Supplementary Material

Methods

Fecal supernatants

Each participant provided fecal samples that were used for fecal supernatant preparation, as previously described.[1] Briefly, fecal material was diluted with PBS (1 g fecal sample/5 mL PBS), homogenized on ice, and centrifuged (10,000 *g*, 10 min) at 4°C. Supernatants were recovered, filtered with 0.22-μm syringe filters to remove bacteria, and stored at −80°C.

Murine colonic organoid culture

Murine colonic crypt isolation and culture were conducted according to the manufacturer's suggested method (https://www.stemcell.com/intestinal-epithelial-organoid-culture-with-intesticult-organoid-growth-medium-mouse-lp.html). In brief, 2 cm colonic tissue was washed with PBS containing 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA). After 3–5 washes, tissue was incubated in cold PBS containing 20 mM EDTA (Lonza, Walkersville, MD) with 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 5 min to remove mucus and in cold PBS containing 20 mM EDTA for 30 min for further digestion. Crypts were released by 1–2 min rhythmic shaking in PBS containing 0.1% BSA. Isolated crypts were centrifuged (300 *g*, 2 min), collected, and plated in 24-well plates with Matrigel (Corning Life Sciences, Corning, NY) polymerized for 15 min at 37°C. Finally, 0.5 mL IntestiCult Organoid Growth Medium (STEMCELL Technologies, Cambridge, MA) was added to the wells. Media were fully exchanged 3 times per week. The organoid cultures were passaged at a split ratio of 1:3 every week to avoid excessive debris accumulation within the organoid lumen. Passages 4–8 were used for this study.

Mast cell culture and reconstitution

Selective reconstitution of mast cells in mast cell–deficient W/Wv mice was conducted according to the method described by Rijnierse.[2] Bone marrow–derived mast cells were obtained from wild-type (C57B16). Bone marrow was aseptically flushed from femurs and cultured for 4 wk in RPMI 1640 medium containing 10% FCS, 4 mM L-glutamine, 0.5 μM 2-ME, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM nonessential amino acids. Recombinant mouse IL-3 (10 ng/mL, #PMC0034, Life Technologies, Rockford, IL) and stem cell factor (SCF/mouse kit ligand; 10 ng/mL; #TP723398, OriGene Technologies, Rockville, MD) were added to the medium to stimulate bone marrow cell development to mature mast cells. Medium was refreshed once a week and fresh IL-3 and stem cell factor were added. Purity of the mast cell population was determined by staining cells with toluidine blue, which indicated that nearly 99% of the viable cells were mast cells after the 4-wk culture. Mast cell–deficient W/Wv mice were injected via the tail vein with 5×10^6 cultured mast cells and the recipients were studied 4–5 weeks later.

Colorectal distension and electromyography recording

The visceromotor responses (VMR) were recorded by quantifying reflex contractions of the abdominal musculature induced by colorectal distension (CRD) The animals were anesthetized with a mixture of xylazine and ketamine (13 and 87 mg/kg body wt, respectively) as described previously.[3] Electromyography electrodes were implanted into the external oblique pelvic muscles 2–3 days before the beginning of the experimental procedures. The skin was sutured over the strain gauge, and the lead wires were looped around the animal's flank and secured with

a single suture in the skin. During the experiment, the strain gauge was connected by way of a shielded cable to a chart recorder to monitor the number of abdominal muscle contractions. A Fogarty catheter balloon (#12TLW804F, Edwards Lifesciences, Irvine, CA) lubricated with glycerol was inserted into the colon through the rectum 30 min before CRD was initiated. Fecal supernatants from healthy controls or IBS-D patients at a volume of 0.3 mL were administered intracolonically, 30 mm proximal to the rectum, 18 h before a series of rectal distensions were performed to generate a pressure-response curve. The responses were considered stable if there was less than 20% variability between two consecutive trials of CRD. The results of electromyography were amplified and filtered (A-M Systems, Sequim, WA), digitized, and integrated by using the SPIKE2/CED 1401 data acquisition interface (Cambridge Electronic Design, Cambridge, UK). Spike bursts higher than 0.3 mV were regarded as significant and were, therefore, used to estimate the response. The increase in the area under the curve of EMG amplitude during CRD over the baseline period before CRD was recorded as the response. The VMR change from the baseline response in each mouse was defined as ΔVMR.

