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Supplemental Information

Microbiota-Sourced Purines Support Wound Healing and Mucous Barrier Function

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SUPPLEMENTAL FIGURE

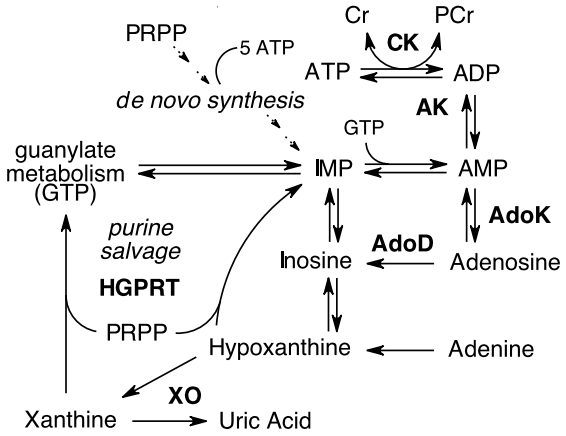

Figure S1. Related to Figures 1F and 2G. The purine metabolic pathway.

Graphical summary of the purine metabolic pathway. IMP, inosine monophosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cr, creatine; PCr, phosphocreatine; PRPP, phosphoribosyl pyrophosphate; GTP, guanosine triphosphate; XO, xanthine oxidase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; AK, adenylate kinase; CK, creatine kinase; AdoK, adenosine kinase; AdoD, adenosine deaminase.

TRANSPARENT METHODS

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Ki-67 Monoclonal	Thermo Fisher Scientific	Cat#14-5698-82; RRID: AB_10854564
BiP/GRP78/HSPA5	Novus Biologicals	Cat#NBP1-06277; RRID: AB_1556186
E. coli	Novus Biologicals	Cat#NB200-579; RRID: AB_10002133
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat#A-11034; AB_2576217
Goat anti-Rat IgG (H+L) Cross Adsorbed Secondary Antobody, Alex Fluor 555	Thermo Fisher Scientific	Cat#A-21434; RRID: AB_2535855
Chemicals, Peptides, and Recombinant Proteins		
Acetonitrile (HPLC)	Fisher Scientific	Cat#A998; CAS: 75-05-8
Methanol (HPLC)	Fisher Scientific	Cat#A452; CAS: 67-56-1
Methanol, Absolute – Acetone free	Millipore Sigma	Cat#M1775; CAS: 67-56-1
Potassium phosphate monobasic for HPLC, LiChropur	Millipore Sigma	Cat#57618; CAS: 7778-77-0
Tetrabutylammonium bisulfate	Millipore Sigma	Cat#86868; CAS: 32503-27-8
Critical Commercial Assays		
TUNEL Assay Kit – HRP-DAB	Abcam	Cat#ab206386

Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat#00664; RRID: IMSR_JAX:000664
Oligonucleotides		
E. coli rpsL mutagenic oligo: G*T*C*A*GACGAACACGGCATACTTTACGCAGCGCG GAGTTCGGTTTTCTAGGAGTGGTAGTATATACACGA GTACATACGCCACGTTTTTGC	This Paper	N/A
Recombinant DNA		
pORTMAGE-3	addgene	Cat#72678; RRID: Addgene_72678
Software and Algorithms		
Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/; RRID: SCR_002798
Agilent OpenLab	Agilent	https://www.agilent.com/en/products/software-informatics/openlab-software-suite#0
Zen2	Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen.html

Experimental Model Details

Vertebrate Animal Use

The University of Colorado Anschutz Medical Campus (AMC) animal management program is accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC) and meets the National Institutes of Health standards as set forth in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23). The institution also accepts as mandatory the PHS Policy on Human Care and Use of Laboratory Animals by Awardee Institutions and NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.

