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

Haruka Tani[a,1], Bo Li[a,1], Takashi Kusu[a], Ryu Okumura[a,b], Junichi Nishimura[a], Daisuke Okuzaki[b,c], Daisuke Motooka[c,d], Shoya Arakawa[e], Asuka Mori[e], Terukazu Yoshihara[f], Takayuki Ogino[f], Shih-Han Tsai[a], Yoki Furuta[a], Masato Muneta[a], Shota Nakamura[c,d], Eiichiro Fukusaki[e], Kimiko Yamamoto[g], Hideo Yagita[h], Hisako Kayama[a,b,i,2], and Kiyoshi Takeda[a,b,c,2]

[a]Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan; [b]The World Premier International Research Center Initiative (WPI) Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan; [c]Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives, Osaka University, Osaka 565-0871, Japan; [d]Genome Information Research Center, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; [e]Department of Biotechnology, Graduate School of Engineering, Osaka University, Osaka 565-0871, Japan; [f]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan; [g]Laboratory of System Physiology, Department of Biomedical Engineering, Graduate School of Medicine, University of Tokyo, Tokyo 113-0033, Japan; [h]Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421, Japan; and [i]Institute for Advanced Co-Creation Studies, Osaka University, Osaka 565-0871, Japan

Extracellular adenosine triphosphate (ATP) released by mucosal immune cells and by microbiota in the intestinal lumen elicits diverse immune responses that mediate the intestinal homeostasis via P2 purinergic receptors, while overactivation of ATP signaling leads to mucosal immune system disruption, which leads to pathogenesis of intestinal inflammation. In the small intestine, hydrolysis of luminal ATP by ectonucleoside triphosphate diphosphohydrolase (E-NTPD)7 in epithelial cells is essential for control of the number of T helper 17 (Th17) cells. However, the molecular mechanism by which microbiota-derived ATP in the colon is regulated remains poorly understood. Here, we show that E-NTPD8 is highly expressed in large-intestinal epithelial cells and hydrolyzes microbiota-derived luminal ATP. Compared with wild-type mice, $Entpd8^{-/-}$ mice develop more severe dextran sodium sulfate–induced colitis, which can be ameliorated by either the depletion of neutrophils and monocytes by injecting with anti–Gr-1 antibody or the introduction of $P2rx4$ deficiency into hematopoietic cells. An increased level of luminal ATP in the colon of $Entpd8^{-/-}$ mice promotes glycolysis in neutrophils through P2x4 receptor–dependent Ca^{2+} influx, which is linked to prolonged survival and elevated reactive oxygen species production in these cells. Thus, E-NTPD8 limits intestinal inflammation by controlling metabolic alteration toward glycolysis via the P2X4 receptor in myeloid cells.

adenosine triphosphate | colitis | E-NTPD8 | immunometabolism | P2X4R

The intestinal microbiota contribute to reinforcing epithelial integrity and shaping the immune system via metabolically derived signaling molecules (1–3). However, alterations in microbial metabolites and their translocation following intestinal dysbiosis are implicated in the pathogenesis of chronic disorders, such as inflammatory bowel diseases (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) (4, 5). Extracellular adenosine triphosphate (ATP) is released by microbes and immune cells in the intestine (6–8) and drives immune responses through the $P2X_{1-7}$ and $P2Y_{1, 2, 11}$ receptors (9). To avoid inappropriate immune reactions in the intestine, luminal ATP is strictly controlled by epithelial ATP-hydrolyzing ectoenzymes, such as ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs) and ectonucleoside triphosphate diphosphohydrolases (E-NTPDases). E-NPP3 on the epithelial cells depresses the apoptosis of plasmacytoid dendritic cells (DCs) in the small intestine and Peyer's patches (10). In addition, E-NTPD7 in small-intestinal epithelial cells hydrolyzes luminal ATP, thus inhibiting excessive T helper 17 (Th17) responses (11). However, how the concentration of luminal ATP produced by commensal bacteria is regulated in the large intestine remains undetermined.

Although intestinal phagocytes such as monocytes, macrophages (Mφ), DCs, and neutrophils have some protective effects, these cells can also function in pathological conditions (12–14). In patients with IBD, inflamed sites of the intestinal mucosa have more inflammatory DCs and Mφ, many of which are dysfunctional (15). In addition, an enhanced neutrophil accumulation in the intestinal mucosa of UC patients correlates with disease severity (16–18). Accordingly, experimental murine colitis can be abrogated by inhibiting the recruitment of monocytes and neutrophils to the intestinal lamina propria by using anti-CCR2 (19) or -Gr-1 (20–22) antibody or by the targeted deletion of CCR2 (23) or β7-integrin (24). Thus, the number of intestinal phagocytes and their physiological functions must be tightly tuned to prevent the intestinal inflammation. However, the mechanisms by which the activity of

Significance

Intestinal bacteria produce extracellular ATP in the lumen during their growth, which drives host immune responses. To avoid excessive immune reactions in the intestinal mucosa, luminal ATP is finely tuned. However, how the concentration of luminal ATP is controlled in the colon remains poorly understood. Here, we discovered that ATP-hydrolyzing enzyme E-NTPD8 acts as an immunomodulator in the colon. $Entpd8$ deficiency led to high sensitivity to DSS-induced colitis in mice, which was due to the sustained survival of colonic neutrophils. Extracellular ATP suppressed apoptosis by inducing metabolic alteration toward glycolysis via P2X4R in neutrophils. These results reveal the mechanism preventing innate intestinal pathology through modulation of myeloid cell metabolism and may serve to identify therapeutic targets for IBD.

monocytes and neutrophils that have infiltrated into the intestinal mucosa are regulated remain poorly understood.

Different immune cell populations have distinct nutrient utilizations and cellular metabolisms (25), which are involved in their differentiation, proliferation, functions, longevity, and epigenetic modification (25–27). Microbial components and metabolites, such as lipopolysaccharide (28) and short chain fatty acids (29), can switch to glycolysis in monocytes and T cells, respectively. The small molecule dimethyl fumarate (DMF), which suppresses glycolysis in lymphocytes and myeloid cells (30), abrogates chemically induced colitis in mice (31, 32), which indicates that inadequate glycolysis activation is involved in the pathogenesis of intestinal inflammation. However, the mechanism underlying the adaptation of intestinal myeloid cells to environmental factors that fuel glycolysis is unclear.

In this study, we investigated the immunomodulatory function of ATP-hydrolyzing ectoenzyme E-NTPD8 in maintenance of the gut homeostasis. Mice with *Entpd8* deficiency had an increased concentration of luminal ATP in their colons, which led to the prolonged survival of neutrophils owing to the facilitation of glycolysis by the P2X4 receptor (P2X4R), thereby exacerbating dextran sodium sulfate (DSS)-induced colitis. Therefore, the clearance of extracellular ATP by E-NTPD8 is essential for the prevention of innate intestinal pathology by inhibiting a metabolic alteration toward glycolysis in myeloid cells.

