

Methods

Animals

Gpr15l-deficient mice (B6-C10orf99Gpr15L^{<em711Npa}, *Gpr15l*^{-/-}) and transgenic mice with heterozygous overexpression of *Gpr15l* (B6-Tg(GPR15L)33Npa, *Gpr15l*-tg) used in acute DSS colitis experiments were provided by Novartis (Basel) under MTA. *Gpr15l*^{-/-} mice were generated using the CRISPR/Cas9-system. The full *Gpr15l* gene (C10orf99, 2610528A11Rik) was deleted by injecting CRISPR tracrRNA and sgRNAs to zygotes from C57Bl/6J mice. The following primers were used for genotyping in PCR reactions: 5'-TTCTAACCACCGAGGCCAAC-3', 5'-AAGGTTGGTGACACCTTTGGA-3', 5'-TGACTGCATGTGGTTGAGGATA-3' (Eurofins Genomics). To generate *Gpr15l*-tg mice, a transgenic cassette including CMV enhancer and chicken *Actb* promoter elements was built and inserted into a custom vector, which was injected into zygotes from C57Bl/6 mice. Transgenic founder animals were screened with PCR (forward primer: 5'-GGGCAACGTGCTGGTTGTTG-3'; reverse primer: 5'-GAAACCACACAAGGCCCTGC-3') to select a mouse with particularly high overexpression for crossing with C57Bl/6 mice to establish the transgenic line. A TaqMan primer assay was used for genotyping by quantitative PCR reaction according to the manufacturers' instructions (Mm01213298_m1 TaqMan Assay, ThermoFisher Scientific).

The *Gpr15l*^{-/-} mice used in the T cell transfer colitis experiments were shared by D. Littman (New York University). The *Gpr15l*^{-/-} mouse strain used in the T cell transfer colitis experiments was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis, an NIH-funded strain repository. Both lines were backcrossed to C57Bl/6J background for 14 generations before use. The *Rag1*^{-/-} (B6.129S7-Rag1^{<tm1Mom>/J}) mice used in the acute DSS and T cell transfer colitis

experiments were purchased from JAX and double knockout mice for *Rag1* and *Gpr15l* were generated by crossing *Rag1*^{-/-} with *Gpr15l*^{-/-} mice. Animals were housed under specific pathogen-free conditions and all experiments were approved by the responsible authorities.

Experimental colitis models

DSS colitis was induced by adding DSS to the drinking water for one week and mice were maintained on normal water for additional three days as previously described [1]. 1.5 % DSS was used unless otherwise indicated. The mice were weighed every 2-3 days and regularly assessed for weight loss, stool consistency, blood discharge and general well-being. Clinical disease activity was assessed as previously described [2] based on semi-quantitative scores for weight loss, stool consistency, and fecal blood.

For T cell transfer colitis, CD4⁺ T cells were enriched from spleen of WT or *Gpr15l*^{-/-} mice by negative selection using a CD4⁺ T cell Isolation Kit (STEMCELL). The enriched CD4⁺ T cells were stained and sorted by flow cytometry using the ARIA III cell sorter (BD Biosciences) to separate naive cells (CD4⁺ CD45RB^{hi} CD25^{lo}). Cells were washed with sterile PBS, and 1 × 10⁶ naive CD4⁺ T cells were transferred i.p. into each *Rag1*^{-/-}*Gpr15l*^{+/+} or *Rag1*^{-/-}*Gpr15l*^{-/-} littermate mice. The mice were weighed biweekly and assessed for weight loss, stool consistency, fecal blood and general well-being. The mice were euthanized at week 6 (or sooner if moribund or weight loss exceeded 20 %) and colon weight and length were assessed.

In co-housing experiments, female *Gpr15l*^{-/-} and WT mice were randomly assigned at weaning (~ 4 weeks of age) to mixed-genotype cages, for 4-5 weeks, with 2-7 mice per cage. In some experiments, mice were subsequently re-separated for an additional 6 weeks. Stool was obtained for 16S rRNA sequencing at the time of weaning, after co-housing and after the re-separation period. Acute DSS colitis was either performed after the co-housing

period under co-housed conditions, after the co-housing period under separated conditions or after the re-separation period under separated conditions.

