The UDP-glucose/P2Y14 receptor axis promotes eosinophil-dependent large intestinal inflammation

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Abstract

Ulcerative colitis (UC) is a chronic disorder of the large intestine with inflammation and ulceration. The incidence and prevalence of UC have been rapidly increasing worldwide, but its etiology remains unknown. In patients with UC, the accumulation of eosinophils in the large intestinal mucosa is associated with increased disease activity. However, the molecular mechanism underlying the promotion of intestinal eosinophilia in patients with UC remains poorly understood. Here, we show that uridine diphosphate (UDP)-glucose mediates the eosinophil-dependent promotion of colonic inflammation via the purinergic receptor P2Y14. The expression of *P2RY14* mRNA was upregulated in the large intestinal mucosa of patients with UC. The P2Y14 receptor ligand UDP-glucose was increased in the large intestinal tissue of mice administered dextran sodium sulfate (DSS). In addition, *P2ry14* deficiency and P2Y14 receptor blockade mitigated DSS-induced colitis. Among the large intestinal immune cells and epithelial cells, eosinophils highly expressed *P2ry14* mRNA. *P2ry14*$^{-/-}$ mice transplanted with wild-type bone marrow eosinophils developed more severe DSS-induced colitis compared with *P2ry14*$^{-/-}$ mice that received *P2ry14*-deficient eosinophils. UDP-glucose prolonged the lifespan of eosinophils and promoted gene transcription in the cells through P2Y14 receptor-mediated activation of ERK1/2 signaling. Thus, the UDP-glucose/P2Y14 receptor axis aggravates large intestinal inflammation by accelerating the accumulation and activation of eosinophils.

INTRODUCTION

Ulcerative colitis (UC), a clinical phenotype of inflammatory bowel diseases (IBD), is a chronic disorder of the large intestine with inflammation and ulceration(1). Although the etiology of UC remains unknown, accumulating evidence suggests that aberrant activation of immune responses causes the initiation of pathologic inflammation in the large intestine(2,3). In patients with UC, production of IL-5 and TNF-α is elevated in the mucosa of large intestine, which is accompanied with the increased number of colonic eosinophils (4,5). Clinical studies revealed that the abundance of eosinophils in the large intestinal mucosa is positively correlated with disease activity in patients with UC(6-8). Moreover, eosinophils induce dysfunction of the enteric neural system in patients with IBD(9). Accordingly, inhibition of eosinophil infiltration into the mucosa improved large intestinal pathology in several murine models of experimental colitis(10-13). These findings strongly suggest that eosinophils are implicated in the pathogenesis of UC. However, the molecular mechanism underlying the modulation of eosinophil activity in the gut remains poorly understood.

P2X purinergic receptors and P2Y G protein-coupled receptors are responsible for the induction of innate and adaptive immune responses(14,15). Increased intestinal luminal ATP caused by *Entpd8* deficiency promotes neutrophil accumulation through the activation of P2X4 receptor and thereby aggravates dextran sodium sulfate (DSS)-induced colitis(16). Autocrine ATP signaling in mast cells and basophils via P2X7 receptor causes the development of intestinal inflammation by facilitating the activation of these cells(17,18). Dysbiotic microbiota-derived lysophosphatidylserines exacerbate Th1-mediated intestinal pathology via P2Y10 receptor(19). These findings indicate that excessive activation of the P2X and P2Y receptors leads to pathological immune responses in the gut. The P2Y14 receptor interacts with uridine diphosphate (UDP)-glucose(14,15), and P2Y14 receptor signaling promotes IL-8 secretion from human endometrial epithelial cells and thereby induces neutrophil migration(20). In the

lung, UDP-glucose accelerates eosinophil chemotaxis via the P2Y14 receptor, which leads to the exacerbation of asthma(21). The P2Y14 receptor in hepatic stellate cells is required for the progression of liver fibrosis(22) and ischemic acute kidney injury(23). The P2Y14 receptor-mediated infiltration of macrophages in the liver leads to insulin resistance in mice fed a high-fat diet(24). In gouty arthritis, P2Y14 receptor signaling accelerates neutrophil extracellular trap-osis in neutrophils(25) and macrophage pyroptosis through the cAMP-dependent activation of NLRP3(26). Although the pathophysiological roles of the P2Y14 receptor were defined in several tissues, the function of the P2Y14 receptor in intestinal homeostasis and the regulation of intestinal pathology remain unknown.

In this study, we explored the immunomodulatory function of the purinergic receptor P2Y14 in the gut. *P2RY14* expression was upregulated at the site of inflammation in the large intestinal mucosa of patients with UC compared with the corresponding normal mucosa of patients with colorectal cancer. In the murine colon, the UDP-glucose concentration was elevated during DSS administration. *P2ry14* deficiency in eosinophils confers protection against DSS-induced colitis. UDP-glucose extended survival and upregulated gene expression in eosinophils through P2Y14 receptor-mediated activation of ERK1/2 signaling. Thus, the UDP-glucose/P2Y14 receptor axis is implicated in the pathogenesis of DSS-induced colitis through the modulation of eosinophil physiology.