Results

Entpd8$^{-/-}$ Mice Have Higher Levels of Luminal ATP in the Colon. The molecular mechanism underlying regulation of the luminal ATP concentration in the large intestine, unlike the small intestine, remains poorly understood. To address this knowledge gap, we performed RNA-sequencing (RNA-seq) on small and large-intestinal epithelial cells and comprehensively analyzed their expression patterns of ectonucleotidases. In large-intestinal epithelial cells, the expression levels of *Enpp5*, *Entpd5*, *Entpd6*, and *Entpd8* were higher than those of other molecules (Fig. 1*A*). Among these candidate targets, E-NTPD8 was identified as capable of hydrolyzing ATP and UTP (33, 34). To evaluate whether E-NTPD8 is involved in the hydrolysis of extracellular ATP, we added an ATP solution to cultured HEK293 cells that had transfected with an *Entpd8*-expression or empty vector and measured the concentration of ATP remaining in the culture supernatants at 5 min after the addition (*SI Appendix*, Fig. S1*A*). E-NTPD8–expressing HEK293 cells had a lower concentration of ATP in their supernatants compared with control cells, which indicates that E-NTPD8 regulates the extracellular ATP concentration. In addition, expression
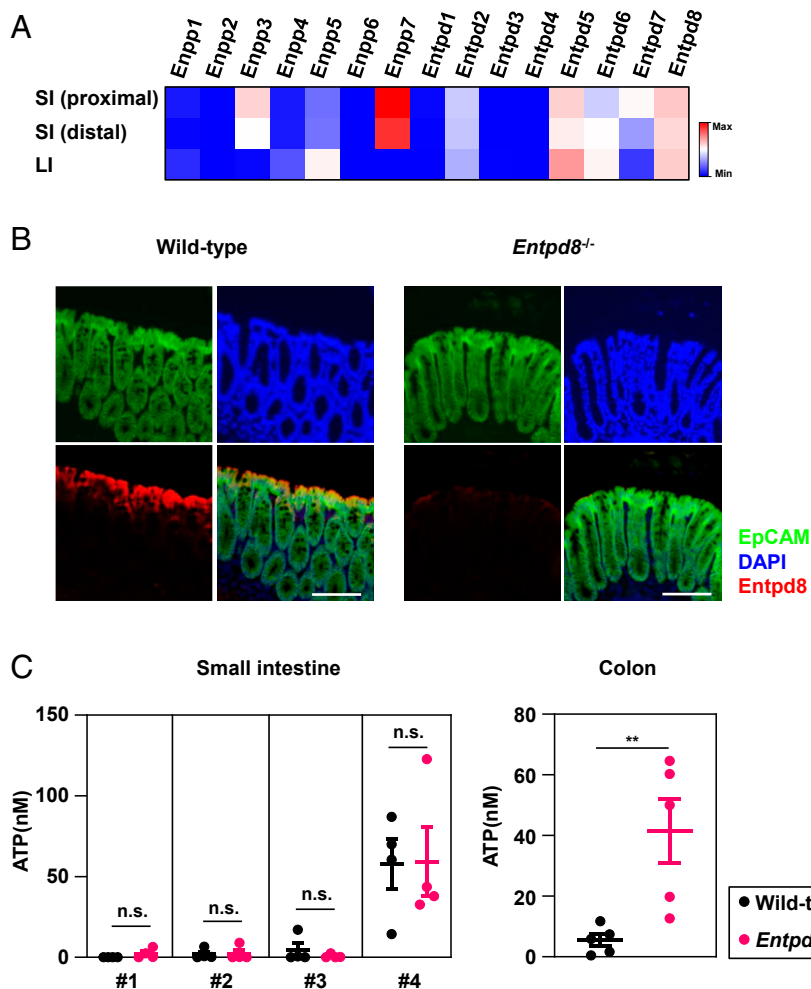
Fig. 1. E-NTPD8 in intestinal epithelial cells hydrolyzes luminal ATP in the colon. (*A*) A heat map of differentially expressed *Enpps* and *Entpds* in epithelial cells isolated from the small or large intestine. (*B*) Expression of E-NTPD8 in the large intestine (red, E-NTPD8; green, EpCAM; and blue, DAPI). (Scale Bar, 100 μm.) Data are representative of two independent experiments. (*C*) Concentrations of luminal ATP in the small intestine (*Left*) and the colon (*Right*). Smaller numbers of the small intestine represent more proximal sites. Mean ± SEM from four (small intestine) or five (colon) independent experiments are shown. **$P < 0.01$; n.s., not significant.

of *ENTPD8* was severely reduced in epithelial cells of patients with UC (*SI Appendix*, Fig. S1 *B* and *C*), indicating the involvement of E-NTPD8 in the pathogenesis of UC.

To assess the physiological roles of E-NTPD8, we generated *Entpd8*−/− mice by using gene targeting (*SI Appendix*, Fig. S1 *D* and *E*). The epithelial cells of the small and large intestines in *Entpd8*−/− mice were confirmed to lack *Entpd8* expression (*SI Appendix*, Fig. S1F). In the colon of wild-type mice, E-NTPD8 protein expression was observed in epithelial cells but not in lamina propria cells (Fig. 1*B*), whereas it was completely undetectable in the colon of *Entpd8*−/− mice. To determine whether E-NTPD8 hydrolyzes extracellular ATP in the intestine, we measured luminal ATP levels in the small and large intestines (Fig. 1*C*). There were no differences in the concentrations of luminal ATP in any of the

four parts of the small intestine between wild-type and *Entpd8*−/− mice. In contrast, a higher level of luminal ATP in the colon was observed for *Entpd8*−/− mice compared with wild-type mice. These findings indicate that E-NTPD8 is essential for maintaining the concentration of luminal ATP in the colon.

***Entpd8* Deficiency Exacerbates Microbiota-Mediated Colitis.** Excessive amounts of extracellular ATP are known to mediate the exacerbation of large-intestinal inflammation in mice (35). Therefore, we examined the sensitivity of *Entpd8*−/− mice to DSS-induced colitis (Fig. 2). Compared with wild-type mice orally administered 3% DSS in drinking water for 7 d, similarly treated *Entpd8*−/− mice exhibited more severe clinical symptoms, including bleeding and pasty stool, which are summarized by their higher disease activity index (DAI) scores (Fig. 2*A*). Additionally, *Entpd8*−/− mice showed
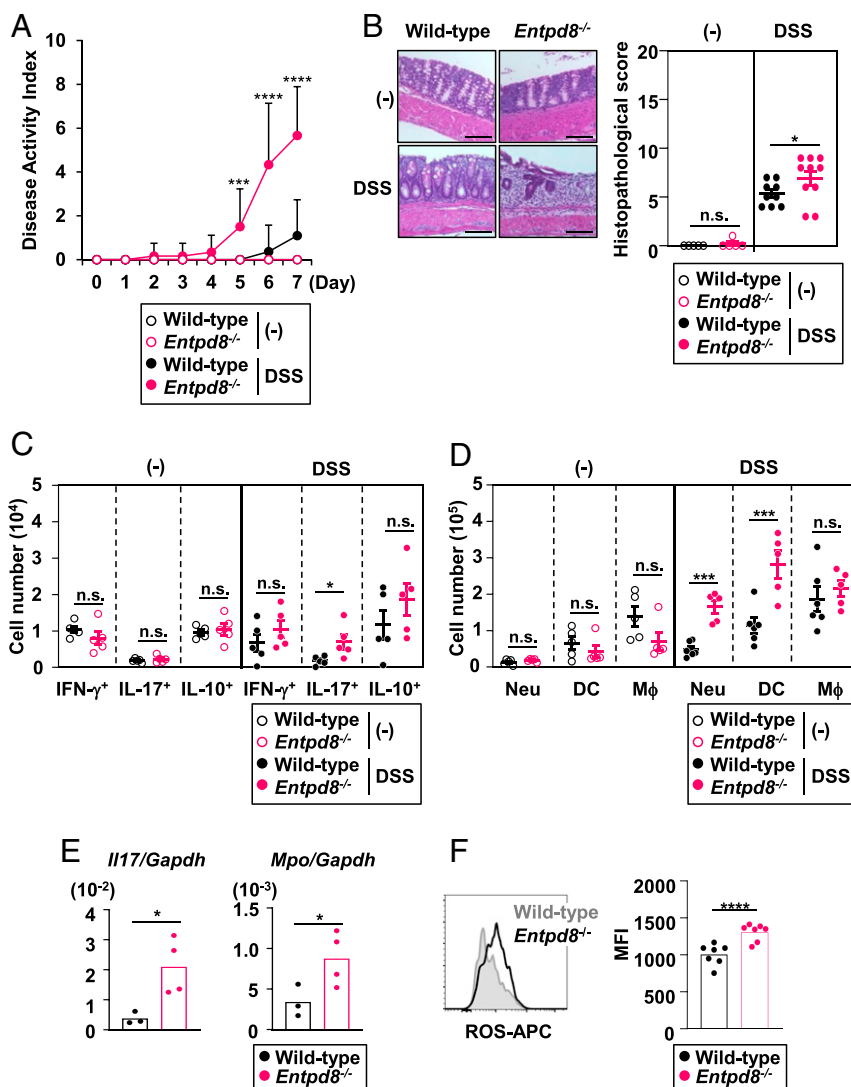
Fig. 2. *Entpd8*−/− mice suffer from severe DSS-induced colitis. Mice were orally administered 3% DSS or left untreated for 7 d. (*A*) The DAI scores of DSS-administered or untreated wild-type (n = 11 or 5, respectively) or *Entpd8*−/− (n = 12 or 5, respectively) mice (mean values ± SEM). ***P < 0.005; ****P < 0.001. (*B*) Hematoxylin-eosin staining (*Left*) and histopathological scores (*Right*) of colons from DSS-administered or untreated wild-type (n = 9 or 5, respectively) or *Entpd8*−/− (n = 10 or 5, respectively) mice (mean values ± SEM). (Scale Bars, 100 μm.) *P < 0.05; n.s., not significant. (*C*) Cell numbers of IFN-γ–, IL-17–, or IL-10–producing CD4+ T cells in the large-intestinal lamina propria from DSS-administered or untreated wild-type or *Entpd8*−/− mice (n = 5 for all groups) (mean values ± SEM). *P < 0.05; n.s., not significant. (*D*) Cell numbers of the indicated innate myeloid cell types in the colonic lamina propria from DSS-administered or untreated wild-type (n = 6 or 5, respectively) or *Entpd8*−/− (n = 6 or 5, respectively) mice (mean values ± SEM). ***P < 0.005; n.s., not significant. (*E*) Expression levels of *Il17* and *Mpo* in the lamina propria cells from the large intestine of DSS-administered wild-type (n = 3) or *Entpd8*−/− (n = 4) mice (mean values ± SD). *P < 0.05. (*F*) Baseline levels of intracellular ROS in colonic Gr-1+ CD11+ cells from DSS-administered wild-type (n = 7) or *Entpd8*−/− (n = 7) mice (mean values ± SD). All data are from at least two independent experiments. ****P < 0.001.