For littermate experiments, *Gpr15^{+/+}* mice were crossed and female *Gpr15^{-/-}* and *Gpr15^{+/+}* mice were selected for experiments. At weaning (4-6 weeks of age), mice were separated according genotype and housed in groups of 2-8 mice per cage. Mice were either separated for 8 weeks before acute DSS colitis was initiated or co-housed until they reached 8 weeks of age and acute DSS colitis was initiated. In both scenarios, stool was obtained for 16S rRNA sequencing at weaning and before DSS colitis.

In rectal supplementation studies, littermate *Gpr15^{-/-}* mice of both sexes were randomly split into two groups (3-4 mice per cage) before induction of acute DSS colitis. Mice were treated with 100 μ L of either 13.5 μ M recombinant human GPR15L (Bachem) or sterile PBS as placebo once daily from day 3 to day 9. To this end, mice were anaesthetized with isoflurane and enemas were applied via a 7F silicon catheter. All enema solutions were sterile-filtered through a 0.2 μ m filter prior to use *in vivo*.

Microbiota depletion followed by fecal microbiota transfer

Wild-type (WT) mice were randomly assigned to experimental groups and housed in groups of four per cage under specific pathogen-free conditions. Mice were treated with a broad-spectrum antibiotic cocktail (ampicillin/sulbactam (1 g/L), metronidazole (1 g/L), vancomycin (500 mg/L), and ciprofloxacin (200 mg/L)) via the drinking water for eleven consecutive days. Antibiotic-containing drinking bottles were replaced every 24 hours. Following antibiotic treatment, mice received regular drinking water for five days. Fecal microbiota transplantation (FMT) was performed three days after cessation of antibiotic treatment. Fresh fecal pellets were collected from *Gpr15^{-/-}* or -proficient donor mice. Fecal suspensions were prepared by homogenizing 100 mg of stool in 1 mL sterile phosphate-buffered saline, followed by centrifugation at 800 $\times$ g for 3 minutes at room temperature. The

supernatant was collected, and 100 μ L was administered per mouse by oral gavage. WT recipient mice received fecal material from *Gpr15l*-deficient or -proficient donors according to random group allocation. Colitis was induced by administration of 2% dextran sulfate sodium in the drinking water for 10 days.

In vivo imaging of reactive oxygen species

The *In vivo* imaging system (IVIS) Lumina II (Caliper Life Sciences) was used to detect reactive oxygen species in mice *in vivo*. To this end, mice received an i.p. injection of 100 μ L of a 20 mM solution of the chemiluminescence probe 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H) dione (L-012, Fujifilm Wako Pure Chemical Corporation), which is activated upon contact with free anionic oxygen radicals, and chemiluminescence was measured 10 minutes later.

Endoscopy

Colonoscopy was performed under anesthesia with isoflurane with a mini-endoscopy system (Karl Storz). Endoscopic signs of intestinal inflammation were scored using the MEICS score as previously described [3].

Hematoxylin/eosin staining and histology

Mouse colon tissue was fixed in ROTI Histofix (4 % formaldehyde, Carl Roth) overnight, dehydrated, and embedded in paraffin. After cutting the tissue using a microtome (SLEE) the tissue was deparaffinized, rehydrated, fixed with ROTI Histol (Merck) and then treated with decreasing concentrations of ethanol. Subsequently, the sections were stained with hematoxylin (Sigma Aldrich) and eosin solution (Merck). Finally, the slides were fixed in ROTI Histol and mounted with Entellan (Merck). Microscopy was performed using a DM6000B Leica microscope.

Histological disease activity was assessed by blinded observers. For acute DSS colitis, the scoring system provided by Erben *et al.* was used [4]. For T cell transfer colitis, a modified version of the scheme described by Chinen *et al.* [5] was used, in which the criteria ‘acute and chronic inflammation’, ‘loss of goblet cells’, ‘thickening of mucosa’, ‘cryptitis/crypt abscesses’, ‘presence of giant cells’ and ‘epithelial erosions’ were used to categorize the severity from 0 (normal) to 3 (severe).

Lamina propria mononuclear cells isolation (LPMCs)

Where indicated, lamina propria mononuclear cells (LPMCs) were isolated using the LP dissociation kit (Miltenyi Biotec), followed by density gradient centrifugation with Percoll (GE Healthcare).