Chemicals

To assess the effects of mast cell degranulation, mast cell degranulation inhibitor cromolyn sodium (30 mg/kg, intraperitoneal [IP], Sigma-Aldrich, St Louis, MO);[4] H1 receptor inhibitor and mast cell degranulation inhibitor ketotifen (1 mg/kg, IP); serine protease inhibitor nafamostat mesylate (10 mg/kg, R&D Systems),[5] H1 inhibitor ebastine (10 mg/kg, Sigma Aldrich), PF04418948, a specific EP2 receptor antagonist (10 mg/kg, R&D systems),[6] or 5-HT3 antagonist ondansetron (5 mg/kg, Sigma Aldrich) were injected intraperitoneally 30 min before intracolonic administration of healthy controls or IBS-D colonic fecal supernatant. PAR2 agonist SLIGKV-

NH2 (100 µg/mouse, R&D Systems, Minneapolis, MN, USA), histamine (300 µg/mouse, R&D Systems), PGE2 (100 µg/kg, Cayman Chemical), and LPS (50 µg/mouse, 0111B:4, phenol extraction, Sigma Aldrich) were administrated intracolonically. For in vitro studies, PGE2 (10 nM/well), SLIGKV-NH2 (100 µM/well), histamine (10 µM/well), LPS (3 or 10 µg/mL), indomethacin, a nonselective COX-1 and COX-2 inhibitor (50 µM/well, Sigma Aldrich) or celecoxib, a specific Cox2 inhibitor (50 µM/well, R&D Systems),[7] were administered to the culture media.

Mediator release from purified colonic mast cells

Purified colonic mast cells ($2–5 \times 10^4$ mast cells/tube) suspended in 3 mL RPMI 1640 media were stimulated with LPS (0, 3, or 10 µg/mL) for 4 h at 37°C. Cell pellets and supernatants were harvested, stored at −70°C and assayed in duplicate for PGE2 (supernatants diluted 1:10) according to manufacturer's instructions.

Mediator release from purified colonic tissue

Colonic specimens were collected and weighed 24 hours after intracolonic injection of FS. The tissues were rapidly immersed in hard plastic tubes containing 1 mL Dulbecco's PBS media and continuously oxygenated (95% O_2/5% CO_2) at 37°C. After a 30-minute incubation, the bathing solution was removed, filtrated, and stored at –80°C. ELISA assays of PGE2 (500141, Cayman Chemical) and histamine (589651, Cayman Chemical) were performed according to the instructions provided by the manufacturer.

Western blot analysis

Colonic samples were homogenized in ice-cold lysis buffer. The homogenate was centrifuged at 14 000 *g* for 10 min. Protein samples were run on Ready Gel 10% Tris-HCl (Bio-Rad, Hercules, CA) for 1.5 h at 80 V. Proteins were transferred to PVDF membranes for 1.5 h at 80 V. The membranes were blocked with TBS supplemented with 0.5% dry milk (Bio-Rad) for 1 h at room temperature, probed with primary antibodies against SERT (Thermo Fisher Scientific, 702076) at 1:500 dilution, TPH1 (Thermo Fisher Scientific, PA1-777) at 1:500 dilution or GAPDH antibody (SC-25778, Santa Cruz Biotechnology) at 1:2000 dilution at 4°C overnight, and then washed in TBS for 1 h. The membranes were probed with corresponding HRP-conjugated secondary antibodies at 1:5000 dilution for 1 h at room temperature and the bands were visualized by electrochemiluminescence (PerkinElmer, Waltham, MA). Signals were quantified using ImageJ (National Institutes of Health, Bethesda, MD) and normalized to controls.

Reverse transcriptase–PCR studies

Total RNA extraction. Gene expressions of SERT, TPH1 and COX-2 were measured by RT-PCR. Colonic tissue samples were lysed in reverse transcriptase buffer. Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA). cDNAs were synthesized with using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using 1 μg of total RNAs; the resultant cDNAs were used for PCR. PCR was performed with a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Green detection.; The PCR conditions were as follows: one cycle at 95°C for 10 min, followed by 40 two-temperature cycles at 95°C for 10 s and 60°C for 30 s. PCR amplifications were performed in a total volume

of 20 μL, containing iQSYBR Green supermix (Bio-Rad, Hercules, CA). Primer sets targeting various mRNAs are listed in Table 1. The housekeeping gene GAPDH served as an internal control.