Tani et al.
The ATP-hydrolyzing ectoenzyme E-NTPD8 attenuates colitis through modulation of P2X4 receptor–dependent metabolism in myeloid cells

PNAS | 3 of 10
https://doi.org/10.1073/pnas.2100594118

worsened large-intestinal histopathology compared with wild-type mice after DSS administration (Fig. 2*B*).

In a steady state, the numbers of interferon (IFN)-γ$^+$, interleukin (IL)-17$^+$, and IL-10$^+$ CD4$^+$ T cells (Fig. 2*C*) and myeloid cells (Fig. 2*D*), such as Ly6G$^+$ CD11b$^+$ neutrophils, CD64$^-$ CD11b$^+$ Ly6C^{intermediate} DCs, and CD64$^+$ CD11b$^+$ Mφ, were normal in the colons of *Entpd8*$^{-/-}$ mice. However, after DSS administration, accumulation of IL-17$^+$ CD4$^+$ T cells, neutrophils, and CD64$^-$ CD11b$^+$ Ly6C^{intermediate} DCs was promoted in *Entpd8*$^{-/-}$ mice compared with wild-type mice (Fig. 2 *C* and *D*). In accordance with increase in the numbers of Th17 cells and neutrophils following DSS administration in *Entpd8*$^{-/-}$ mice, the expression levels of *Il17a* and *Mpo* were higher in the large-intestinal lamina propria of these mice than in wild-type mice (Fig. 2*E*). Furthermore, the level of reactive oxygen species (ROS) production in neutrophils was elevated in the colon of DSS-treated *Entpd8*$^{-/-}$ mice relative to that in wild-type mice (Fig. 2*F*).

To examine whether E-NTPD8 in nonhematopoietic or hematopoietic cells influences sensitivity to colitis, we generated bone marrow (BM) chimeric mice. Wild-type and *Entpd8*$^{-/-}$ recipients were received either wild-type or *Entpd8*-deficient BM cells, after which they were administered 3% DSS (*SI Appendix*, Fig. S2). Similar DAI scores and levels of colon histopathology were observed in wild-type recipients transplanted with wild-type or *Entpd8*-deficient BM cells (*SI Appendix*, Fig. S2 *A* and *B*). Regardless of whether the BM cells were from a wild-type or *Entpd8*$^{-/-}$ donor, the *Entpd8*$^{-/-}$ recipients had elevated DAI scores associated with more severe large-intestinal pathology compared with those of wild-type recipients, which indicates that E-NTPD8 in nonhematopoietic cells, possibly epithelial cells, is essential for suppression of colitis.
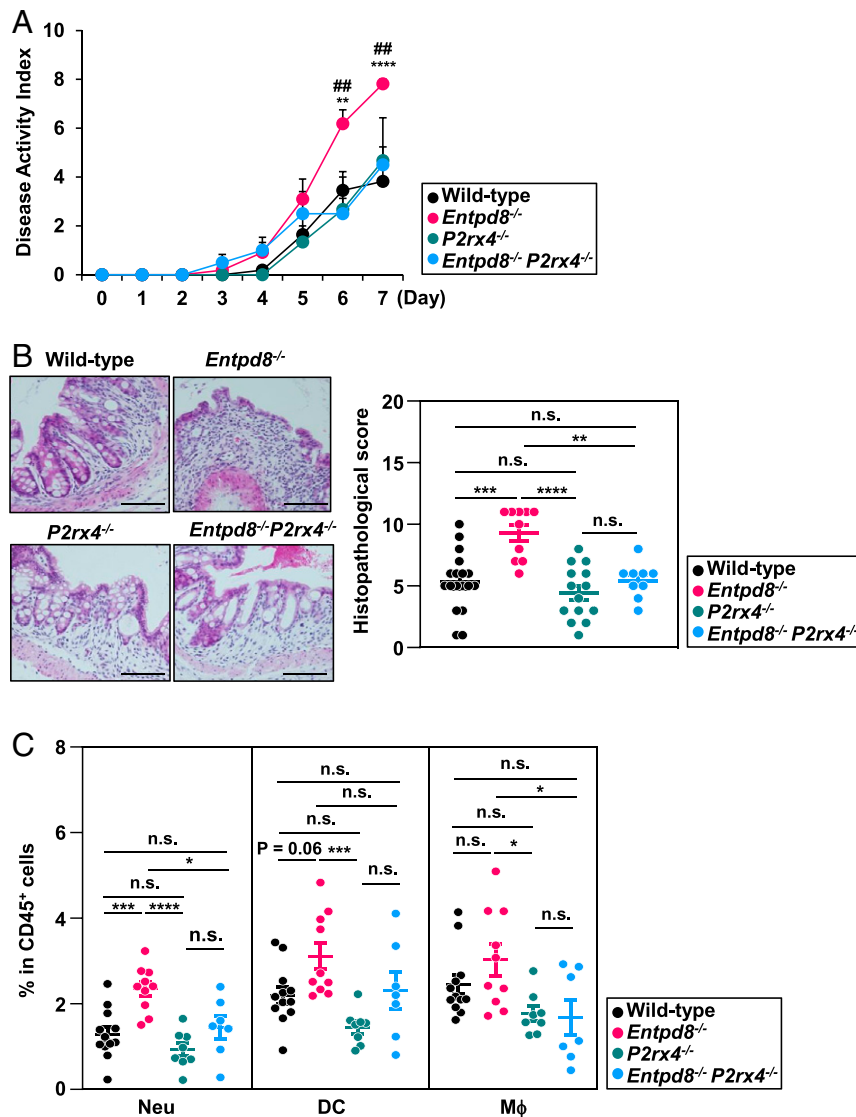

Fig. 3. DSS-induced colitis is mitigated in *P2rx4*$^{-/-}$ *Entps8*$^{-/-}$ mice. Wild-type, *Entpd8*$^{-/-}$, *P2rx4*$^{-/-}$, or *P2rx4*$^{-/-}$ *Entpd8*$^{-/-}$ mice were administered 3% DSS for 7 d. (*A*) The DAI scores of wild-type (*n* = 16), *Entpd8*$^{-/-}$ (*n* = 11), *P2xr4*$^{-/-}$ (*n* = 8), and *P2xr4*$^{-/-}$ *Entpd8*$^{-/-}$ (*n* = 8) mice (mean values ± SD). **$P < 0.01$, ****$P < 0.001$. ##$P < 0.01$. *P denotes significance between wild-type and *Entpd8*$^{-/-}$ mice. #P denotes significance between *Entpd8*$^{-/-}$ and *P2xr4*$^{-/-}$ *Entpd8*$^{-/-}$ mice. All data are from three independent experiments. (*B*) Representative sections (*Left*) and histopathological scores (*Right*) of the colon from wild-type (*n* = 18), *Entpd8*$^{-/-}$ (*n* = 10), *P2rx4*$^{-/-}$ (*n* = 14), and *P2rx4*$^{-/-}$ *Entpd8*$^{-/-}$ (*n* = 9) mice. All data are mean values ± SEM from three independent experiments. **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$; n.s., not significant. (Scale Bars, 100 µm.) (*C*) Frequencies of the indicated innate myeloid cell types in the large-intestinal lamina propria of wild-type (*n* = 12), *Entpd8*$^{-/-}$ (*n* = 10), *P2rx4*$^{-/-}$ (*n* = 8), and *P2rx4*$^{-/-}$ *Entpd8*$^{-/-}$ (*n* = 7) mice (mean values ± SEM). All data are from two independent experiments. *$P < 0.05$, ***$P < 0.005$, ****$P < 0.001$; n.s., not significant.

Intestinal bacteria have been reported to release ATP in the lumen (6–8, 35). To investigate whether E-NTPD8 mediates the clearance of commensal bacteria–derived ATP, mice were administered an antibiotics mixture (ABX) in their drinking water for 12 wk, and the ATP level in their feces was measured (*SI Appendix*, Fig. S3*A*). In untreated *Entpd8*−/− mice, the fecal ATP concentration was significantly higher than that in wild-type mice. However, ABX treatment reduced the fecal concentration of ATP in *Entpd8*−/− mice to a level similar to that in wild-type mice. In accordance with the reduced level of fecal ATP in ABX-treated *Entpd8*−/− mice, these mice had markedly lower DAI score with less severe large-intestinal pathology following DSS administration

compared with untreated *Entpd8*−/− mice (*SI Appendix*, Fig. S3 *B and C*). These findings suggest that E-NTPD8 in epithelial cells controls the concentration of luminal ATP released by commensal bacteria in the colon and thereby provides resistance to DSS-induced colitis.