Immunofluorescence of mouse colon tissue

Cryopreserved colon sections were fixed with 4 % paraformaldehyde (PFA). In experiments involving detection with the TSA amplification kit endogenous peroxidase activity was quenched using 30 % hydrogen peroxide in methanol/water. Sections were permeabilised with 0.1 % Triton X-100 in phosphate-buffered saline (PBS) and non-specific binding was blocked using an Avidin/Biotin Blocking Kit (Vector Laboratories), ROTI ImmunoBlock (Carl Roth), 20 % goat serum (PAN-Biotech), and a protein-blocking reagent (Carl Roth). Sections were incubated overnight at 4 °C with primary antibodies against myeloperoxidase (MPO) (rabbit polyclonal anti-MPO, 1:100, Invitrogen), CD4 (rat anti-CD4, RM4-5, 1:100, BioLegend), Foxp3 (rat anti-Foxp3, NRRF-30, 1:100, Invitrogen), EpCAM (rabbit, EPR20533-266, 1:1000, Abcam), or CD11b (rat anti-CD11b Alexa Fluor 488, M1/70, 1:100, eBioscience). Rat IgG2b κ Alexa Fluor 488 (1:100, eB149/10H5, Invitrogen) was used as an isotype control for CD11b staining. Following primary antibody incubation, sections were

washed with PBS and incubated for 1 hour at room temperature with the appropriate secondary antibodies: goat anti-rabbit biotin (1:200, polyclonal, Jackson ImmunoResearch) for MPO, goat anti-rat biotin (1:200, polyclonal, BioLegend) for CD4 and Foxp3, and goat anti-rabbit Cy3 (1:200, polyclonal, Abcam) for EpCAM. Streptavidin-Cy3 (1:200, BioLegend) or the TSA Cy3 Kit (PerkinElmer) was used to detect biotinylated secondary antibodies. Sections were washed with PBS between each step to remove unbound antibodies. Sections not incubated with primary antibodies served as negative controls for MPO, CD4, Foxp3 and EpCAM. Terminal deoxynucleotidyl transferase nick end labelling (TUNEL) staining was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Merck). Nuclei were counterstained with Hoechst 33342 dye (Thermo Fisher) and slides were embedded with Vectashield Mounting Medium (Vector Laboratories). Microscopy was performed using a fluorescence microscope (DM600B, Leica) and a confocal microscope (SP8, Leica). Data analysis and quantification was performed using Fiji software v1.52n (NIH).

GPR15L Treatment of Colonic Organoid Monolayers

Briefly, 3D large intestinal organoids were collected using ice-cold PBS and dissociated into single cells with TrypL Express Enzym (ThermoFisher Scientific) for 10–15 minutes at 37 °C. 48-well plates were pre-coated with diluted Matrigel (1:40 in cold basal medium) for 1 hour. Cells were counted and seeded onto the Matrigel-coated plates at a density of 3×10^4 cells per well in complete organoid medium supplemented with mEGF (ImmunoTools), B27 supplement (ThermoFisher Scientific), N2 supplement (ThermoFisher Scientific), N-acetylcysteine (Merck), and puromycin (Merck). Cells were cultured until 2D monolayers reached 80–90 % confluence (typically after 48–72 hours) prior to treatment. For GPR15L stimulation, recombinant human GPR15L (ThermoFisher Scientific) was added to the culture medium at a final concentration of 150 nM. Cells were treated with PBS

(control) or GPR15L for 48 hours, after which monolayer cells were harvested for qPCR analysis. Gene expression was assessed using primers for *Cldn4*, *Muc5b*, *Defa6*, and *Areg*, with *Hprt* used as the housekeeping control. Relative mRNA expression levels were calculated using the $\Delta\Delta C_t$ method.

RNA isolation, cDNA transcription and qPCR

The NucleoSpin RNA Kit for RNA isolation (Machery Nagel) was used to isolate RNA from tissues and cells in accordance with the manufacturer's instructions. RNA was transcribed into cDNA using poly d(T) 12-18 primer HP27, in accordance with the instructions provided by the Agilent AffinityScript Kit. Quantitect Primer Assays and SybrSelect MasterMix (Thermo Fisher Scientific) were used for the qPCR analysis. For housekeeping controls, Qiagen's HPRT Quantitect Primer Assays for humans and mice were utilized. The CFX Connect real-time PCR machine (BioRad) was used to measure the plates.

