0.01. n.s., not significant. **(B)** Frequencies of Annexin V$^+$ cells in neutrophils (left) or monocytes (right). Data are pooled from three independent experiments. *p < 0.05. n.s., not significant.

SI References

1. Yamamoto K*, et al.* (2006) Impaired flow-dependent control of vascular tone and remodeling in P2X4-deficient mice. *Nat Med* 12(1):133-137.
2. Nemoto Y*, et al.* (2008) Negative feedback regulation of colitogenic CD4+ T cells by increased granulopoiesis. *Inflamm Bowel Dis* 14(11):1491-1503.
3. Sato A*, et al.* (2015) The Wnt5a-Ror2 axis promotes the signaling circuit between interleukin-12 and interferon-gamma in colitis. *Sci Rep* 5:10536.
4. Rais R*, et al.* (2016) FOLH1/GCPII is elevated in IBD patients, and its inhibition ameliorates murine IBD abnormalities. *JCI Insight* 1(12).
5. Van der Sluis M*, et al.* (2006) Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* 131(1):117-129.
6. Kusu T*, et al.* (2013) Ecto-nucleoside triphosphate diphosphohydrolase 7 controls Th17 cell responses through regulation of luminal ATP in the small intestine. *J Immunol* 190(2):774-783.
7. Robledo-Avila FH*, et al.* (2018) Dysregulated Calcium Homeostasis in Cystic Fibrosis Neutrophils Leads to Deficient Antimicrobial Responses. *J Immunol* 201(7):2016-2027.
8. Atarashi K*, et al.* (2008) ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455(7214):808-812.
9. Luo B, Groenke K, Takors R, Wandrey C, & Oldiges M (2007) Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography-mass spectrometry. *J Chromatogr A* 1147(2):153-164.
10. Choi J, Grossbach MT, & Antoniewicz MR (2012) Measuring complete isotopomer distribution of aspartate using gas chromatography/tandem mass spectrometry. *Anal Chem* 84(10):4628-4632.