Depletion of Monocytes and Neutrophils but Not Adaptive Immune Cells Abrogates DSS-Induced Colitis in *Entpd8*−/− Mice. During DSS administration, *Entpd8*−/− mice suffered from severe colitis accompanied by increased numbers of IL-17+ CD4+ T cells, neutrophils, and DCs in the lamina propria of the colon. To determine whether E-NTPD8 is required for limiting Th17 cell–mediated intestinal
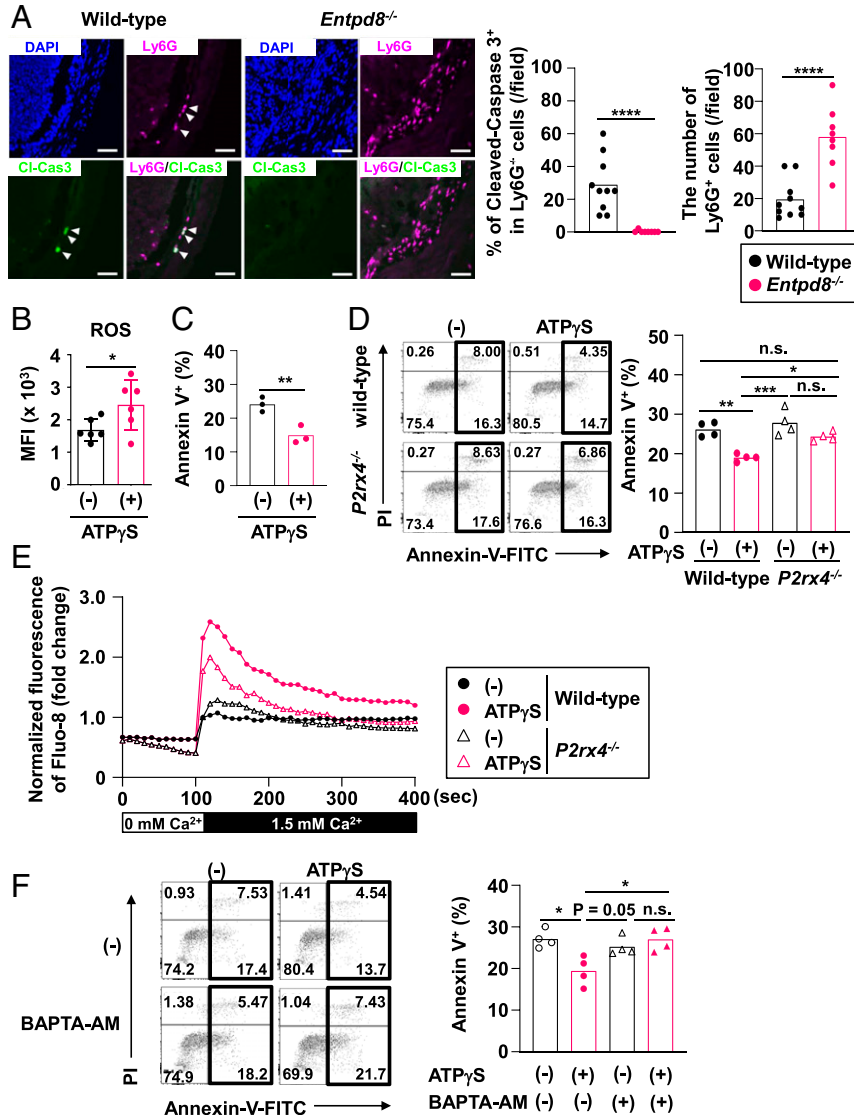
Fig. 4. Sustained Ca2+ influx via P2X4 receptor mediates the inhibition of apoptosis in Gr-1+ CD11b+ cells. (A) Immunohistochemistry of Ly6G+ cleaved-Caspase 3 (Cl-Cas3)+ cells in the colon from mice administered 3% DSS for 7 d (*Left*), the number of Ly6G cells (*Right*), and the frequency of Cl-Cas3+ cells in Ly6G+ cells (*Middle*) in a field (wild-type: 10 fields and *Entpd8*−/−: 8 fields). Images are representative of three independent experiments (blue, DAPI; green, Cl-Cas3; and magenta, Ly6G (Scale bar, 50 μm). ****P < 0.001. (B) Flow cytometry–based analysis of ROS production in colonic Gr-1+ CD11b+ cells stimulated with or without ATP-γS for 3 h (mean values ± SD). *P < 0.05. Data were pooled from six independent experiments. (C) The frequencies of annexin V+ cells among Gr-1+ CD11b+ cells from the colon following stimulation with or without ATP-γS for 5 h. All data are from three independent experiments. **P < 0.01. (D) Flow cytometric dot plots (*Left*) and the frequencies of annexin V+ cells (*Right*) among Gr-1+ CD11b+ cells from the colonic lamina propria of wild-type or *P2rx4*−/− mice after their stimulation with or without ATP-γS for 5 h. All data are from four independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005; n.s., not significant. (E) Cytosolic Ca2+ level in Gr-1+ CD11b+ cells from the large-intestinal lamina propria of wild-type or *P2rx4*−/− mice. All data are representative of two independent experiments. (F) Flow cytometric dot plots (*Left*) and the frequencies of annexin V+ cells (*Right*) in Gr-1+ CD11b+ cells from the colons of BALB/c mice following treatment with or without ATP-γS in the presence or absence of BAPTA-AM for 5 h. All data are from four independent experiments. *P < 0.05; n.s., not significant.

Tani et al.
The ATP-hydrolyzing ectoenzyme E-NTPD8 attenuates colitis through modulation of P2X4 receptor–dependent metabolism in myeloid cells

PNAS | 5 of 10
https://doi.org/10.1073/pnas.2100594118

pathology, we generated $Entpd8^{-/-}$ $Rag2^{-/-}$ mice and administered 2.5% DSS to these mice for 5 d. Compared with $Rag2^{-/-}$ mice, $Entpd8^{-/-}$ $Rag2^{-/-}$ mice had higher DAI scores (*SI Appendix*, Fig. S4A) and higher numbers of neutrophils and DCs in the large-intestinal lamina propria (*SI Appendix*, Fig. S4B). We also depleted CD4$^+$ T cells in wild-type and $Entpd8^{-/-}$ mice using anti-CD4 antibody before and during DSS administration (*SI Appendix*, Fig. S4C). The numbers of CD4$^+$ T cells in the colonic lamina propria of both wild-type and $Entpd8^{-/-}$ mice were severely reduced by anti-CD4 antibody treatment (*SI Appendix*, Fig. S4D). However, DAI scores and histopathological scores of the colon remained higher in $Endpd8^{-/-}$ mice with anti-CD4 antibody injection compared with wild-type mice (*SI Appendix*, Fig. S4 *E* and *F*), indicating that CD4$^+$ T cells are dispensable for exaggeration of colitis in $Entpd8^{-/-}$ mice.

Previous studies have demonstrated that the depletion of neutrophils and monocytes by using anti–Gr-1 antibody reduces inflammatory manifestations in chemically induced colitis (20–22, 36). To define whether a promoted accumulation of neutrophils and DCs is involved in development of severe colitis in $Entpd8^{-/-}$ mice, anti–Gr-1 antibody or control IgG was intraperitoneally injected into mice (*SI Appendix*, Fig. S5). The $Entpd8^{-/-}$ mice injected with control IgG displayed more severe disease manifestations with more profound histopathology of the colon compared with similarly treated wild-type mice (*SI Appendix*, Fig. S5 *A* and *B*). However, the ablation of neutrophils and monocytes by treatment with anti–Gr-1 antibody mitigated the severity of colitis in $Entpd8^{-/-}$ mice, as shown by the lower DAI scores and less severe large-intestinal pathology (*SI Appendix*, Fig. S5 *A* and *B*). These results indicate that the hydrolysis of luminal ATP by E-NTPD8 prevents innate intestinal pathology.

The ATP–P2X4R Axis in Myeloid Cells Is Involved in the Exacerbation of Colitis in *Entpd8*$^{-/-}$ Mice. To determine the signaling pathway responsible for the induction of pathological innate immune responses in the colon of $Entpd8^{-/-}$ mice, we investigated the expression patterns of ATP receptors in intestinal epithelial cells (*SI Appendix*, Fig. S6A) and colonic myeloid cells (*SI Appendix*, Fig. S6B) by performing an RNA-seq analysis. Among P2 purinergic receptors, $P2rx4$, an ATP-gated Ca^{2+} channel, was expressed at a high level in both colonic epithelial cells and myeloid cells. Thus, we attempted to examine whether P2X4R signaling contributes to the development of severe colitis in $Entpd8^{-/-}$ mice by generating $Entpd8^{-/-}$ $P2rx4^{-/-}$ mice. Under homeostatic condition, the numbers of neutrophils, DCs, and Mφ in the large-intestinal lamina propria of $P2rx4^{-/-}$ mice were similar to those of wild-type mice (*SI Appendix*, Fig. S6C). In addition, the permeability of intestinal epithelial cells to fluorescein isothiocyanate-conjugated dextran was normal in $P2rx4^{-/-}$ mice (*SI Appendix*, Fig. S6D). During DSS administration, either the clinical parameters or histopathology of the colon in $P2rx4^{-/-}$ mice were similar to those in wild-type mice (Fig. 3 *A* and *B*). However, the introduction of a $P2rx4$ deficiency into $Entpd8^{-/-}$ mice lessened the level of disease activity and ameliorated the large-intestinal histopathology (Fig. 3 *A* and *B*). In this context, $Entpd8^{-/-}P2rx4^{-/-}$ mice showed lower levels of neutrophils and Mφ accumulation in the colon compared with $Entpd8^{-/-}$ mice (Fig. 3C), as well as partial reduction in the accumulation of DCs.