LPMC re-stimulation and multiplexed cytokine quantification

Cells were cultured in flat-bottom 96-well plates (Corning) pre-coated with anti-mouse CD3 antibodies (1:850, 145-2C11, BioLegend) for 1 hour at 37 °C. Cells were resuspended at a final concentration of 2.5×10^6 cells/mL (corresponding to 5×10^5 cells per well) in culture medium (RPMI containing GlutaMAX (Gibco), 10 % fetal bovine serum (FBS, Gold Fote, PAN Biotech) and 1 % Penicillin/Streptomycin (P/S, Thermo Fisher Scientific)) supplemented with anti-CD28 (1:1000, 37.51, eBioscience). Cells were incubated for 48 h at 37 °C and 5 % CO₂. After stimulation, supernatants were collected and used for multiplex analysis. Cytokine concentrations for IL-1 β , TNF- α and IL-13 were determined using a bead-based multiplex immunoassay according to the manufacturer's instructions (eBioscience ProcartaPlex Mouse) and analysed on a Luminex platform. Plates were read on the Luminex instrument and analyte concentrations were calculated from standard curves generated with

recombinant cytokine standard. Quality controls and sample replicates were included on each plate. Data were analysed using the ProcartaPlex Analyst Software and exported for statistical analysis.

Fluorescence in situ hybridisation (FISH)

Mouse colon samples were collected and fixed overnight at room temperature in Carnoy's solution (60 % methanol, 30 % chloroform, 10 % glacial acetic acid). Following fixation, tissues were dehydrated in an ethanol dilution series (70 %, 90 %, 95 %, and 100 %) and subsequently embedded in paraffin. Paraffin blocks were sectioned at 5 µm thickness using a microtome (SLEE). Tissue sections were deparaffinized by incubation at 65 °C for at least 1 hour, cleared in Roti-Histol (15 minutes), and rehydrated through a descending ethanol dilution series (100 %, 96 %, and 70 %). For fluorescence in situ hybridization (FISH), sections were hybridized with the pan-bacterial probe EUB338 (5'-Cy3-GCT-GCC-TCC-CGT-AGG-AGT-3'). On each slide, one section was hybridized with the negative control probe NON338 (5'-Cy3-CGA-CGG-AGG-GCA-TCC-TCA-3') under identical conditions (90 minutes at 46 °C).

Following hybridization, sections were post-fixed in 4 % paraformaldehyde (PFA) and incubated with Ulex europaeus agglutinin I (UEA I, Vector Laboratories), a fluorescent dye-conjugated lectin (1:100 dilution). Nuclei were counterstained with Hoechst 33342 (Thermo Fisher Scientific), and slides were mounted with Mowiol (Sigma-Aldrich). Fluorescence imaging was performed using a Leica DM6000 B fluorescence microscope and a Leica SP8 confocal microscope. Image processing and quantitative analysis were carried out using Fiji software v1.52n (NIH). To quantify bacterial mucus infiltration, the distance bacteria infiltrated the mucus layer was measured using LAS X software (Leica) and normalized to mucus layer thickness.

Bulk RNA sequencing

Bulk RNA sequencing was performed on mRNA isolated from LPMCs and full colon tissue of *Gpr15^{-/-}* and WT mice with acute DSS colitis. Libraries were created using the Illumina TrueSeq Stranded mRNA Kit and sequenced on an Illumina HiSeq 4000 platform. Reads were checked for quality and normalized against transcript length. Transcripts expressed low or in few samples were excluded to minimize false positive results. Transcripts were quantified for each gene, and a two-stage test was used to check false positive results. The overall false discovery rate was set at 5 %. All p-values were corrected using the Benjamini-Hochberg procedure.

DNA isolation from stool and stool suspensions

Stool and cultured stool suspensions were immediately stored at -80 °C until DNA extraction. Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions.

16S ribosomal RNA gene amplification

The V4 regions of the 16S ribosomal RNA genes were amplified using 10 ng of bacterial template DNA with region-specific primers (515F forward primer: 5'-GTGYCAGCMGCCGCGGTAA-3'; 806R reverse primer: 5'-GGACTACNVGGGTWTCTAAT-3'), containing 12 bp barcodes on the forward primer (<https://earthmicrobiome.org/protocols-and-standards/16s/>), in a reaction consisting of 25 PCR cycles using the NEBNext Q5 Hot Start Hifi PCR Master Mix (New England Biolabs). PCR products were purified with AMPure XP Beads (Beckmann Coulter), pooled in equimolar ratios and analysed by 2 × 151 paired-end sequencing on an Illumina MiSeq device.