Ribosomal Protein L32S

Mice

C57BL/6J, FVB/N, and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) or CLEA Japan (Tokyo, Japan). *Rag1*$^{-/-}$ mice (Strain #:032562) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). *P2ry14*$^{-/-}$ mice were generated using the CRISPR-Cas 9 system. *P2ry14*$^{-/-}$ mice (BALB/c background) were backcrossed to C57BL/6 mice for at least 10 generations. Eight- to twelve-week-old mice were used for the experiments. 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 (03-083).

Human samples

Normal sites of the colon were obtained from six patients with colorectal cancer (CRC) and inflamed sites of the colons were obtained from six patients with UC (Supplementary Table 1). UC was diagnosed in accordance with the established diagnostic criteria(27). These studies were approved by the Ethical Committees of Osaka University School of Medicine (approval number: 549 and 15435).

Reagents

Dextran sulfate sodium salt (DSS) (36–50 kDa) was purchased from MP Biomedicals (cat no: 160110, Kaysersberg, France). 7-AAD (cat no: 420404), TruStain FcX™ PLUS (anti-mouse CD16/32) (cat no: 156604), anti-CD45-Pacific Blue (cat no: 103126), anti-CD45-FITC (cat no: 103107), anti-CD45-APC (cat no: 103112), anti-CD3-PE/Cy7 (cat no: 100219), anti-CD19-PE (cat no: 115508), anti-B220-FITC (cat no: 103206), anti-CD11b-FITC (cat no: 101206), anti-CD11c-APC (cat no: 117309), anti-FcεRIα-APC (cat no: 134315), anti-c-kit-PE/Cy7 (cat no: 105813),

anti-CD3ε-FITC (cat no: 100306), anti-CD4-FITC (cat no: 100406), anti-CD317-PE (cat no: 127103), and anti-CD8α-FITC (cat no: 100706) were purchased from BioLegend (San Diego, CA, USA). Phosflow Fix Buffer I (cat no: 557870), Phosflow Perm Buffer III (cat no: 558050), anti-ERK1/2-Alexa Fluor 647 (pT202/pY204, cat no: 612593), anti-CD11b-PE/Cy7 (cat no: 552850), anti-CD64-PE (cat no: 558455), anti-Ly6G-FITC (cat no: 551460) and anti-Siglec-F-PE (cat no: 552126) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). P2Y14 receptor antagonist 4-[4-(4-Piperidinyl)phenyl]-7-[4-(trifluoromethyl)phenyl]-2-naphthalenecarboxylic acid (PPTN hydrochloride) (cat no: 4862/10) was purchased from Tocris Bioscience (Bristol, UK). UDP-α-D-Glucose (cat no: 15602) was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). ERK1/2 inhibitor U0126 (cat no: U120-1MG) and lipopolysaccharides O55:B5 (cat no: L4005) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MEBCYTO® Apoptosis Kit (Annexin V-FITC Kit) (cat no: 4700) was purchased from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). Murine GM-CSF (cat no: 315-03-20UG) and murine IL-5 (cat no: 215-15-25UG) were purchased from PEPROTECH (Rocky Hill, NJ, USA). Murine IL-33 (cat no: 3626-ML) and murine IFN-β (cat no: 8234-MB-010) were purchased from R&D Systems (Minneapolis, MN, USA).

Colitis induction

Acute colitis was induced in 8- to 12-week-old mice by administering DSS (36–50 kDa) in their drinking water. C57BL/6 background *P2ry14*$^{-/-}$ mice and their wild-type littermates were administered 1.5% DSS for 5-7 days. The disease activity index scores, which incorporate the weight loss score and the bleeding stool score, were used to evaluate the severity of DSS-induced colitis, as reported previously(28). In brief, (i) weight loss (0 points: none; 1 point: 1-5 % weight loss; 2 points: 5-10 % weight loss; 3 points: 10-20 % weight loss; 4 points: more than 20 % weight loss); (ii) bleeding (0

points: no bleeding; 2 points: slight bleeding; 4 points: gross bleeding). DAI scores were calculated as the total of these scores. In addition, to analyze changes in large intestinal pathology, the colons were collected from mice, fixed in 4% paraformaldehyde and then embedded in paraffin. Paraffin-embedded sections mounted on glass slides were used for hematoxylin and eosin (H&E) staining. Each section of the colon was evaluated using the inflammation scores, as described previously(29). Images of H&E staining were taken by using Biozero (BZ-X710, Keyence, Osaka, Japan).

Splenic $CD4^+$ cells isolated with the MACS® technology (Miltenyi Biotec). Naive $CD4^+$ T cells (5×10^5) were transferred into *Rag1*$^{-/-}$ mice (8 weeks old) by an intraperitoneal injection; 28 days later, the colons were collected and analyzed concentration of UDP-glucose.