We further investigated whether P2X4R activation in either hematopoietic or nonhematopoietic cells contributes to the aggravation of colitis in $Entpd8^{-/-}$ mice. $Entpd8^{-/-}$ recipients were transplanted with $P2rx4^{+/+}$ or $P2rx4^{-/-}$ BM cells and then administered 3% DSS for 7 d (*SI Appendix*, Fig. S7). The DSS-administered $Entpd8^{-/-}$ recipients reconstituted with $P2rx4^{-/-}$ BM cells showed less severe bleeding and better stool consistency, as presented by their lower DAI scores, relative to DSS-administered mice reconstituted with $P2rx4^{+/+}$ BM cells (*SI Appendix*, Fig. S7A). Accordingly, the severity of colonic histopathology was alleviated in

$P2rx4^{-/-}$ BM cell recipients (*SI Appendix*, Fig. S7B). Moreover, the frequencies and numbers of neutrophils, DCs, and Mφ were drastically reduced in the colonic lamina propria of $P2rx4^{-/-}$ BM cell recipients (*SI Appendix*, Fig. S7C). These findings demonstrate that activation of P2X4R signaling pathway in hematopoietic cells is responsible for the exaggeration of colitis in $Entpd8^{-/-}$ mice. To assess the effect of microbial communities on P2X4R-mediated aggravation of colitis in $Entpd8^{-/-}$ mice, we analyzed the fecal microbiota composition (*SI Appendix*, Fig. S8). Results of 16S ribosomal RNA gene sequencing analysis revealed that the phylum-level fecal bacterial composition in $Entpd8^{-/-}$ mice was not changed compared with those in wild-type and $Entpd8^{-/-}$ $P2rx4^{-/-}$ mice, which indicates that development of severe innate intestinal pathology in $Entpd8^{-/-}$ mice is not due to dysbiosis.

Extracellular ATP Inhibits the Apoptosis of Colonic Gr-1$^+$ CD11b$^+$ Cells. In DSS-treated wild-type mice, the number of Ly6G$^+$ neutrophils with cleaved-caspase 3 was increased, indicating that many neutrophils undergo apoptosis during DSS treatment (Fig. 4A). However, the number of cleaved-caspase 3–positive neutrophils was barely detected in the colonic lamina propria of DSS-treated $Entpd8^{-/-}$ mice. In addition, the injection of anti-Gr1 antibody alleviated DSS-induced colitis in $Entpd8^{-/-}$ mice (*SI Appendix*, Fig. S5). Therefore, we analyzed the effects of extracellular ATP on the activities of Gr-1$^+$ CD11b$^+$ cells. To evaluate the effector functions, colonic Gr-1$^+$ CD11b$^+$ cells were cultured for 3 h in the presence or absence of ATP-γS, which is a nonhydrolysable ATP. ATP-γS treatment promoted ROS production in colonic Gr-1$^+$ CD11b$^+$ cells (Fig. 4B). We further analyzed the resistance to cell death of colonic Gr-1$^+$ CD11b$^+$ cells that had been stimulated with or without ATP-γS for 5 h by staining the cells with propidium iodide and annexin V. The frequency of annexin V–positive cells was lower in the ATP-γS–stimulated cells (Fig. 4C).

We then analyzed whether the effect of ATP-γS on colonic Gr1$^+$ CD11b$^+$ cells is dependent on P2X4R. An ATP-γS–dependent inhibition of apoptosis was not observed in $P2rx4^{-/-}$ Gr-1$^+$ CD11b$^+$ cells (Fig. 4D), which indicates that P2X4R signaling is responsible for prolonging the lifespan of colonic neutrophils. Previous studies have demonstrated that P2X4R is essential for sustaining the ATP-evoked Ca^{2+} signaling in Mφ (37), neutrophils (38), and T cells (39). We observed that the peak level of cytosolic Ca^{2+} was higher in ATP-γS–stimulated Gr-1$^+$ CD11b$^+$ cells from the colon of wild-type mice compared with those of $P2rx4^{-/-}$ mice (Fig. 4E). Furthermore, the increased Ca^{2+} level was sustained in wild-type cells but not $P2rx4^{-/-}$ cells. Thus, we investigated whether activation of Ca^{2+} signaling is required for the acquisition of resistance to cell death by colonic Gr-1$^+$ CD11b$^+$ cells following extracellular ATP stimulation. Loading colonic Gr-1$^+$ CD11b$^+$ cells with the Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) prevented the ATP-γS–induced reduction of apoptotic cell death in these cells (Fig. 4F). These results suggest that ATP-induced Ca^{2+} mobilization via P2X4R mediates prolonged survival of colonic Gr-1$^+$ CD11b$^+$ cells.

Extracellular ATP–Induced Promotion of Glycolysis Results in the Inhibition of Apoptosis in Colonic Neutrophils. Previous studies have demonstrated that the Ca^{2+} signaling promotes glycolytic metabolism in neutrophils (39, 40), which is linked to the reduction of apoptosis in these cells (40). Therefore, we examined whether extracellular ATP influences cellular metabolism in Gr-1$^+$ CD11b$^+$ cells. An RNA-seq analysis revealed that ATP-γS stimulation in colonic Gr-1$^+$ CD11b$^+$ cells up-regulated their expression of molecules involved in the glycolytic pathway (Fig. 5A). In agreement with those data, the level of maximum glycolysis following phorbol myristate acetate (PMA) stimulation, as determined by measuring extracellular acidification rates (ECAR), was increased in colonic Gr-1$^+$ CD11b$^+$ cells pretreated with ATP-γS compared with that