Bioinformatic processing of the 16S sequencing data

For bioinformatic processing, FASTQ files were used in two pipelines:

DADA2 pipeline: Raw fastq files were imported and analysed in QIIME2 v2025.4 [6] with DADA2 [7] as the method for quality control, dereplication and amplicon sequence variant (ASV) table generation. The SILVA small subunit (SSU) database release 138.2 was used at a 99% similarity cut-off for taxonomic classification. ASV and taxonomic tables were imported into R (version 4.5.1) as a phyloseq object and the “vegan” (2.6.4) package was used for diversity analysis and ordination. Ggplot2 [8] was used for the generation of graphical illustrations. Before alpha diversity calculation, sample counts were repeatedly (512 times) rarefied to the lowest library size for normalization, ensuring that diversity comparisons are made on a consistent basis [9]. Before beta diversity calculation, libraries were normalized by log-transformed variance stabilization. For functional pathway analysis, software PICRUST2 2.0 was used as described earlier [10].

USEARCH pipeline: Operational taxonomic units (OTUs) and zero-radius OTUs (ZOTUs) clustering was performed in parallel with the USEARCH v11 algorithm [11] via the IMNGS server [12]. Taxonomic classification of the identified ZOTUs was done with SINA v1.7.2 [13] using the SILVA SSU database release 138.2 and NCBI nucleotide BLAST [14]. After that, the data were imported into R v4.5.1 and filtered using the phyloseq package [15]. Differential abundance analyses were performed using the limma-voom linear model [16,17] for pairwise comparisons within the microbiomeMarker package [18]. Data was visualized in heat trees with the metacoder package [19].

Radial diffusion assay

Radial diffusion assays were performed as previously described [20,21]. 1 µg of recombinant human GPR15L was used to test the anti-microbial properties of GPR15L.

Bacterial growth kinetics with GPR15

Bacterial Strain and Culture Conditions

All experiments were performed using *Salmonella enterica* serovar Typhimurium strain NCTC 12023. Bacteria were streaked from cryo stocks onto LB agar plates and incubated overnight at 37 °C. Plates were subsequently stored at 4 °C and used within a maximum of one week. For anaerobic experiments an anaerobic jar was used, media were pre-reduced prior to use for 16h at 37°C.

Antimicrobial Compounds and Serial Dilutions

Polymyxin B (#0235.1, Carl Roth, Karlsruhe, Germany) and recombinant GPR15L were prepared as 10× stock solutions at 640 µM. Two-fold serial dilutions were generated ranging from 640 µM to 1.25 µM (640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 µM).

Preparation of Bacterial Inoculum

A single colony was inoculated into 3 ml pre-reduced Müller-Hinton broth (MHB) and incubated at 37 °C until mid-logarithmic phase in the anaerobic jar. Bacterial cells were harvested by centrifugation at 900 × g for 10 minutes at 4 °C. The supernatant was discarded and the pellet washed once with 10 mM sodium phosphate buffer pH7.4 + 1 % (w/v) MHB, followed by a second centrifugation step under the same conditions. The pellet was resuspended in sodium phosphate buffer pH7.4 + 1 % MHB. Optical density at 600 nm was measured, and cultures were adjusted to a final concentration corresponding to 1.11×10^4 CFU per 100 µl (10,000 bacteria per well).

Determination of Initial Inoculum (T0)

To determine the initial bacterial count, 15 µl of the standardized bacterial suspension were added to 135 µl phosphate buffer containing 0.2 % (w/v) PVP-40 (#SIALPVP40, Sigma-

Aldrich, Schnelldorf, Germany), followed by serial dilutions (10^{-1} and 10^{-2}). Subsequently, 50 μ l of each dilution were plated in duplicate onto Müller-Hinton agar plates and incubated overnight at 37 °C.

Incubation and Plating Assay

A total of 90 μ l bacterial suspension and 10 μ l of 10-fold concentrated antimicrobial compound was mixed in a well of a 96-well plate. For anaerobic conditions, each well was covered with 100 μ l paraffin oil. The 96-well plates were incubated for 3 hours at 37 °C in an anaerobic jar. For quantification of surviving bacteria, 15 μ l of each bacterial suspension were transferred into 135 μ l phosphate buffer containing 0.2 % PVP-40, and serial dilutions (10^{-1} - 10^{-3}) were prepared. From each dilution, 50 μ l were plated in duplicate onto Müller-Hinton agar plates and incubated overnight at 37 °C. Colonies were counted the following day and CFU/ml were calculated.