Measurement of UDP-glucose by LC-MS/MS

To quantify the concentration of UDP-glucose, the colons were collected from C57BL/6 mice treated with or without 1.5% DSS for 7 days. These colons were washed with phosphate-buffered saline (PBS) and stored at -80 °C. Quantification of UDP-glucose was performed using an LCMS-8060 (Shimadzu, Kyoto, Japan) in the laboratory of Shimadzu Techno-Research, Inc. (Shimadzu, Kyoto, Japan).

Cell isolation from the large intestinal lamina propria

Myeloid cells were isolated from the large intestinal lamina propria using a previously described protocol(30) with slight modifications. In brief, the large intestines were opened longitudinally, washed with PBS to remove the feces, then placed in Hank's balanced salt solution with 5 mM EDTA and incubated at 37 °C for 15 min in a shaking water bath. After washing in PBS, the tissues were cut into small pieces and incubated in RPMI 1640 medium containing 1 mg/ml collagenase D (Roche, Basel, Switzerland), 40 μg/ml DNase I (Sigma Aldrich), 0.5 mg/ml dispase (Thermo Fisher Scientific, MA,

USA), and 4% fetal bovine serum (FBS) for 45 min at 37 °C in a shaking water bath. The digested large intestinal tissues were passed through a 40 μm-cell strainer, and the isolated cells were washed in PBS containing 2% FBS. The $7AAD^{-}CD45^{+}CD3^{+}$ T cells, $7AAD^{-}D45^{+}CD19^{+}B220^{+}$ B cells, $7AAD^{-}CD45^{+}CD11b^{-}CD11c^{+}$ dendritic cells, $7AAD^{-}CD45^{+}CD11b^{-}CD11c^{intermediate}B220^{+}BST2^{+}$ plasmacytoid dendritic cells, $7AAD^{-}CD45^{+}CD11b^{+}CD64^{+}$ macrophages, $7AAD^{-}CD45^{+}CD11b^{+}Ly6G^{+}$ neutrophils, $7AAD^{-}CD45^{+}CD11b^{+}$Siglec-$F^{+}$ eosinophils, and $7AAD^{-}CD45^{+}CD11b^{+}$c-$kit^{+}Fc\varepsilon RI\alpha^{+}CD3^{-}CD4^{-}CD8^{-}B220^{-}$ mast cells were purified from murine lamina propria cells using a FACS Aria flow cytometer (BD Biosciences). Furthermore, the $7AAD^{-}CD45^{+}Ly6G^{+}CD11b^{+}$ neutrophils, $7AAD^{-}CD45^{+}CD64^{+}$ $CD11b^{+}$ macrophage, $7AAD^{-}CD45^{+}CD11c^{+}CD11b^{-}$ dendritic cells, $7AAD^{-}CD45^{+}CD11b^{+}$c-$kit^{+}Fc\varepsilon RI\alpha^{+}CD3^{-}CD4^{-}CD8^{-}B220^{-}$ mast cells, and $7AAD^{-}CD45^{+}CD11b^{+}$Siglec-$F^{+}$ eosinophils were analyzed with a FACSCanto II flow cytometer (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR, USA). The instrumental compensation was set in each experiment using four-, five-, six-, or seven-color-stained samples.

Real-time RT-PCR

Total RNA was isolated using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and cDNA was generated using 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; WI, USA) using GoTaq qPCR Master Mix (Promega; Madison, Wisconsin, United States). The following primer sets were used: *P2ry14*: 5′-CCATGCAAAATGGAAGTCTG-3′ and 5′-CGGAAAGACTGGGTGTCTTC-3′, *Gapdh*: 5′-CCTCGTCCCGTAGACAAAATG-3′ and 5′-TCTCCACTTTGCCACTGCAA-3′, *Dusp6*: 5′-ATAGATACGCTCAGACCCGTG-3′ and

5′-ATCAGCAGAAGCCGTTCGTT-3′, *Ifit3*: 5′-AGACAGGGTGTGCAACCAGG-3′ and 5′-GGTGACCAGTCGACGAATTTCTGATTGATC-3′, *Cxcl16*: 5′-CCTTGTCTCTTGCGTTCTTCC-3′ and 5′-TCCAAAGTACCCTGCGGTATC-3′, *Ribosomal protein l32* (*Rpl32*): 5′-ATCAGGCACCAGTCAGACCGAT-3′ and 5′-GTTGCTCCCATAACCGATGTTGG-3′. 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).

scRNA-seq data analysis

To define the expression pattern of *P2RY14* mRNA in human colonic lamina propria cells, the public scRNA-seq dataset(31) was reanalyzed using Python (v.3.10.11) and Scanpy (v.1.9.3) software. In brief, the aligned transcriptome matrix data for immune cells were downloaded, then low-quality cells (defined as cells with fewer than 200 genes) and genes (defined as genes expressed in fewer than 3 cells) were removed. The cells were filtered based on the number of genes by count (less than 2250) and the percentage of mitochondrial reads (less than 15%). Following normalization and log transformation, principal component analysis, dimensionality reduction with uniform manifold approximation and projection (UMAP), and Leiden graph clustering were performed. Annotation was carried out manually using differentially expressed genes, and a bar chart of the cell compartment of each cluster was created with matplotlib (v.3.7.1). The mRNA expression levels of *P2RY14* and *SIGLEC8* were plotted onto the UMAP graph with Scanpy functions.