Immunohistochemistry

Colonoids were fixed in 10% formalin for 2 hours at 22°C, and then in 25% sucrose in PBS (0.1 mol/L) overnight at 4°C. Colonoids were frozen embedded in Tissue-Tek O.C.T., sectioned with a cryostat at 6-μm thickness, and subsequently immunofluorescence stained. After blocking with 5% Donkey serum in PBS with 0.3% Triton X-100, sections were incubated with rabbit monoclonal primary antibodies against SERT (Thermo Fisher Scientific, 702076) at 1:200 dilution and goat polyclonal primary antibody against E-cadherin (R&D, AF748) at 1:200 dilution. Alexa Fluor 488-conjugated Donkey anti-Rabbit IgG (H+L) secondary antibody (1:500; Molecular Probes, Life Sciences Solutions, A-21206) and Cyanine Cy3-conjugated donkey anti-goat antibody (1:500; Jackson ImmunoResearch, West Grove, PA, AB_2340411) were used as secondary antibodies.

Fecal supernatant and plasma preparation for LPS measurement.

Fecal samples were collected and stored at –80°C. Based on our recent studies, fecal samples were diluted (1 g fecal sample/5 mL PBS), homogenized on ice, and centrifuged (10,000*g*, 10 minutes, 4°C). The supernatants were recovered, filtered on 0.22 μm filters to remove bacteria, and then stored at –80°C. Blood was collected by intracardiac puncture and plasma separated by centrifugation (3000 g for 15 min at room temperature). LPS levels were measured with a

quantitative chromogenic limulus amoebocyte lysate (LAL) QCL-1000 test kit (Lonza), following the manufacturer's protocols.

References

1. Zhou SY, Gillilland M, 3rd, Wu X, et al. FODMAP diet modulates visceral nociception by lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. J Clin Invest 2018;128:267-280.

2. Rijnierse A, Koster AS, Nijkamp FP, et al. Critical role for mast cells in the pathogenesis of 2,4-dinitrobenzene-induced murine colonic hypersensitivity reaction. *J Immunol*. 2006;176(7):4375–4384.

3. Grabauskas G, Heldsinger A, Wu X, et al. Diabetic visceral hypersensitivity is associated with activation of mitogen-activated kinase in rat dorsal root ganglia. *Diabetes*. 2011;60(6):1743–1751.

4. Carroll SY, O'Mahony SM, Grenham S, et al. Disodium cromoglycate reverses colonic visceral hypersensitivity and influences colonic ion transport in a stress-sensitive rat strain. *PLoS One*. 2013;8:e84718.

5. Mori S, Itoh Y, Shinohata R, et al. Nafamostat mesilate is an extremely potent inhibitor of human tryptase. *J Pharmacol Sci*. 2003;92(4):420–423.

6. af Forselles KJ, Root J, Clarke T, et al. In vitro and in vivo characterization of PF-04418948, a novel, potent and selective prostaglandin EP_2 receptor antagonist. *Br J Pharmacol*. 2011;164(7):1847–1856.

7. Mestre T, Péllisier T, Fialip J, et al. A method to perform direct transcutaneous intrathecal injection in rats. *J Phamacol Toxicol Methods*. 1994:32(4):197–200.

Supplementary Table 1. Primer sets targeting various mRNAs

Gene	Primer assay ID
GAPDH	Mm.PT.39a.1
SERT	Mm.PT.58.43910045
TPH1	Mm.PT.58.8815029
COX2	Mm.PT.58.9154407
MAOA	Mm.PT.58.8802827
Chga	Mm.PT.58.41352258
Tpsab1	Mm.PT.58.28396863
Tpsb2	Mm.PT.58.31550141.g
Tlr4	Mm.PT.58.41643680
TNF	Mm.PT.58.12575861

PrimeTime qPCR assays were used for RT-PCR analysis (Integrated DNA Technologies, Coralville, Iowa). The housekeeping gene GAPDH served as an internal control.