in untreated cells (*SI Appendix*, Fig. S9*A*). In addition, the maximum levels of the oxygen consumption rate (OCR) were augmented in ATP-γS–stimulated colonic Gr-1$^+$ CD11b$^+$ cells (*SI Appendix*, Fig. S9*A*). Gr-1$^+$ CD11b$^+$ cells are further divided into Ly6G$^+$ CD11b$^+$ neutrophils and Ly6C$^+$ CD11b$^+$ monocytes (41, 42). To define whether neutrophils or monocytes are sensitive to ATP, we isolated Ly6G$^+$ CD11b$^+$ neutrophils and Ly6C$^+$ CD11b$^+$ monocytes from the colonic lamina propria and analyzed their responses to ATP-γS (*SI Appendix*, Fig. S9*B*). Colonic Ly6G$^+$ CD11b$^+$ neutrophils exhibited ATP-γS–dependent elevation of the maximum ECAR and OCR (*SI Appendix*, Fig. S9*C*), but Ly6C$^+$ CD11b$^+$ monocytes did not (*SI Appendix*, Fig. S9*D*). ATP-γS–dependent up-regulation of the maximum ECAR and OCR in neutrophils was inhibited by BAPTA-AM treatment (*SI Appendix*, Fig. S9*E*). We next explored the abundance of glycolytic intermediate glucose-6-phosphate (G6P) in colonic neutrophils cultured in the presence of U-^{13}C$_6$ glucose by liquid chromatography-mass spectrometry (40) (Fig. 5 *A* and *B*). Consistent with the up-regulation of ECAR, relative abundances of Glu-6-P m+6, in which all six carbons are ^{13}C, and total Glu-6-P, which includes both unlabeled Glu-6-P and all mass isotopomers of Glu-6-P (m+1 to m+6), were increased in colonic neutrophils stimulated with ATP-γS. These findings indicate that extracellular ATP promotes neutrophil glycolysis in the colon through activation of the Ca^{2+} signaling. To address whether the extracellular ATP–mediated promotion of glycolysis contributes to the suppression of apoptosis, glycolytic activity was inhibited by 2 deoxy-D-glucose (2DG) in colonic Gr-1$^+$ CD11b$^+$ cells cultured in the presence or absence of ATP-γS, and the apoptosis rates of these cells were analyzed (*SI Appendix*, Fig. S10*A*). Treatment with 2DG abrogated the ATP-γS–induced decrease in the frequency of annexin-positive cells among Gr-1$^+$ CD11b$^+$ cells. The ATP-γS–dependent elevation of glycolytic pathway was more evident in Ly6G$^+$ CD11b$^+$ neutrophils than Ly6C$^+$ CD11b$^+$ monocytes (*SI Appendix*, Fig. S9 *C* and *D*). Accordingly, ATP-γS–dependent
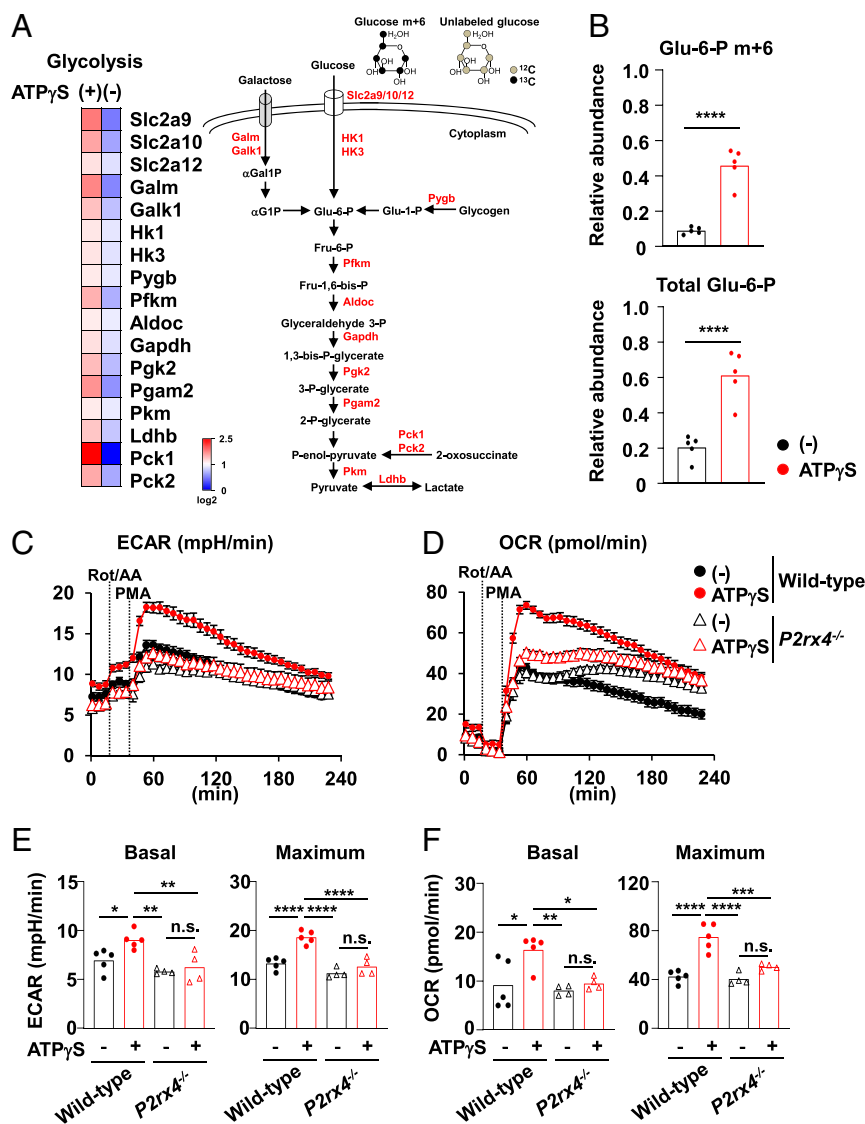
Fig. 5. Promotion of glycolysis in neutrophils through P2X4 receptor. (*A*) A heat map (*Left*) of the genes related with glycolysis in the Gr-1$^+$ CD11b$^+$ cells stimulated with or without ATP-γS for 2.5 h. Scheme of glycolytic pathway (*Right*). (*B*) Relative abundances of Glu-6-P m+6 and total Glu-6-P in colonic Ly6G$^+$ CD11b$^+$ neutrophils cultured in the presence of U-^{13}C$_6$ glucose with or without 100 μM ATP-γS for 3 h. Glu-6-P; glucose 6-phosphate. These data are pooled from five independent experiments. ****$P < 0.001$. (*C–F*) The ECAR (*C* and *E*) and OCR (*D* and *F*) in Gr-1$^+$ CD11b$^+$ cells from the colons of wild-type or *P2rx4*$^{-/-}$ mice following pretreatment with or without ATP-γS for 3 h. The bar graphs in *E* and *F* show the average values of basal and maximum ECAR (*E*) and OCR (*F*) from four wells. All data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$; n.s., not significant.

Tani et al.
The ATP-hydrolyzing ectoenzyme E-NTPD8 attenuates colitis through modulation of P2X4 receptor–dependent metabolism in myeloid cells

PNAS | 7 of 10
https://doi.org/10.1073/pnas.2100594118

suppression of apoptosis was observed in Ly6G^{+} CD11b^{+} neutrophils, but not in Ly6C^{+} CD11b^{+} monocytes (*SI Appendix*, Fig. S10B). These results suggest that an increased level of glycolysis is crucial for the inhibition of apoptosis by extracellular ATP in colonic neutrophils.

To investigate whether P2X4R provides the signal for promoting glycolysis, Gr-1^{+} CD11b^{+} cells from the colons of wild-type or *P2rx4*^{−/−} mice were stimulated with ATP-γS for 3 h, after which their ECAR and OCR were measured (Fig. 5 C–F). We observed ATP-γS–dependent elevations in the basal and maximum values of ECAR in wild-type Gr-1^{+} CD11b^{+} cells, whereas these changes were not induced in *P2rx4*^{−/−} cells (Fig. 5 C and E). In addition, higher basal and maximum OCR values were observed in ATP-γS–stimulated wild-type cells but not *P2rx4*^{−/−} cells compared with unstimulated cells (Fig. 5 D and F). These findings indicate that a P2X4R-induced Ca^{2+} influx is required for promotion of glycolysis in colonic neutrophils.

Discussion

In this study, we showed that E-NTPD8 in epithelial cells exerts an immunomodulatory function. The clearance of microbiota-derived ATP by E-NTPD8 is essential for inhibiting the prolonged survival of neutrophils and monocytes by discouraging the P2X4R-mediated promotion of glycolysis, thereby abrogating innate intestinal pathology.

Extracellular ATP released by intestinal commensals suppresses the production of IgA and down-regulates its affinity for commensal bacteria (43, 44). We found a lower frequency of IgA^{+} plasma cells among the CD45^{+} hematopoietic cells in the colon of *Entpd8*^{−/−} mice, as compared with wild-type mice ($7.97 \pm 0.86\%$ and $10.82 \pm 4.04\%$; $P = 0.031$) under steady state conditions. This finding indicates that E-NTPD8–mediated hydrolysis of ATP might be necessary for inhibiting the reduction of IgA^{+} plasma cells in the colon. Although we found a higher number of IL-17^{+} CD4^{+} T cells and lower number of IgA^{+} plasma cells in the colon of *Entpd8*^{−/−} mice compared with wild-type mice, more severe large-intestinal pathology was induced in *Entpd8*^{−/−} mice, even in mice lacking adaptive lymphocytes as a result of a *Rag2* gene deletion, which suggests that altered adaptive immune responses do not account for the pathological intestinal inflammation found in *Entpd8*^{−/−} mice at least during the acute phase of DSS-induced colitis.

Previous studies showed that augmentation of the intracellular Ca^{2+} level boosts glycolysis in T lymphocytes by inducing glycolytic enzyme expression through the activation of transcription factors, such as HIF1-α and NFAT (45, 46). Similarly, enhanced HIF1-α expression in neutrophils resulting from either a *Phd2* deficiency or hypoxia up-regulates glycolysis (40, 47), leading sustained neutrophil survival (40). In the present study, we demonstrated that ATP-γS induces the expression of glycolysis-related molecules in colonic Gr-1^{+} CD11b^{+} cells (Fig. 5A), suggesting that extracellular ATP-induced metabolic alteration toward glycolysis in colonic neutrophils might be initiated by transcriptional modification via the P2X4R-mediated activation of the Ca^{2+} signaling.

Several studies have demonstrated that a metabolic switch to glycolysis in neutrophils precipitates ROS production by up-regulating NADPH oxidase, which is required for pathogen clearance (48, 49). In accordance with the promoted glycolysis in ATP-γS–exposed neutrophils, ROS production was also elevated in these cells in the current study. Furthermore, a higher level of ROS production during DSS-induced colitis was observed in the colonic neutrophils from *Entpd8*^{−/−} mice than in those from wild-type mice. Thus, in *Entpd8*^{−/−} mice, the activation of glycolysis by microbiota-derived ATP may be linked to the enhancement of ROS production other than the inhibition of apoptosis in colonic neutrophils.

In this study, we showed that, in addition to *P2rx4*, colonic Gr-1^{+} CD11b^{+} cells also express *P2rx7* and *P2ry2*. *P2rx4*^{−/−} Gr-1^{+} CD11b^{+} cells stimulated with extracellular ATP showed transient elevation of intracellular Ca^{2+} level, which may depend on either

P2X7R or P2Y2R. However, the introduction of a *P2rx4* deficiency into *Entpd8*^{−/−} mice completely mitigated the myeloid cell-mediated exaggeration of colitis, which indicates that P2X4R is the most relevant P2 purinergic receptor for the acquisition of immunopathological phenotypes in colonic neutrophils in *Entpd8*^{−/−} mice. The present study found that colonic epithelial cells, likely myeloid cells, express *P2rx4*. In fecal microbiota of *P2rx4*^{−/−} mice, the relative abundance of *Bacteroidetes* was higher than those in wild-type mice, while the relative abundance of *Proteobacteria* was lower. These results suggest that epithelial cell P2X4R contributes to the maintenance of gut microbial community.