Murine Hypoxanthine Enemas

Male 15 – 16-week-old mice were depleted of purine producing bacteria by administration of streptomycin in the drinking water (5 g/L) for 3 days. Mice were anesthetized using 3% isoflurane by inhalation dispensed from an anesthesia machine and administered 100 μL of sterile phosphate buffered saline (PBS, pH 7.4) as a control or sterile PBS with 1 mM hypoxanthine by enema ~18 and 5 h before sacrifice. A distal piece of colon tissue (~15 mg) was placed in 500 μL of 80% methanol, flash frozen by immersion in liquid nitrogen, and stored at -80 °C until processed for HPLC metabolite analysis.

Murine K12 Colonizations

Bacterial Preparation for Oral Gavage: A glycerol stock of the streptomycin resistant K12 strain used for the colonization experiments was streaked on LB agarose plates containing streptomycin (50 μg/mL) and grown at 37 °C overnight. A single colony was used to inoculate 2 mL of streptomycin containing LB broth, and the culture grown overnight at 37 °C with shaking. A 1.5 mL aliquot of the culture was centrifuged at 3000 g for 5 min to pellet the bacteria, the supernatant removed, and the bacteria then resuspended in 1.5 ml of PBS in preparation for oral gavage.

Germ-free Mouse Monocolonization: Germ-free mice were provided by the University of Colorado AMC gnotobiotic core. Female 7 – 8 week old gnotobiotic mice were administered 100 μL of the K12 bacterial preparation by oral gavage and allowed one week for colonization and complete turnover of the colonic epithelium, as described previously (Alexeev et al., 2018). Mice subjected to dextran sodium sulfate (DSS)-induced colitis were administered 3% DSS in drinking water for 5 days and sacrificed on day 7. K12 colonization was verified before and after DSS treatment by streaking fecal extracts on MacConkey agarose plates containing streptomycin (50 μg/mL) and bacteria grown at 37 °C overnight. A distal segment of colon tissue (~15 mg) was placed in 500 μL of 80% methanol, flash frozen by immersion in liquid nitrogen, and stored at -80 °C until processed for HPLC metabolite analysis. Fecal pellets were also flash frozen and similarly stored until HPLC analysis.

Conventionally raised Mouse Purine Depletion and K12 Reconstitution: Female 8-week-old C57BL/6J mice were ordered from The Jackson Laboratory and adapted to the University of Colorado AMC animal facility for one week. Mice receiving streptomycin were administered the antibiotic in the drinking water (5 g/L), and those being colonized with K12 were done so the next day by administration of 100 μL of the K12 bacterial preparation by oral gavage. All mice were then equilibrated to the treatments for one week to allow for complete turnover of the colonic epithelium. Mice receiving streptomycin were maintained on the antibiotic throughout all experimentation. Mice submitted to dextran sodium sulfate (DSS)-induced colitis were administered 3% DSS in drinking water for 5 days and sacrificed on day 7. K12 colonization was verified before and after DSS treatment by streaking fecal extracts on MacConkey agarose plates containing streptomycin and bacteria grown at 37 °C overnight. A piece of colon tissue encompassing the most distal fecal pellet was excised and fixed in methacarn (60% absolute methanol, 30% chloroform, 10% glacial acetic acid, v/v) for histology, as described previously (Kuhn et al., 2018). A distal piece of colon tissue (~15 mg) was placed in 500 μL of 80% methanol, flash frozen by immersion in liquid nitrogen, and stored at -80 °C until processed for HPLC metabolite analysis.

Method Details

Streptomycin-resistant K12 Preparation

The streptomycin-resistant *rpsL* R86S *E. coli* mutant strain was generated through a single cycle of multiplex automated genome engineering (MAGE) using the pORTMAGE-3 plasmid. pORTMAGE-3 was a gift from Csaba Pál. The BW25113 parent strain was transformed with the pORTMAGE-3 plasmid and MAGE recombineering was performed as described previously (Nyerges et al., 2016). The *E. coli* rpsL mutagenic oligonucleotide was synthesized with four phosphorothioate linkages at the 5′ terminus (Wang et al., 2012). The *rpsL* R86S mutants were selected through plating on LB agar with kanamycin and streptomycin. Curing of the pORTMAGE-3 plasmid was accomplished through growth at 37 °C. Verification that the mutant colonies had lost the pORTMAGE-3 plasmid was performed through replicate plating on single and dual selection LB agar.