Supplementary Figure 1. (**A**) 5-HT level in platelet-poor serum in mice 18 h after intracolonic infusion of FS from HC or IBS-D patients, with or without pretreatment with cromolyn sodium (administered 30 min before FS infusion, IP). Results are expressed as mean ± SEM, one-way ANOVA, n = 10-16 in each group with duplicated measurements. (**B**) RT–PCR of SERT in distal colon from mice treated with intracolonic infusion of PBS or fecal supernatant from HC. Results are expressed as mean ± SEM, Student *t* test, *n* = 4–7 in each group.

Supplementary Figure 2. Release of 5-HT from distal colonic mucosa in mice 18 h after intracolonic administration of FS from HC or IBS-D patients, with or without pretreatment of cromolyn sodium or PF-04418948 (administered 30 min before FS infusion, IP). Results are

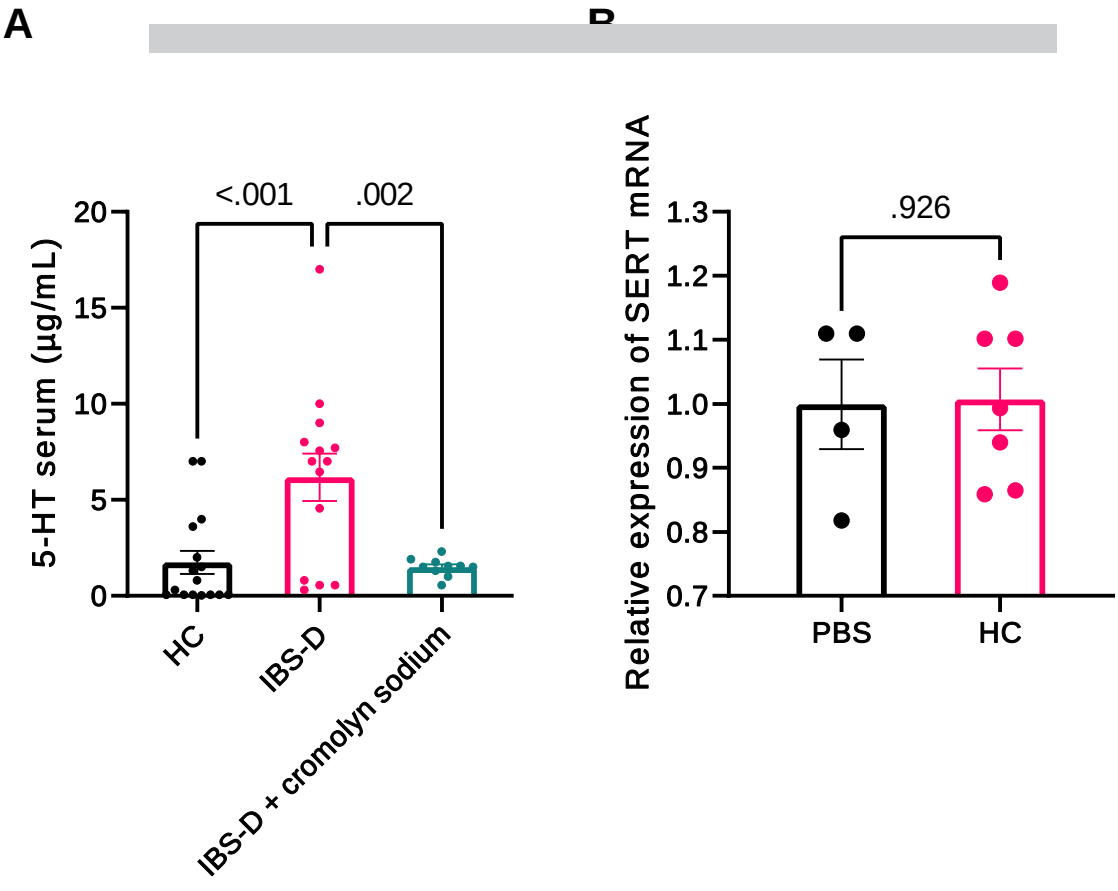
expressed as mean ± SEM, one-way ANOVA, *n* = 8-10 in each group with duplicated measurements.

Supplementary Figure 3. Platelet-poor serum 5-HT level in mice treated with intracolonic infusion of PBS or PGE2 (100 µg/kg) for 18 h. Results are expressed as mean ± SEM, from control, Student *t* test, n = 8–12 in each group with duplicated measurements.