16S RNA analysis

Each library was prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide with a primer set 27Fmod/338R targeting the V1–V2 region of 16S rRNA genes. 301-bp paired end sequencing of the amplicons was performed on a MiSeq system (Illumina) using a MiSeq Reagent v3 600 cycle kit. The paired end

sequences obtained were merged, filtered, and denoised using DADA2. Taxonomic assignment was performed using QIIME2 feature-classifier plugin with the Greengenes 13_8 database. The QIIME2 pipeline, version 2020.2, was used as the bioinformatics environment for the processing of all relevant raw sequencing data (https://qiime2.org).

RNA-seq analysis

For mouse samples, total RNA was extracted from mouse colon using a RNeasy Micro Kit (Qiagen, Valencia, CA, USA). Full-length cDNA was prepared using a SMART-Seq HT Kit (Takara Bio, Japan) according to the manufacturer's instructions. According to the SMARTer kit instructions, an Illumina library was then prepared using a NexteraXT DNA Library Preparation Kit (Illumina). Sequencing was performed on NovaSeq 6000 platform in a 101-base single-end mode. Generated reads were mapped to the mouse (mm10) reference genome using TopHat v2.1.1 in combination with Bowtie2 ver. 2.2.8 and SAMtools ver. 0.1.18. Fragments per kilobase of exon per million mapped fragments (FPKMs) were calculated using Cuffdiff 2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/).

For human samples, total RNA was extracted from human colon using a RNeasy Mini Kit. RNA libraries were prepared using TruSeq stranded mRNA Library Prep Kit (Illumina). Sequencing was performed on NovaSeq 6000 platform in a 101-base single-end mode. Generated reads were mapped to the human (hg19) reference genome using TopHat v2.1.1. FPKMs were calculated using Cuffdiff 2.2.1. Heatmap of the expression levels of the P2X and P2Y receptors (Figure 1A) were generated from the FPKM values. In addition, heatmap of differentially expressed genes (Figure 4A) were generated by BioJupies (https://maayanlab.cloud/biojupies/).

Culture of bone marrow eosinophils

Eosinophils isolated from bone marrow were cultured in RPMI 1640 medium

containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM 2-mercaptoethanol in the presence of 2 ng/ml GM-CSF and 10 ng/ml IL-5 at 37 °C for 36 h to induce the expression of the P2Y14 receptor. The cells were then treated with 5μM U0126 treatment for 15 min, followed by stimulation with 10 μM UDP-glucose for 2 h, and the mRNA expression levels of *Dusp6*, *Ifit3*, *Cxcl16*, and *Rpl32* were analyzed.

Cytokine analysis

The concentrations of IL-5, IL-6, and TNF-α in the culture supernatants of the colon from mice administered 1.5% DSS for 5 days and untreated mice were determined by using Cytometric Bead Array (CBA) kit (BD Biosciences).

Measurement of pERK1/2 in eosinophils

Colonic lamina propria cells isolated from C57BL/6 mice were treated with or without 10 μM UDP-glucose. After 15 min, surface staining was performed with anti-CD45, CD11b, and Siglec-F antibodies and intracellular cytokine staining was performed with anti-mouse ERK1/2 (pT202/pY204)-Alexa Fluor 647 antibody (BD Biosciences). by using BD Phosflow Fix Buffer I and Perm Buffer III in accordance with the manufacturer's instructions.

Apoptosis assay

The lamina propria cells were isolated from the colon of wild-type or *P2ry14*$^{-/-}$ mice and cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM 2-mercaptoethanol at 37 °C for 2h. After which, the cells were stimulated with 10 μM UDP-glucose in the presence or absence of 5 μM U0126 (Sigma-Aldrich) or 10 μM PPTN (Tocris Bioscience). After 2 h, the cells were washed with PBS and stained with annexin V and propidium iodide. The frequencies of

$7AAD^{-}$annexin $V^{+}CD45^{+}CD11b^{+}$Siglec-F^{+} eosinophils were analyzed by flow cytometry.

Statistical analysis

Differences between the control and experimental groups were analyzed by performing a two-tailed unpaired Student's *t*-test or one-way ANOVA followed by a Tukey's multiple comparisons test with GraphPad Prism version 8.4.3 (GraphPad Software, La Jolla, CA, USA), except for the shotgun-seq analysis. Differences with a calculated *p*-value of <0.05 were considered statistically significant.