HPLC Analyses

Extracellular Fecal Metabolites: Fecal pellets were removed from -80 °C and dispersed in 500 μL of ice-cold Milli-Q (mq) water. All extractions were performed on ice. The sample was vortexed for ~10 s and then centrifuged at 18,000 g and 4 °C for 10 min. The supernatant was then transferred to a new tube, another 500 μL of mq water added to the fecal matter, and the matter resuspended. The extraction process was repeated two more times, producing a total of 1.5 mL of extract. The extract was dried using and Eppendorf Vacufuge at room temperature. The dried extract was dissolved in 400 μL of mobile phase A (details below), filtered through a Vivaspin 5,000 MWCO PES column (Sartorius Stedim Biotech), and submitted to HPLC analysis.

Colon Tissue Metabolites: Colon tissue metabolites extracted as previously described with minor variations (Lee et al., 2018). Approximately ~15 mg of whole, distal colon was quickly placed in 500 μl of 80% MeOH and flash-frozen under liquid nitrogen to quench metabolic activity. The frozen tissue was stored at −80 °C, if necessary, before continuing on with the metabolite extraction procedure. All extractions were performed on ice. For metabolite extraction, the tissue was first sonicated 3 × 10 s (BioLogics Inc., 150 V/T Ultrasonic Homogenizer, power output ~20). The sample was then placed in

liquid nitrogen until frozen, removed and thawed, and vortexed for ~10 s. The sample was centrifuged for 10 min at 18,000 g and 4 °C, and the supernatant transferred to a new Eppendorf tube. Another 500 µl of 80% MeOH was then added to the sample, the tissue resuspended, and the extraction process repeated twice more. The resulting 1.5 ml of extract was dried via an Eppendorf Vacufuge at room temperature. The dried extract was dissolved in 400 µl of mobile phase A (details below) and filtered (Whatman Puradisc 4, 0.45 µm, nylon) into vials for HPLC injection.

HPLC Protocol: Metabolites were analyzed by HPLC as previously described with minor variations (Lee et al., 2018). Analyses were performed on an Agilent Technologies 1260 Infinity HPLC using a Phenomenex Luna 3 µm C18(2) column (100 Å, 150 × 4.6 mm) (mobile phase A, 50 mM KH_2PO_4, 5 mM tetrabutylammonium bisulfate, pH 6.25; mobile phase B, acetonitrile; column temperature, 30 °C; flow rate, 1 ml/min). Chromatographic separation of the metabolites was performed using a combination of isocratic and gradient methods, including column washing and equilibration periods at the end (0 min, 100% A; 7 min, 100% A; 10 min, 97% A; 18 min, 97% A; 45 min, 86% A; 60 min, 50% A; 80 min, 50% A; 90 min, 100% A; 135 min, 100% A). The metabolites were detected by absorption at wavelengths of 210, 254, and 280 nm, with their absorbance spectra and retention times verified by co-injection with authentic standards. Metabolites were quantitated from calibration curves developed from the injection of standards ranging from 100 nM to 5 mM.

Histological Analyses

Paraffin embedding of tissue and slide preparation (blank and H&E) was performed by the University of Colorado AMC Department of Pathology histology laboratory. Slides were deparaffinized through a series of washes: xylene, 2 × 3 min; xylene:ethanol::1:1, 3 min; ethanol, 2 × 3 min; 95% ethanol, 3 min; 70% ethanol, 3 min; 50% ethanol, 3 min; rinse in cold tap water.