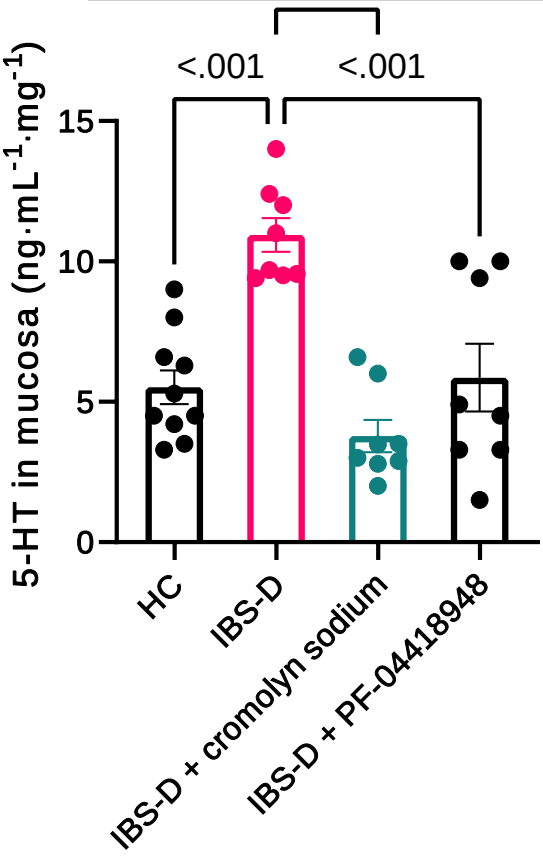
Supplementary Figure 4. (**A**) SERT in distal colon from mice treated with intracolonic infusion of PBS or LPS (50 µg/mouse). Results are expressed as mean ± SEM, n = 4–6 in each group, Student *t* test. (**B**) SERT expression in colonic mucosa of mice treated with HC FS, IBS-D FS, or IBS-D FS pretreated with heat (95°C for 5 min), soybean trypsin inhibitor (STI, 2.5 mg/mL), or bacterial protease inhibitor cocktail (BPIC, 0.1 mL/mL). Results are expressed as mean ± SEM, one-way ANOVA, n = 3–4 in each group.

Supplementary Figure 5. (**A**) Intracolonic injection of IBS-D FS caused mast cell activation as evidenced by increased mucosal levels of mast cell mediators such as histamine and PGE2. Results are expressed as mean ± SEM, from control, Student *t* test, n = 6 in each group. (**B**) Tryptase-1 (Tpsab1) and Tryptase-2 (Tpsb2) expression in colonic mucosa of mice treated with PBS, HC FS, and IBS-D FS. Results are expressed as mean ± SEM, one-way ANOVA, n = 6 in each group. (**C**) Higher LPS levels in IBS-D patients' fecal samples and the plasma of IBS-D FS injected mice. Results are expressed as mean ± SEM, from HC, Student *t* test, n = 6 in each group. (**D**) TLR4 expression in colonic mucosa of mice treated with PBS, HC FS, and IBS-D FS. Results are expressed as mean ± SEM, one-way ANOVA, n = 8 in each group. (E) TNF-α (TNF)