RESULTS

P2ry14 deficiency ameliorates DSS-induced colitis

Among P2X and P2Y receptors, *P2RY14* mRNA was highly expressed in inflamed sites of the large intestinal mucosa from Japanese patients with UC compared with those in normal sites of the mucosa from patients with colorectal cancer (Figure 1A and B). To evaluate the role of the P2Y14 receptor in the intestine, we generated *P2ry14*$^{-/-}$ mice using the CRISPR-Cas9 system (Supplementary Data 1A and B). We observed that the concentration of UDP-glucose as well as inflammatory cytokines, such as IL-5, IL-6, and TNF-α, was increased in the colon of C57BL/6J mice during DSS administration (Figure 2A and Supplementary Data 2A). The concentration of UDP-glucose was also elevated in the colon of a naive CD4^{+} T cell adoptive transfer model of colitis (Supplementary Data 2B). These findings indicate that production of UDP-glucose is promoted in the intestine during inflammation. To determine the impact of the P2Y14 receptor on the regulation of intestinal inflammation, wild-type and *P2ry14*$^{-/-}$ mice were administered DSS. Compared with wild-type mice, *P2ry14*$^{-/-}$ mice displayed less severe clinical symptoms, including body weight loss and bleeding, which are summarized by the disease activity index scores (Figure 2B). In addition, *P2ry14*$^{-/-}$ mice showed less severe large intestinal pathology at 7 days after the initiation of DSS administration (Figure 2C). The numbers of eosinophils, CD64^{+} macrophages, mast cells, and neutrophils were decreased in the colon of *P2ry14*$^{-/-}$ mice administered DSS for 5 days compared with those in wild-type mice (Figure 2D), whereas the numbers of these cells in the colon of *P2ry14*$^{-/-}$ mice were comparable to those of wild-type mice at steady state (Supplementary Data 3A). Under homeostatic conditions, *Burkholderiales* in the fecal microbiota was reduced in *P2ry14*$^{-/-}$ mice (wild-type: 1.458 ± 0.246%; *P2ry14*$^{-/-}$ mice: 0.895 ± 0.157%, $p = 0.0017$) (Supplementary Data 3B).

To further validate the colitogenic effect of the P2Y14 receptor, C57BL/6J mice were peritoneally injected P2Y14 receptor antagonist PPTN(21) during

DSS-induced colitis. P2Y14 receptor blockade by PPTN treatment led to a decreased level of disease activity and abrogated the large intestinal histopathology (Figure 2E and F). These findings indicate that activation of the P2Y14 receptor leads to the progression of DSS-induced colitis.

P2Y14 receptor signaling in eosinophils exacerbates colitis

To determine how P2Y14 receptor signaling regulates large intestinal pathology, we analyzed the expression of *P2ry14* mRNA in immune cells and epithelia cells from the large and small intestines of healthy C57BL6/J mice. Among these cell populations, eosinophils highly expressed *P2ry14* mRNA in both the large and small intestines (Figure 3A). scRNA-seq analysis data(31) showed that a cluster, including eosinophils ($SIGLEC8^+$ cells), basophils, and mast cells, expresses *P2RY14* mRNA in human colon biopsies (Figure 3B and Supplementary Data 4). Moreover, this population was increased in colon biopsies from patients with UC compared with healthy controls (Figure 3B). In addition to the colon, eosinophils in the lung, but not the bone marrow, spleen, or liver, expressed *P2ry14* mRNA in C57BL6/J mice (Figure 3C). To investigate what factor induces the expression of *P2ry14* mRNA, eosinophils isolated from bone marrow were stimulated with IL-5, IL-33, IFN-β, and lipopolysaccharide. Among these molecules, IL-5 elicited *P2ry14* transcription in bone marrow eosinophils (Figure 3D). Previous studies demonstrated that the promotion of eosinophil infiltration is implicated in the pathogenesis of DSS-induced colitis(10,12,13,32). Therefore, to determine whether P2Y14 receptor signaling in eosinophils contributes to the promotion of large intestinal pathology, $P2ry14^{-/-}$ mice were transplanted with eosinophils isolated from the bone marrow of wild-type or $P2ry14^{-/-}$ mice at 3 days after the initiation of DSS administration. Compared with $P2ry14^{-/-}$ mice transplanted with *P2ry14*-deficient eosinophils, $P2ry14^{-/-}$ mice that received wild-type eosinophils exhibited more severe disease manifestations (Figure 3E). Additionally, the transfer of wild-type eosinophils

was linked to more profound histopathology of the large intestine in *P2ry14*$^{-/-}$ mice administered DSS (Figure 3F). These results suggest that P2Y14 receptor signaling in eosinophils is involved in the aggravation of colitis during DSS administration independently of microbiota composition.

The UDP-glucose/P2Y14 receptor axis modulates eosinophil physiology through the activation of ERK1/2 signaling