Collectively, our results demonstrate that regulation of luminal ATP by E-NTPD8 is required for the metabolic adaptation of myeloid cells in the colon, where microbiota constitutively secrete ATP, and that disruptions to this regulation lead to severe intestinal inflammation. Growing evidence suggests that targeting the metabolic reprograming occurring within immune cells has therapeutic potential for treating autoimmune diseases and inflammatory disorders (50). The anti-inflammatory drug DMF, which is used in the treatment of psoriasis and multiple sclerosis, down-regulates glycolysis in lymphocytes and myeloid cells via inactivation of the glycolytic enzyme GAPDH (30). Interestingly, DMF suppresses dinitrobenzene sulfuric acid–induced colitis by preventing neutrophil accumulation and activation (31). As in *Entpd8*^{−/−} mice, the clinical disease activity in UC patients is correlated with an elevated number of infiltrated neutrophils (16), which exhibit higher ROS production (51) and increased survival time (52–55). Although genome-wide association study did not indicate any UC-related mutations of the *ENTPD8* gene, there remains the possibility that neutrophil-mediated pathology in UC patients is associated with a reduced expression of *ENTPD8* resulting from the perturbation of epigenetic or posttranscriptional modification. Thus, it is important that future research investigates the expression of E-NTPD8 at the protein level in colonic epithelial cells as well as the fecal/luminal concentration of ATP in UC patients, which will provide insights for developing immunotherapeutic interventions targeting the modification of metabolic processes in colonic myeloid cells.

Materials and Methods

Detailed information on the materials, methods, and associated references can be found in *SI Appendix, SI Materials and Methods*.

Human Samples. Colon samples were obtained from five patients with UC and seven patients with colorectal cancer. Patient characteristics are provided in *SI Appendix*, Fig. S1B. The colon was washed in phosphate-buffered saline (PBS) to remove feces and placed in Hank's balanced salt solution (HBSS) containing 20 mM ethylenediaminetetraacetic acid (EDTA) and then incubated at room temperature. After 3 min, epithelial cells were peeled off from the colon and washed in PBS, after which they were isolated total RNA to analyzed expression of ATP-hydrolyzing enzymes. This study was approved by the Ethical Committee of Osaka University School of Medicine (10261-12). We obtained written informed consent from all patients to use their samples and data.

Generation of *Entpd8*^{−/−} Mice. To generate *Entpd8*^{−/−} mice, a targeted vector was constructed by replacing exons 4 to 9 of *Entpd8* with a neomycin-resistance gene cassette, and a gene encoding herpes simplex virus thymidine kinase driven by a phosphoglycerate kinase promoter was inserted into the genomic fragment for use in negative selection. After V6.5 embryonic stem cells were transfected with the targeted vector, the resulting colonies that were doubly resistant to G418 and ganciclovir were selected and screened with PCR and Southern blot analyses. Homologous recombinants were microinjected into blastocysts from female C57BL/6 mice, and the heterozygous F1 progeny were intercrossed to obtain *Entpd8*-deficient mice. The *Entpd8*-deficient mice and their wild-type and heterozygous littermates from these intercrosses were identified from the results of Southern blot, Northern blot, and real-time RT-PCR analyses and then used for subsequent experiments. The *Entpd8*-deficient mice were backcrossed to BALB/c mice for at least 10 generations, and the *Entpd8*-deficient mice and their wild-type

littermates from the intercrosses of heterozygous mice were used for the experiments.

Metabolic Analysis. The ECAR and OCR were measured with a XFe96 Extracellular Flux Analyzer (Agilent Technologies). Gr-1$^+$ CD11b$^+$, Ly6G$^+$ CD11b$^+$, or Ly6C$^+$ CD11b$^+$ cells were isolated from the colons of wild-type or *P2rx4*$^{-/-}$ mice that had been administered 3% DSS for 6 d. Cell-Tak–coated XF96 cell culture microplate was seeded with 1×10^5 cells that were then stimulated with or without 100 μM ATP-γS for 3 h. The culture medium was replaced with Agilent Seahorse XF Roswell Park Memorial Institute medium (Agilent Technologies) supplemented with 1 mM pyruvate (Agilent Technologies), 10 mM glucose (Agilent Technologies), and 2 mM L-glutamine (Agilent Technologies), after which the ECAR and OCR were analyzed under basal conditions and upon stimulation with 1 μg/mL PMA following treatment with 0.5 μM rotenone and antimycin A (Rot/AA).

RNA-seq Analysis. To isolate large and small-intestinal epithelial cells from C57BL/6J mice, murine intestines were opened longitudinally and washed with PBS to remove feces. The extracted intestines were placed in HBSS containing 5 mM EDTA and incubated at 37 °C for 20 min in a shaking water bath. After removing the intestinal tissues, the suspended epithelial cells were centrifuged at $780 \times g$ for 5 min at 4 °C and washed with PBS. To analyze the expression levels of ATP receptors, myeloid cells, such as CD64$^-$ DCs, CD64$^+$ Mφ, and Gr-1$^+$ CD11b$^+$ cells, were isolated from the colons of healthy BALB/c mice. Gr-1$^+$ CD11b$^+$ cells were isolated from BALB/c mice that had been administered 3% DSS for 6 d, and these cells were stimulated with 100 μM ATP-γS for 2.5 h to identify extracellular ATP-inducible genes. Total RNA was extracted from these cells with an RNeasy Mini kit (Qiagen) in accordance with the manufacture's protocol. Full-length complementary DNA was generated by using a SMART-Seq HT Kit (Takara Bio). An Illumina library was then prepared by using a Nextera DNA Library Preparation Kit (Illumina). Sequencing was performed on an Illumina HiSEq 2500 sequencer (Illumina) in 75-base single-end mode. Sequenced reads were mapped to the mouse reference genome sequence (mm10) with TopHat version 2.0.12. Fragments per kilobase of exons per million mapped fragments (FPKMs) were calculated using Cufflinks version 2.1.1. Among calculated genes with a normalized FPKM value of >1.0 in ATP-γS–stimulated Gr-1$^+$ CD11b$^+$ cells, 7,126 were up-regulated >1.3-fold from ATP-γS–stimulated Gr-1$^+$ CD11b$^+$ cells compared with untreated cells (Fig. 5*A*). A heat map of *Enpp1-7* and *Entpd1-8* (Fig. 1*A*) and graphs showing the expression levels of purinergic P2 receptors (*SI Appendix*, Fig. S6 *A* and *B*) were generated from the FPKM values.

Statistical Analysis. Differences between the control and experimental groups were evaluated by a two-tailed unpaired Student's *t* test or two-way ANOVA followed by Tukey's multiple comparisons test using GraphPad Prism 8.4.3 for Windows (GraphPad Software Inc.). Differences where $P < 0.05$ were considered statistically significant.

Data Availability. Gene data have been deposited in the National Center Biotechnology Information Gene Expression Omnibus (GSE160379, GSE160380, and GSE160381). All other study data are included in the article and/or *SI Appendix*.