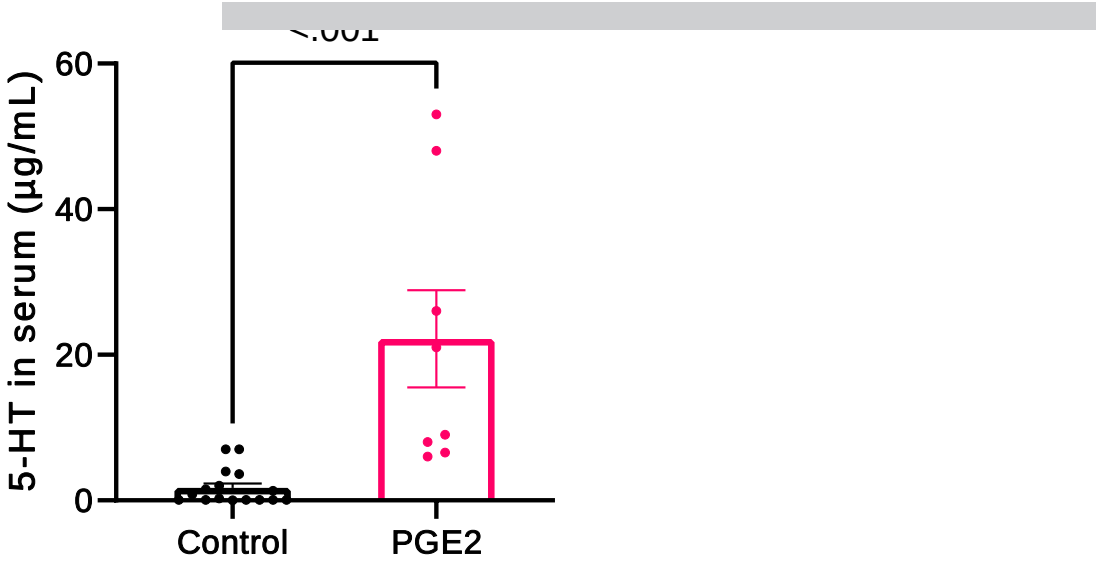
expression in colonic mucosa of mice treated with PBS, HC FS, and IBS-D FS. Results are expressed as mean ± SEM, one-way ANOVA, n = 6 in each group. (**F**) Protease activity in HC FS and IBS-D FS. Results are expressed as mean ± SEM, from HC FS, Student *t* test, n = 6 in each group.