Immunofluorescence: Deparaffinized slides were placed in Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) for heat-induced epitope retrieval (HIER) (Biocare Medical Decloaking Chamber, DC2008US). HIER was performed at 80 °C for 1 h (Ki67) or 125 °C for 30 s (BiP) and the slides then equilibrated to room temperature. The slides were washed 2 × 5 min in TBS wash buffer (50 mM Tris, 150 mM NaCl, 0.025% Triton X-100, pH 7.6), the tissue permeabilized by immersion in TBS wash buffer containing 0.2% Triton X-100 for 8 min, then the slides washed again 2 × 5 min in TBS wash buffer. No HIER or permeabilization was performed for the *E. coli* immunofluorescent analyses, and the washes were performed in TBS only. The tissue was blocked using 10% normal goat serum and 1% bovine serum albumin (BSA) in Tris buffered saline (TBS, 50 mM Tris, 150mM NaCL, pH 7.6) for 2 h at room temperature. No HIER or permeabilization was performed on slides for *E. coli* immunofluorescence, and washes were performed in TBS only. Primary antibody in TBS containing 1% BSA (Ki67, 1:100; BiP, 1:50; *E. coli*, 1:10) was then added to the tissue and the slides incubated overnight at 4 °C. The slides were then washed 2 × 5 min in TBS wash buffer and secondary antibody added (1:500 in 1% BSA TBS) and incubated for 1 h at room temperature. Then the slides were rinsed 3 × 5 min in TBS, a coverslip added using ProLong Diamond Mountant with DAPI, and the slides cured for 24 h. Fluorescent images of the tissue were taken using a Zeiss AxioCam MRc 5 at 100X or 200X magnification. Multiple, non-overlapping images of each tissue section from each mouse group (n = 3 – 5) were taken, and all images used for quantification. Images were processed and quantitated using the Zeiss Zen2 program. Immunofluorescence was quantified by drawing appropriate regions of interest for the target being analyzed and normalized to DAPI (target/DAPI, fluorescence sum/sum).

Mucosal Depth: Measurements (n = 21 - 24) of the mucosa depth were determined from representative images (n = 7 – 8) of the H&E stains that span the tissue sections from each mouse cohort (n = 4 – 5). Images were taken using a Zeiss AxioCam MRc 5 at 200X magnification, and measurements performed using the Zeiss Zen2 program.

TUNEL Staining: An Abcam TUNEL HRP-DAB Assay Kit (ab206386) was purchased and applied to deparaffinized slides obtained from each mouse cohort (n = 4) as per the manufacturer instructions. The slides were dehydrated by soaking in 70%, 95%, and 100% ethanol for 3 min each, cleared in xylenes for 3 min, and then mounted with coverslip using Cytoseal 60. The number of TUNEL positive cells in each colon cross section was counted and divided by the mucosal depth to normalize.

Mucin Staining: Deparaffinized slides were placed in Alcian Blue solution (1% in 3% acetic acid, pH 2.5) for 30 min. The slides were then washed in deionized water for 2 min, counterstained with nuclear fast red for 5 min, and washed for 1 min in deionized water. The slides were dehydrated by soaking in 70%, 95%, and 100% ethanol for 3 min each, cleared in xylenes for 3 min, and then mounted with coverslip using Cytoseal 60. Multiple, non-overlapping images of each tissue section from each mouse group (n = 5) were taken using a Zeiss AxioCam MRc 5 at 100X magnification, as described previously (Campbell et al., 2014).

Inner Mucus Layer Depth Measurements: The sterile, inner mucus region of the epithelia was measured through *E. coli* immunofluorescence analyses on colonic tissue sections, as described in the past (Kuhn et al., 2018). Representative images across each tissue section were taken using a Zeiss AxioCam MRc 5 at 100X magnification from each mouse group (n = 5). Multiple measurements distributed across each image (n = 5) from the surface of the epithelium to the luminal *E. coli* were performed to determine the depth of the inner mucus layer.

Statistical Analyses

Statistical analyses were performed in GraphPad Prism 8 using an unpaired two-tailed parametric t-test for direct comparisons. Statistical differences were considered significant when $p \leq 0.05$.

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