To elucidate the mechanism by which P2Y14 receptor signaling activates eosinophils, we analyzed the gene expression profiles of eosinophils from the colon of wild-type and *P2ry14*$^{-/-}$ mice administered DSS. Gene set enrichment analysis within BioJupies based on the results of an RNA-seq analysis revealed that expression of molecules involved in the ERK1 and ERK2 cascade was upregulated in large intestinal eosinophils from wild-type mice compared with *P2ry14*$^{-/-}$ mice during DSS-induced colitis (Figure 4A and Supplementary Table 2). Therefore, we analyzed ERK1/2 phosphorylation in eosinophils derived from the colon of healthy C57BL6J mice. FACS analyses displayed that the level of ERK1/2 phosphorylation was elevated in colonic eosinophils stimulated with UDP-glucose (Figure 4B). Because we found P2Y14 receptor-dependent accumulation of eosinophils in the colon during inflammation, we investigated the impact of UDP-glucose-induced activation of the P2Y14 receptor on the survival of eosinophils. Colonic eosinophils were cultured in the presence or absence of UDP-glucose for 2 h and stained with annexin V and propidium iodide. The frequency of annexin V-positive cells was reduced in UDP-glucose-stimulated eosinophils (Figure 4C), whereas it was not observed in the cells pretreated with ERK1/2 pathway inhibitor U0126. In addition, UDP-glucose-mediated suppression of apoptosis was not evident in eosinophils in the presence of P2Y14 receptor antagonist PPTN (Figure 4D). Moreover, there was no difference in the frequency of annexin-positive cells between *P2ry14*-deficient colonic eosinophils treated with and without UDP-glucose (Figure 4E).

These results indicate that P2Y14 receptor signaling endows colonic eosinophils with an extended lifespan through activation of the ERK1/2 signaling pathway. Next, to examine whether the UDP-glucose/P2Y14 receptor axis is involved in the induction of gene transcription, eosinophils isolated from bone marrow were cultured in the presence of IL-5 and their survival factor GM-CSF(11) for 36 h to induce the expression of P2Y14 receptor, and were stimulated with UDP-glucose. The mRNA expression levels of *Dusp6*, *Ifit3*, and *Cxcl16*, which were decreased in colonic eosinophils from *P2ry14*$^{-/-}$ mice (Supplementary Table 2), were upregulated following UDP-glucose stimulation (Figure 4F). However, UDP-glucose-induced mRNA expression of *Dusp6*, *Ifit3*, and *Cxcl16* was suppressed by U0126 treatment. These findings demonstrate that activation of the P2Y14 receptor by UDP-glucose in eosinophils leads to modulation of their lifespan and gene transcription in an ERK1/2 signaling-dependent manner.

DISCUSSION

In this study, we showed that the P2Y14 receptor in eosinophils exerts an immunomodulatory effect in the colon. The UDP-glucose/P2Y14 receptor axis in eosinophils induces prolonged survival and enhances gene expression through the activation of ERK1/2 signaling, thereby exacerbating large intestinal pathology. Several studies have shown that the P2Y14 receptor is expressed in hematopoietic stem cells(33,34). A previous study demonstrated that the lack of *P2ry14* has no effect on stem cell physiology in steady state and influences senescence of the cells after chemotherapy, radiation stress, and serial bone marrow transplantation(33). In accordance with these results, the numbers of myeloid cells were normal in the colon of *P2ry14*$^{-/-}$ mice under homeostatic conditions. Colonic eosinophils from DSS-treated *P2ry14*$^{-/-}$ mice showed reduced expression of *Cxcl9*, *Cxcl16*, *Il12b*, *Gzma*, *Gzmb*, and *Gzmc* compared with those from DSS-treated wild-type mice (Supplementary Table 2), suggesting that UDP-glucose/P2Y14 receptor axis initiates expression of inflammatory mediators in eosinophils. A previous study exhibited that CCL11 (Eotaxin) derived from Ly6C^{high} CCR2^{+} monocytes and macrophages facilitates eosinophil infiltration in the colon during DSS-induced colitis(12). This suggests that UDP-glucose acts on eosinophils recruited into the colon in a CCL11-dependent manner(12,35,36), which induces production of inflammatory mediators and thereby exacerbates colitis. Accumulating evidence suggests that perturbations of stromal cell activity are involved in the pathogenesis of UC(37). We cannot exclude the possibility that perturbations of stromal cell homeostasis through the overactivation of the P2Y14 receptor causes exacerbation of DSS-induced colitis. In addition, there is a possibility that the UDP-glucose/P2Y14 receptor axis in stromal cells under homeostatic condition regulates microbiota composition because *P2ry14*$^{-/-}$ mice harbored dysbiotic microbiota without changes in myeloid cell composition (Supplementary Data 3A and B). Thus, it would be important to analyze the impact of *P2ry14* deficiency on stromal cell activity

in a future study to precisely understand the colitogenic effect of the P2Y14 receptor.

UDP-glucose was increased in the colon tissue of mice administered DSS compared with untreated mice. It would be interesting to investigate whether the level of UDP-glucose is increased at inflamed sites within the mucosa of patients with UC. Previous studies demonstrated that goblet-like Calu-3 cells and human bronchial epithelial cells secrete UDP-glucose together with mucin(38-40). In addition, injured cells(22,41) and apoptotic cells(40) were identified as the source of UDP-glucose. Therefore, UDP-glucose may be released by damaged cells and goblet cells as a damage-associated molecular pattern during intestinal inflammation.