1. Z. Zhang, H. Tang, P. Chen, H. Xie, Y. Tao, Demystifying the manipulation of host immunity, metabolism, and extraintestinal tumors by the gut microbiome. *Signal Transduct. Target. Ther.* **4**, 41 (2019).
2. A. Lavelle, H. Sokol, Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat. Rev. Gastroenterol. Hepatol.* **17**, 223–237 (2020).
3. H. Kayama, R. Okumura, K. Takeda, Interaction between the microbiota, epithelia, and immune cells in the intestine. *Annu. Rev. Immunol.* **38**, 23–48 (2020).
4. M. Levy, A. A. Kolodziejczyk, C. A. Thaiss, E. Elinav, Dysbiosis and the immune system. *Nat. Rev. Immunol.* **17**, 219–232 (2017).
5. H. Tilg, N. Zmora, T. E. Adolph, E. Elinav, The intestinal microbiota fuelling metabolic inflammation. *Nat. Rev. Immunol.* **20**, 40–54 (2020).
6. I. Hironaka *et al.*, Glucose triggers ATP secretion from bacteria in a growth-phase-dependent manner. *Appl. Environ. Microbiol.* **79**, 2328–2335 (2013).
7. T. Iwase *et al.*, Isolation and identification of ATP-secreting bacteria from mice and humans. *J. Clin. Microbiol.* **48**, 1949–1951 (2010).
8. R. Mempin *et al.*, Release of extracellular ATP by bacteria during growth. *BMC Microbiol.* **13**, 301 (2013).
9. F. Di Virgilio, D. Dal Ben, A. C. Sarti, A. L. Giuliani, S. Falzoni, The P2X7 receptor in infection and inflammation. *Immunity* **47**, 15–31 (2017).
10. Y. Furuta *et al.*, E-NPP3 controls plasmacytoid dendritic cell numbers in the small intestine. *PLoS One* **12**, e0172509 (2017).
11. T. Kusu *et al.*, Ecto-nucleoside triphosphate diphosphohydrolase 7 controls Th17 cell responses through regulation of luminal ATP in the small intestine. *J. Immunol.* **190**, 774–783 (2013).
12. T. Németh, M. Sperandio, A. Mócsai, Neutrophils as emerging therapeutic targets. *Nat. Rev. Drug Discov.* **19**, 253–275 (2020).
13. M. Honda, P. Kubes, Neutrophils and neutrophil extracellular traps in the liver and gastrointestinal system. *Nat. Rev. Gastroenterol. Hepatol.* **15**, 206–221 (2018).
14. V. Papayannopoulos, Neutrophil extracellular traps in immunity and disease. *Nat. Rev. Immunol.* **18**, 134–147 (2018).
15. C. Caër, M. J. Wick, Human intestinal mononuclear phagocytes in health and inflammatory bowel disease. *Front. Immunol.* **11**, 410 (2020).
16. A. Bressenot *et al.*, Comparing histological activity indexes in UC. *Gut* **64**, 1412–1418 (2015).
17. L. Rosenberg *et al.*, Histologic markers of inflammation in patients with ulcerative colitis in clinical remission. *Clin. Gastroenterol. Hepatol.* **11**, 991–996 (2013).
18. H. S. de Souza, C. Fiocchi, Immunopathogenesis of IBD: Current state of the art. *Nat. Rev. Gastroenterol. Hepatol.* **13**, 13–27 (2016).
19. E. Zigmond *et al.*, Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. *Immunity* **37**, 1076–1090 (2012).
20. J. Zhou *et al.*, Targeting EZH2 histone methyltransferase activity alleviates experimental intestinal inflammation. *Nat. Commun.* **10**, 2427 (2019).
21. Y. F. Yang *et al.*, Ficolin-A/2, acting as a new regulator of macrophage polarization, mediates the inflammatory response in experimental mouse colitis. *Immunology* **151**, 433–450 (2017).
22. X. Sun, B. Liu, R. B. Sartor, C. Jobin, Phosphatidylinositol 3-kinase-γ signaling promotes *Campylobacter jejuni*-induced colitis through neutrophil recruitment in mice. *J. Immunol.* **190**, 357–365 (2013).
23. C. C. Bain *et al.*, Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol.* **6**, 498–510 (2013).
24. A. Schippers *et al.*, β7-Integrin exacerbates experimental DSS-induced colitis in mice by directing inflammatory monocytes into the colon. *Mucosal Immunol.* **9**, 527–538 (2016).
25. L. Makowski, M. Chaib, J. C. Rathmell, Immunometabolism: From basic mechanisms to translation. *Immunol. Rev.* **295**, 5–14 (2020).
26. X. Li, G. Egervari, Y. Wang, S. L. Berger, Z. Lu, Regulation of chromatin and gene expression by metabolic enzymes and metabolites. *Nat. Rev. Mol. Cell Biol.* **19**, 563–578 (2018).
27. R. J. Kishton, M. Sukumar, N. P. Restifo, Metabolic regulation of T cell longevity and function in tumor immunotherapy. *Cell Metab.* **26**, 94–109 (2017).
28. E. Lachmandas *et al.*, Microbial stimulation of different Toll-like receptor signalling pathways induces diverse metabolic programmes in human monocytes. *Nat. Microbiol.* **2**, 16246 (2016).
29. M. Luu, A. Visekruna, Short-chain fatty acids: Bacterial messengers modulating the immunometabolism of T cells. *Eur. J. Immunol.* **49**, 842–848 (2019).
30. M. D. Kornberg *et al.*, Dimethyl fumarate targets GAPDH and aerobic glycolysis to modulate immunity. *Science* **360**, 449–453 (2018).
31. G. Casili *et al.*, Dimethyl fumarate reduces inflammatory responses in experimental colitis. *J. Crohn's Colitis* **10**, 472–483 (2016).
32. X. Liu *et al.*, Dimethyl fumarate ameliorates dextran sulfate sodium-induced murine experimental colitis by activating Nrf2 and suppressing NLRP3 inflammasome activation. *Biochem. Pharmacol.* **112**, 37–49 (2016).
33. M. Fausther *et al.*, Cloning, purification, and identification of the liver canalicular ecto-ATPase as NTPDase8. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292**, G785–G795 (2007).
34. S. C. Robson, J. Sévigny, H. Zimmermann, The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal.* **2**, 409–430 (2006).
35. K. Atarashi *et al.*, ATP drives lamina propria T(H)17 cell differentiation. *Nature* **455**, 808–812 (2008).
36. Y. Nemoto *et al.*, Negative feedback regulation of colitogenic CD4$^+$ T cells by increased granulopoiesis. *Inflamm. Bowel Dis.* **14**, 1491–1503 (2008).
37. J. A. Layhadi, J. Turner, D. Crossman, S. J. Fountain, ATP evokes Ca^{2+} responses and CXCL5 secretion via P2X$_4$ receptor activation in human monocyte-derived macrophages. *J. Immunol.* **200**, 1159–1168 (2018).
38. C. Ledderose *et al.*, Purinergic P2X4 receptors and mitochondrial ATP production regulate T cell migration. *J. Clin. Invest.* **128**, 3583–3594 (2018).
39. Y. Bao *et al.*, Mitochondria regulate neutrophil activation by generating ATP for autocrine purinergic signaling. *J. Biol. Chem.* **289**, 26794–26803 (2014).
40. P. Sadiku *et al.*, Prolyl hydroxylase 2 inactivation enhances glycogen storage and promotes excessive neutrophilic responses. *J. Clin. Invest.* **127**, 3407–3420 (2017).

Tani et al.
The ATP-hydrolyzing ectoenzyme E-NTPD8 attenuates colitis through modulation of P2X4 receptor–dependent metabolism in myeloid cells

PNAS | 9 of 10
https://doi.org/10.1073/pnas.2100594118

41. S. Jaillon *et al.*, Neutrophil diversity and plasticity in tumour progression and therapy. *Nat. Rev. Cancer* **20**, 485–503 (2020).

42. S. Gordon, P. R. Taylor, Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* **5**, 953–964 (2005).

43. M. Proietti *et al.*, ATP released by intestinal bacteria limits the generation of protective IgA against enteropathogens. *Nat. Commun.* **10**, 250 (2019).

44. L. Perruzza *et al.*, T follicular helper cells promote a beneficial gut ecosystem for host metabolic homeostasis by sensing microbiota-derived extracellular ATP. *Cell Rep.* **18**, 2566–2575 (2017).

45. S. Kahlfuss *et al.*, STIM1-mediated calcium influx controls antifungal immunity and the metabolic function of non-pathogenic Th17 cells. *EMBO Mol. Med.* **12**, e11592 (2020).

46. M. Vaeth *et al.*, Store-operated Ca^{2+} entry controls clonal expansion of T cells through metabolic reprogramming. *Immunity* **47**, 664–679.e6 (2017).

47. S. R. Walmsley *et al.*, Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J. Exp. Med.* **201**, 105–115 (2005).

48. G. Soto-Heredero, M. M. Gómez de Las Heras, E. Gabandé-Rodríguez, J. Oller, M. Mittelbrunn, Glycolysis–A key player in the inflammatory response. *FEBS J.* **287**, 3350–3369 (2020).

49. H. S. Jun, D. A. Weinstein, Y. M. Lee, B. C. Mansfield, J. Y. Chou, Molecular mechanisms of neutrophil dysfunction in glycogen storage disease type Ib. *Blood* **123**, 2843–2853 (2014).

50. E. M. Pålsson-McDermott, L. A. J. O'Neill, Targeting immunometabolism as an anti-inflammatory strategy. *Cell Res.* **30**, 300–314 (2020).

51. S. Sedghi *et al.*, Increased production of luminol enhanced chemiluminescence by the inflamed colonic mucosa in patients with ulcerative colitis. *Gut* **34**, 1191–1197 (1993).

52. M. Lampinen, P. Sangfelt, Y. Taha, M. Carlson, Accumulation, activation, and survival of neutrophils in ulcerative colitis: Regulation by locally produced factors in the colon and impact of steroid treatment. *Int. J. Colorectal Dis.* **23**, 939–946 (2008).

53. H. Hanai *et al.*, Leukocyte adsorptive apheresis for the treatment of active ulcerative colitis: A prospective, uncontrolled, pilot study. *Clin. Gastroenterol. Hepatol.* **1**, 28–35 (2003).

54. A. E. Brannigan *et al.*, Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. *Shock* **13**, 361–366 (2000).

55. D. A. McCarthy, D. S. Rampton, Y. C. Liu, Peripheral blood neutrophils in inflammatory bowel disease: Morphological evidence of in vivo activation in active disease. *Clin. Exp. Immunol.* **86**, 489–493 (1991).

10 of 10 | **PNAS**
https://doi.org/10.1073/pnas.2100594118

Tani et al.
The ATP-hydrolyzing ectoenzyme E-NTPD8 attenuates colitis through modulation of P2X4 receptor–dependent metabolism in myeloid cells