NaCl conditions

For testing the effect of NaCl on GPR15L, 10 mM sodium phosphate buffer (pH 7.4) + 1 % (w/v) MHB was supplemented with 10 to 100 mM NaCl. For the NaCl titration assay a GPR15L concentration of 32 μ M was used.

Growth Kinetics

For kinetic growth analysis, lid-covered plates with the remaining 85 μ l bacterial suspension were transferred to a M-PLEX plate reader (Tecan, Grödig, Austria). Absorbance at 600 nm was measured over 18 h at 37 °C with readings taken every 20 minutes following 20 seconds of shaking.

Data Analysis

All statistical analyses were performed in R version 4.5.1. For calculation of minimum inhibitory concentrations (MIC) the endpoint of the kinetic growth measurements after 21 hours of incubation was used. Smoothing splines were fitted to the scaled absorbance data using the *stinepack* package. The MIC was defined as the AMP concentration at which

the fitted spline crossed a threshold defined by 10 % of the maximum absorbance. Maximum growth rates ($\mu_{\max}$) were calculated from the full kinetic growth curves. After scaling, the logarithmic growth phase of each curve was identified, and smoothing splines were fitted using the *growthrates* package to estimate the maximum specific growth rate ($\mu_{\max}$). The $\mu_{\max}$ data were further fitted against anti-microbial compound concentrations using a four-parameter log-logistic model (LL.4) implemented in the *drc* package. If the maximum effect exceeded 10 % inhibition, the inhibitory concentrations corresponding to 50 % growth reduction (IC₅₀) were deduced from the model.

Anaerobic culture and treatment of human stool suspensions

Human stool samples were collected in sterile containers and stored at -80 °C until processing. On average, approximately 3 hours elapsed between sampling and freezing and no medium was added to the stool samples prior to freezing. For experimental analyses, aliquots of the same sample were treated either with GPR15L or without GPR15L, enabling direct assessment of GPR15L-dependent effects. During the experiment, stool samples were thawed inside an anaerobic chamber. All buffers and culture media were pre-incubated in the anaerobic chamber for at least 24 hours prior to the experiment. To prepare the initial suspension, stool samples were re-suspended in 10 mM phosphate buffer containing 0.1 % cysteine and 0.1 % Gifu anaerobic medium (GAM) to a final concentration of 100 mg/mL. CFU counting of this suspension ranged from 1.65×10^9 to 3.9×10^9 CFU/mL across the included samples, confirming the presence of viable bacteria at the start of the experiment. Under sterile conditions, 0.5 mL aliquots of the homogenized stool suspension were pre-incubated with recombinant human GPR15 ligand (GPR15L, Novartis) at a final concentration of 100 μ g/mL, or with 0.1 % acetic acid as vector control for 2 hours at 37 °C under anaerobic conditions. Following the pre-incubation, samples were transferred to fresh

Gifu anaerobic medium (GAM; HyServe) and further incubated for 16 hours at 37 °C in an anaerobic chamber (Don Whitley Scientific, UK). All treatments were performed in duplicate.

Shotgun metagenomic sequencing and data processing

Sequencing libraries were prepared from genomic DNA from human or mouse fecal samples and sequenced on an Illumina NovaSeq X Plus platform by Novogene to generate 150 bp paired-end reads.

For bioinformatic processing, raw fastq reads were processed with fastp and bbdup for quality control and adapter trimming and filtering. Human host DNA was then depleted by aligning the quality-filtered reads to the human reference genome (GRCh38) with Bowtie2. For mouse samples, host DNA was removed by aligning the quality-filtered reads to the mouse reference genome (GRCm39). Taxonomic profiling was conducted on cleaned, host-depleted reads using Kraken2 for classification against the PlusPF, followed by Bracken for estimation of species level abundances. For functional profiling of metabolic pathways MetaPhlAn4.1 using the mpa_vOct22_CHOCOPhIAnSGB_202403 index followed by HUMAnN4 analysis was used. Statistical analyses were performed in R (version 4.5).