Our results demonstrate that the P2Y14 receptor in eosinophils exerts a colitogenic function through ERK1/2 activation under inflammatory conditions. We showed that IL-5 upregulates the expression of *P2ry14* mRNA in bone marrow eosinophils, which might serve as a host defense during enteric bacterial infection. IL-5 production is enhanced in the intestinal mucosa of patients with UC(4,42-44), like DSS-treated mice (Supplementary Data 2A), which might be associated with the promoted accumulation of *P2RY14*-expressing eosinophils in the mucosa of patients with UC. A recent study identified the heterogeneity of eosinophils in the murine colon, such as active eosinophils with a regulatory function, which are recruited in an IL-33 and IFN-γ-dependent manner, and basal eosinophils(45). Interestingly, active eosinophils with high levels of CD80 and PD-L1 expression were reduced in number at inflamed sites within the mucosa of patients with UC(45). In a mouse model of chronic intestinal inflammation, depletion of eosinophils using an anti-IL-5 antibody or an anti-GM-CSF antibody improved intestinal pathology(11). In the treatment of eosinophilic asthma, anti-IL-5 neutralizing antibodies are widely used(46). In the current study, we revealed that blockade of the interaction of the P2Y14 receptor by UDP-glucose treatment mitigates eosinophil-mediated colitis. As UC is a multifactorial disorder related to an increased risk of colorectal cancer and fibrosis(1,47), there is a

need to develop an optimized and personalized approach to facilitate long-lasting remission. Based on our data, the P2Y14 receptor as well as IL-5 could be therapeutic targets for eosinophilic UC. Moreover, further elucidation of the diverse roles of eosinophils in tissue repair and the induction of an anti-inflammatory response or pathogenic inflammatory response may uncover putative diagnostic biomarkers and potential targets for therapeutic interventions for UC.

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Author contributions:

L.L., T.I., B.L., H.T., K.T., and H.K. designed and performed the experiments, analyzed data, and revised the manuscript. H. M. and T.O. supervised the human study and analyzed data. D.O., D.M., and S.N. designed the experiments, analyzed data, and supervised the RNA-seq analysis and the 16S rRNA analysis. K.T. and H.K. supervised the study and wrote the manuscript.

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Data and materials availability:

RNA-seq data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE242641 and GSE242644 for Figure 1 and Figure 4A, respectively).

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Figure Legends

Figure 1: Expression patterns of the P2X and P2Y receptors in the human colon

(A) A heatmap of the gene encoding the P2X and P2Y receptors at normal sites of the colon from colorectal cancer patients (n = 6) or inflamed sites of the colon from patients with ulcerative colitis (UC) (n = 6). **(B)** The graphs show the FPKM values of *P2RY14* mRNA for normal sites of the colon from colorectal cancer patients (n = 6) or inflamed sites of the colon from patients with ulcerative colitis (UC) (n = 6) (mean ± SD). *$p <$ 0.05.

Figure 2: *P2ry14* deficiency attenuates the aggravation of DSS-induced colitis

(A) Concentration of UDP-glucose in the colon of C57BL/6 mice treated with or without 1.5% DSS for 7 days (mean ± SD). **$p < 0.01$. **(B)** Disease activity index scores of wild-type mice (n = 12) and *P2ry14*$^{-/-}$ mice (n = 12). All data are from three independent experiments (mean ± SD). *$p < 0.05$, ****$p < 0.001$. **(C)** Left: Representative distal colon sections. Right: Histological scores of the colon from wild-type mice (n = 12) and *P2ry14*$^{-/-}$ mice (n = 12). All data are from three independent experiments (mean ± SD). *$p < 0.05$. **(D)** Cell numbers of eosinophils, macrophages, mast cells, neutrophils, and dendritic cells from the colon of wild-type and *P2ry14*$^{-/-}$ mice administered DSS for 5 days. All data are from two independent experiments (mean ± SD). **$p < 0.01$, n.s., not significant. **(E)** Disease activity index scores of wild-type mice treated with vehicle (n = 6) or PPTN (10 mg/kg (body weight)) (n = 6) during DSS administration (mean ± SD). ****$p < 0.001$. **(F)** Left: Representative distal colon sections. Right: Histological scores of the colon from mice treated with or without PPTN during DSS administration (mean ± SD). *p < 0.05.

Figure 3: P2Y14 receptor signaling in eosinophils is implicated in the pathogenesis of colitis

(A) mRNA expression of *P2ry14* in the indicated cells from the mouse colon. **(B)** UMAP plot of FACS-sorted $CD45^+$ cells from the human colon. Data(31) from healthy controls and patients with ulcerative colitis were merged. Left: Expression patterns of *P2RY14* mRNA and *SIGLEC8* mRNA in human colonic $CD45^+$ cells. Middle: UMAP plot of FACS-sorted $CD45^+$ cells from the human colon colored by cluster. Right: The frequency of each subpopulation. HC, healthy control; UCn, non-inflamed sites of the colon from patients with ulcerative colitis; UCi, inflamed sites of the colon from patients with ulcerative colitis. Eos, eosinophil; Baso, basophil; Mast, mast cell. **(C)** *P2ry14* mRNA expression in eosinophils isolated from the bone marrow, lung, colon, spleen, and liver of C57BL/6 mice. Data are pooled from three independent experiments (mean ± SD). $^{**}p < 0.01$, $^{***}p < 0.005$. **(D)** *P2ry14* mRNA expression in bone marrow-derived eosinophils stimulated with 10 ng/ml IL-5, 10 ng/ml IL-33, 25 ng/ml IFN-β, or 100 ng/ml LPS for 36 h. Date are pooled from three independent experiments (mean ± SD). $^{**}p < 0.01$, n.s., not significant. **(E)** Disease activity index scores of *P2ry14*$^{-/-}$ mice who received 4×10^5 bone marrow eosinophils from wild-type mice (n = 8) or *P2ry14*$^{-/-}$ mice (n = 7) during DSS administration. Data are pooled from three independent experiments (mean ± SD). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.001$. **(F)** Left: Representative distal colon sections. Right: Histological scores. Data are pooled from three independent experiments (mean ± SD). $^{*}p < 0.05$.