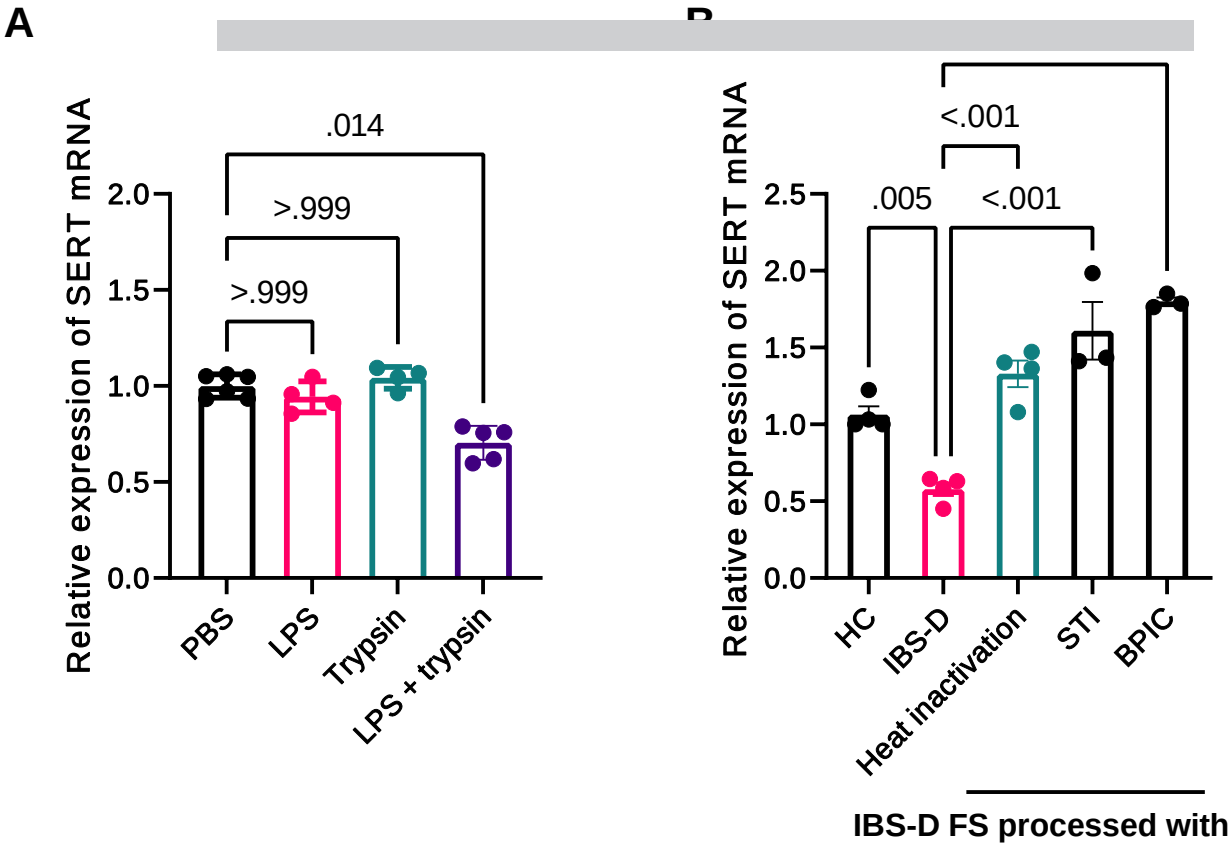
Supplementary Figure 1



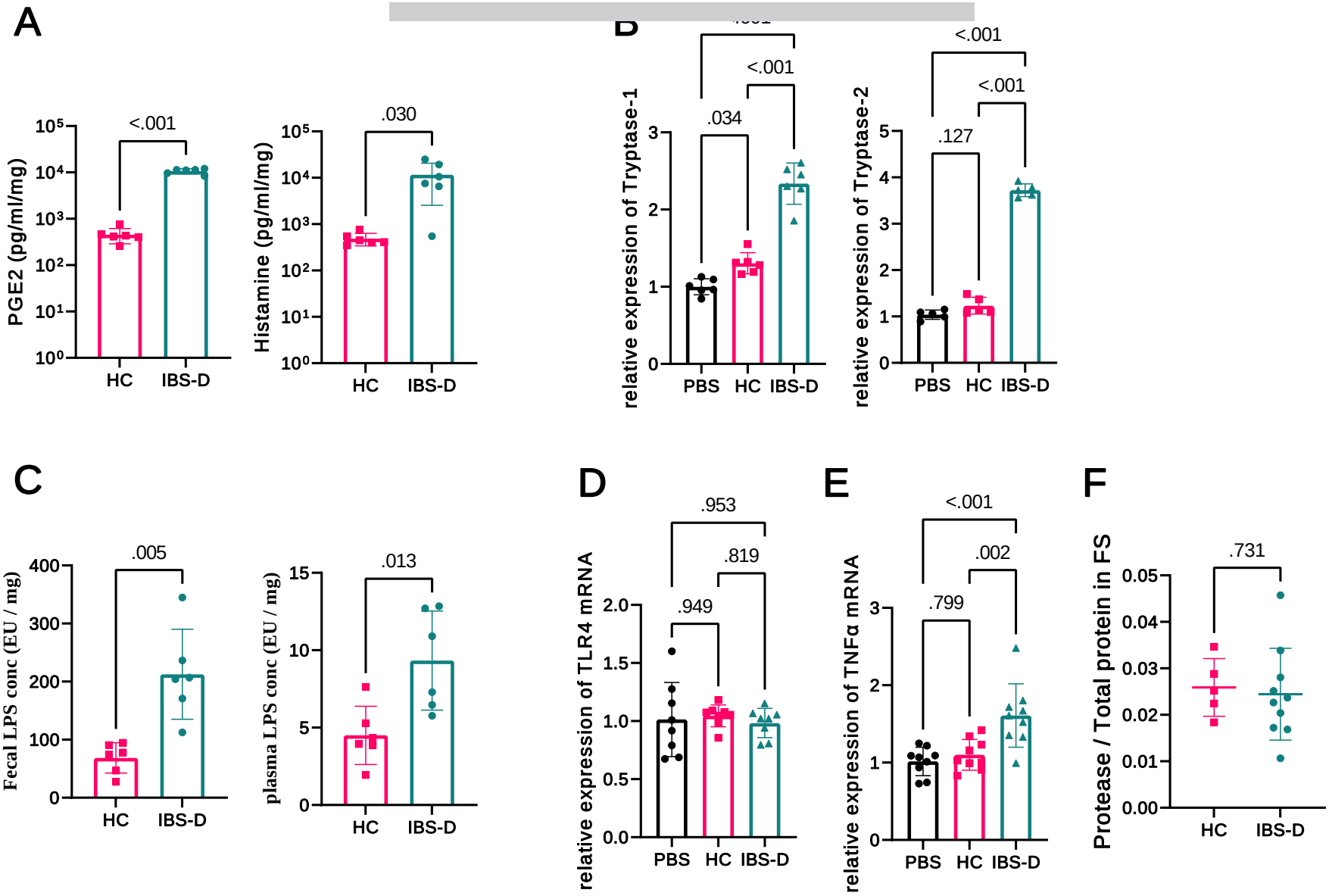
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5