Stool samples and intestinal biopsies from human donors

All material from human donors was obtained after written informed consent and according to approval of the responsible Ethics Committee and in compliance with all relevant ethical regulations. Baseline characteristics are summarized in *Suppl. Tables 1-6*. For RNA samples and tissue sections from the biobank of the Department of Medicine 1 of the University Hospital Erlangen, samples were obtained from patients with IBD and non-IBD controls during routine colonoscopy. Sample acquisition for the Switzerland and the IBDome cohort have been described elsewhere [22,23]. For the analysis of flare-free survival,

patients were prospectively followed up and flares were defined as any increase in HBI score for CD patients or partial Mayo score for UC patients that triggered treatment intensification/switch or an increase of at least 4 and 2 points, respectively.

ELISA for GPR15L in human stool samples

Fresh stool samples were immediately stored at -80 °C. For the quantification of GPR15L, samples were thawed and further processed on ice. 100 mg per sample were added to 2 mL 70 % ethanol and a homogenous suspension was prepared by shaking for 30 seconds twice on a Qiagen TissueLyser II at a frequency of 30/second. Following centrifugation at 20600 rcf for 10 minutes at 4 °C, supernatants were obtained and used for ELISA.

Monoclonal anti-GPR15L (clone 1020903, 1:500, R&D Systems) was coated to wells of a 96 well-plate as capture antibody overnight at room temperature. After three washing steps with Wash Buffer (R&D Systems), wells were blocked with Reagent Diluent (Novus Biologicals) for 2 hours, followed by three additional washing steps. 100 µL of a standard serial dilution, blank and samples were pipetted to the wells in doublets and incubated overnight at 4 °C. Subsequently, wells were washed three times and an HRP-conjugated monoclonal anti-GPR15L detection antibody (clone 1020904, 1 µg/mL, Novus Biologicals) was added. After incubation for 2 hours at room temperature in the dark and three further washing steps, 100 µL Substrate Solution (Novus Biologicals) was added and incubated for 20 minutes at room temperature in the dark. Finally, 50 µL of Stop Solution (Novus Biologicals) were added and a TECAN Infinite F50 ELISA reader was used to measure absorbance at 450 nm.

Immunofluorescence in IBD patients and controls

Cryosections of colon tissue from UC and CD patients as well as non-IBD controls were provided by the biobank of the Department of Medicine 1 at the University Hospital Erlangen.

Sections were fixed with 4% PFA, permeabilized with 0.1% Triton-X in PBS and blocked using ROTI ImmunoBlock (Carl Roth) with 20 % goat serum (PAN-Biotech). The primary antibody targeting GPR15L (1:200, polyclonal, Abcam) was incubated overnight at 4 °C, followed by a secondary antibody incubation for 1 hour at room temperature (goat anti-rabbit Cy3, 1:200, Merck). Afterwards, cryosections were stained in a second round with a primary antibody targeting CD326 (EpCAM, 1:4000, 9C4, BioLegend) for 2 hours at room temperature followed by a secondary antibody incubation for 1 hour at room temperature (goat anti-mouse AF488, 1:200, ThermoFischer Scientific). Sections treated with secondary antibodies alone served as negative control. Nuclei were stained with Hoechst 33342 (ThermoFisher Scientific) and slides were embedded in Vectashield Mounting Medium (Vector Laboratories). Microscopy was performed using a confocal SP8 microscope (Leica). The mean fluorescence intensities (MFI) of GPR15L and EpCAM were quantified using Fiji software v1.52n (NIH). The MFI of the negative control was subtracted from the respective value and the MFI of GPR15L was normalized to the MFI of EpCAM. Samples with severe epithelial erosions were excluded to ensure normalization validity.

Statistics

GraphPad Prism 10.2.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and results are shown as box plots or scatter dot plots and violin plots with mean and standard error of the mean (SEM) with mean and standard error of the mean. Outliers were identified with the ROUT test (Q = 1 %) and excluded from the analyses. Shapiro-Wilk test was used to determine normal distribution. Regularly distributed data was examined using paired or unpaired t-tests as applicable. The Mann-Whitney test was used for non-regularly distributed data. For more than two groups with non-regularly distributed data Kruskal-Wallis test was used. A two-way ANOVA with Tukey's multiple comparisons

test was computed for groups dependent on an extra variable. P-values < 0.05 were considered significant.