Figure 4: UDP-glucose/P2Y14 receptor axis modulates life span and gene expression in eosinophils through promotion of the ERK1/2 signaling pathway

(A) Left: A heat map of differentially expressed genes in colonic eosinophils from wild-type and *P2ry14*$^{-/-}$ mice administered DSS for 5 days. Right: Enriched gene ontology (GO) pathway in eosinophils from the colon of wild-type mice administered DSS for 5 days. **(B)** Left: Histogram of pERK1/2 (pT202/pY204) in colonic eosinophils treated with or without UDP-glucose for 15min. Right: Graphs show the average mean

fluorescence intensity of pERK1/2 in eosinophils (mean ± SD). Data are pooled from four independent experiments. ***$p < 0.005$. **(C)** Flow cytometric pseudo-color plots (left) and the frequency of annexin V^+ cells in colonic eosinophils stimulated with UDP-glucose for 2 h following U0126 treatment for 15 min (right). Data are from four independent experiments (mean ± SD). *$p < 0.05$, n.s., not significant. **(D)** Flow cytometric pseudo-color plots (left) and the frequency of annexin V^+ cells in colonic eosinophils stimulated with UDP-glucose for 2h in the presence or absence of PPTN (right). Data are from four independent experiments (mean ± SD). *$p < 0.05$, n.s., not significant. **(E)** Flowcytometric pseudo-color plots (left) and the frequency of annexin V^+ cells in eosinophils from the colon of wild-type and *P2ry14*$^{-/-}$ mice following UDP-glucose stimulation for 2h (right). Data are from four independent experiments (mean ± SD). *$p < 0.05$, n.s., not significant. **(F)** mRNA expression of the indicated genes in eosinophils cultured in the presence of UDP-glucose with or without U0126.

Supplementary Data Legends

Supplementary Data 1: Generation of *P2ry14*$^{-/-}$ mice

(A) Scheme of the Cas9/gRNA-targeting sites around the second exon of the *P2ry14* gene. Upper: Maps of the wild-type *Otud3* locus. Black boxes, coding exons; white boxes, noncoding exons. Lower: The sequence of *Otud3* wild-type allele. Light blue, genotyping forward primer sequence; yellow and green, sgRNA targeting sequences; pink, genotyping reverse primer sequence; gray, sequence of the deleted region in *P2ry14*$^{-/-}$ mice. **(B)** PCR analysis of genomic tail DNA using the primer sets shown in (A).

Supplementary Data 2: Increased production of cytokines and UDP-glucose during large intestinal inflammation

(A) The colons of C57BL/6 mice treated with or without 1.5% DSS for 5 days were cultured for 24 h and concentrations of IL-5, IL-6, and TNF-α in the colonic explant supernatants were analyzed (mean ± SD). *$p < 0.05$, ***$p < 0.005$. **(B)** Concentration of UDP-glucose in the colon of *Rag1*$^{-/-}$ mice transferred naive CD4^{+} T cells (n = 3) and untreated *Rag1*$^{-/-}$ mice (n = 3) (mean ± SD). ***$p < 0.005$**.**

Supplementary Data 3: The numbers of myeloid cells in the colon under homeostatic conditions

(A) Cell numbers of eosinophils, macrophages, mast cells, neutrophils, and dendritic cells in healthy wild-type mice (n = 6) and *P2ry14*$^{-/-}$ mice (n = 5). Data are pooled from two independent experiments (mean ± SD). n.s., not significant. **(B)** The average frequencies of the relative abundance of bacteria at the order level as determined by fecal DNA sequencing of 16S ribosomal DNA in healthy wild-type (n = 6) and *P2ry14*$^{-/-}$ (n = 5) mice. n.s., not significant.

Supplementary Data 4: P2RY14 mRNA is expressed in cluster of Eosinophil/Basophil/Mast cell, but not neutrophil, among human colonic CD45^{+} cells.

(A and B) Expression patterns of marker genes of the indicated cells in human colonic CD45^{+} cells.

Supplementary Table 1: Characteristics of the individuals participating in RNA-seq analysis

Supplementary Table 2: The list of upregulated genes in eosinophils from the large intestine of wild-type mice compared with *P2ry14*$^{-/-}$ mice