References for Methods

- 1 Wirtz S, Popp V, Kindermann M, *et al.* Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc.* 2017;12:1295–309. doi: 10.1038/nprot.2017.044
- 2 Park YH, Kim N, Shim YK, *et al.* Adequate Dextran Sodium Sulfate-induced Colitis Model in Mice and Effective Outcome Measurement Method. *J Cancer Prev.* 2015;20:260–7. doi: 10.15430/JCP.2015.20.4.260
- 3 Wirtz S, Popp V, Kindermann M, *et al.* Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc.* 2017;12:1295–309. doi: 10.1038/nprot.2017.044
- 4 Erben U, Loddenkemper C, Doerfel K, *et al.* A guide to histomorphological evaluation of intestinal inflammation in mouse models. *Int J Clin Exp Pathol.* 2014;7:4557–76.
- 5 Chinen T, Komai K, Muto G, *et al.* Prostaglandin E2 and SOCS1 have a role in intestinal immune tolerance. *Nat Commun.* 2011;2:190. doi: 10.1038/ncomms1181
- 6 Bolyen E, Rideout JR, Dillon MR, *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* 2019;37:852–7. doi: 10.1038/s41587-019-0209-9
- 7 Callahan BJ, McMurdie PJ, Rosen MJ, *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods.* 2016;13:581–3. doi: 10.1038/nmeth.3869
- 8 Wickham H. *ggplot2*. Cham: Springer International Publishing 2016.
- 9 Schloss PD. Rarefaction is currently the best approach to control for uneven sequencing effort in amplicon sequence analyses. *mSphere.* 2024;9:e0035423. doi: 10.1128/msphere.00354-23
- 10 Douglas GM, Maffei VJ, Zaneveld JR, *et al.* PICRUST2 for prediction of metagenome functions. *Nat Biotechnol.* 2020;38:685–8. doi: 10.1038/s41587-020-0548-6
- 11 Edgar RC. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. 2016;081257.
- 12 Lagkouvardos I, Joseph D, Kapfhammer M, *et al.* IMNGS: A comprehensive open resource of processed 16S rRNA microbial profiles for ecology and diversity studies. *Sci Rep.* 2016;6:33721. doi: 10.1038/srep33721
- 13 Pruesse E, Peplies J, Glöckner FO. SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics.* 2012;28:1823–9. doi: 10.1093/bioinformatics/bts252
- 14 Altschul SF, Gish W, Miller W, *et al.* Basic local alignment search tool. *J Mol Biol.* 1990;215:403–10. doi: 10.1016/S0022-2836(05)80360-2
- 15 McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One.* 2013;8:e61217. doi: 10.1371/journal.pone.0061217

- 16 Ritchie ME, Phipson B, Wu D, *et al.* limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43:e47. doi: 10.1093/nar/gkv007
- 17 Law CW, Chen Y, Shi W, *et al.* voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol.* 2014;15:R29. doi: 10.1186/gb-2014-15-2-r29
- 18 Cao Y, Dong Q, Wang D, *et al.* microbiomeMarker: an R/Bioconductor package for microbiome marker identification and visualization. *Bioinformatics.* 2022;38:4027–9. doi: 10.1093/bioinformatics/btac438
- 19 Foster ZSL, Sharpton TJ, Grünwald NJ. Metacoder: An R package for visualization and manipulation of community taxonomic diversity data. *PLoS Comput Biol.* 2017;13:e1005404. doi: 10.1371/journal.pcbi.1005404
- 20 Schroeder BO, Wu Z, Nuding S, *et al.* Reduction of disulphide bonds unmasks potent antimicrobial activity of human β -defensin 1. *Nature.* 2011;469:419–23. doi: 10.1038/nature09674
- 21 Puértolas-Balint F, Schroeder BO. Intestinal α -Defensins Play a Minor Role in Modulating the Small Intestinal Microbiota Composition as Compared to Diet. *Microbiol Spectr.* 2023;11:e0056723. doi: 10.1128/spectrum.00567-23
- 22 Yilmaz B, Juillerat P, Øyås O, *et al.* Microbial network disturbances in relapsing refractory Crohn's disease. *Nat Med.* 2019;25:323–36. doi: 10.1038/s41591-018-0308-z
- 23 Trajanoski Z, Plattner C, Sturm G, *et al.* IBDome: An integrated molecular, histopathological, and clinical atlas of inflammatory bowel diseases. *Res Sq.* 2025;rs.3.rs-6443303. doi: 10.21203/rs.3.rs-6443303/v1