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Original research

The endogenous peptide GPR15L shapes the intestinal microbiota to counteract colitis

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ABSTRACT

Background The peptide GPR15L is produced by colonic epithelial cells and has been implicated in T cell recruitment to the large intestine. However, its role in chronic colitis has been unclear so far.

Objective To explore the role of GPR15L in the pathogenesis of experimental colitis and IBD.

Design We studied how genetic deletion or overexpression of *Gpr15l* as well as rectal application of recombinant GPR15L alters the course of acute dextran sodium sulfate colitis and T cell transfer colitis. The impact of GPR15L on microbiota was explored with co-housing, littermate and faecal microbiota transfer studies, by 16S rRNA sequencing as well as anti-microbial assays and shotgun metagenomics. The expression of GPR15L was evaluated across three independent cohorts of patients with IBD and correlated to microbial diversity and flare-free survival.

Results GPR15L clearly mitigated experimental colitis, but this was independent of T cell recruitment and GPR15. Instead, we observed that the effects of GPR15L were mediated by altered microbiomes in the large intestine and, consistently, showed that GPR15L acts as an antimicrobial peptide under anaerobic conditions and shapes microbial communities towards a homeostatic phenotype. Rectal supplementation of GPR15L counteracted experimental colitis. In patients with IBD, GPR15L expression was decreased in active inflammation, correlated with microbial diversity and was associated with flare-free survival.

Conclusions GPR15L is a host-defence peptide that plays a beneficial role in the pathogenesis of intestinal inflammation. It seems promising to further evaluate its potential as a future therapeutic approach in IBD.

INTRODUCTION

Despite the multifactorial pathogenesis of IBD that crucially involves the interaction of environmental factors with genetic susceptibility,¹ clinically established therapeutic approaches are confined to strategies mitigating downstream immunological

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ The peptide GPR15L is produced by the large intestinal epithelium.
- ⇒ GPR15L interacts with GPR15 to recruit T cells to the large intestine.

WHAT THIS STUDY ADDS

- ⇒ GPR15L counteracts experimental colitis independent of its role in T cell recruitment.
- ⇒ GPR15L acts as an antimicrobial peptide to shape large intestinal microbial communities towards homeostasis.
- ⇒ GPR15L is downregulated in patients with active IBD and GPR15L expression is associated with flare-free survival.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Topical application of GPR15L emerges as a potential therapeutic approach to interfere with dysregulated host-microbiota communication in IBD.

responses.² Over the recent years, the number of such options approved for UC and/or Crohn's disease (CD) and tailored to targets such as $\alpha 4\beta 7$ integrin, IL12/23p40, IL23p19, Janus kinases and sphingosine-1-phosphate receptors has substantially increased.³ However, the pre-existing therapeutic ceiling with durable remission rates in clinical trials reaching up to 30% has not yet been surpassed.⁴

Environmental factors that have been associated with an increased risk of IBD include, among others, antibiotics use in childhood, mode of birth, breast-feeding, diet, air pollution and hygienic conditions.⁵ Many of them directly or indirectly impact on the composition of the intestinal microbiota, a huge consortium of estimated 10^{13} – 10^{14} bacteria, viruses and fungi that populate the gastrointestinal tract, where they fulfil important functions for digestion and promote epithelial homeostasis.^{6,7}

Accordingly, evolution has instructed the immune system of the host to tolerate a eubiotic environment, while restraining and combating pathogenic organisms.^{8,9} However, dysbiosis featured by a decrease in the overall diversity as well as an enrichment of specific bacteria is a hallmark of IBD^{10–12} and it has been postulated that translocation of bacterial antigens over a leaky barrier triggers inflammation in the gut.¹ One of the endogenous mechanisms to counteract such dysbiosis is antimicrobial peptides that are secreted by epithelial cells in the gut and protect the mucosa from the invasion of pathogens.¹³ While their role for mucosal homeostasis is well established,¹⁴ the functional relevance and translational potential for chronic intestinal inflammation remain largely enigmatic.

At the same time, an important and translationally relevant process contributing to initiation and maintenance of intestinal inflammation in IBD is the recruitment of lymphocytes to the tissue.¹⁵ In 2013, the orphan receptor GPR15 had been identified as a molecule that specifically directs T cells to the large intestine, thereby modulating the activity of experimental colitis.^{16,17} In 2017, GPR15 was deorphanised and GPR15L identified as the so far only known ligand,^{18,19} binding to the receptor with high specificity and promoting lymphocyte gut homing upstream of surface integrins.²⁰ Under homeostatic conditions, GPR15L is predominantly produced by colonic epithelial cells.¹⁹ Here, we set out to explore the initial hypothesis that GPR15L is involved in the pathogenesis of experimental and human colitis by directing such T cell recruitment.

We show that GPR15L counteracts intestinal inflammation in mice and is clearly downregulated in IBD, while high expression is associated with favourable prognosis. Unexpectedly, however, these effects were not dependent on lymphocyte recruitment and GPR15. Instead, GPR15L acted as an antimicrobial peptide and promoted a homeostatic phenotype of mouse and human intestinal microbiota. Therapeutic application in mice clearly reduced disease activity, nominating GPR15L as a promising future therapeutic approach for IBD.

METHODS

Methods are available in online supplemental file 1.

RESULTS

GPR15L counteracts experimental colitis

To understand the impact of GPR15L on experimental colitis, we initially induced acute dextran sodium sulfate (DSS) colitis in *Gpr15l*-deficient and wild-type mice housed under the same specific pathogen-free conditions. While we did not expect major findings based on our hypothesis that GPR15L controls lymphocyte recruitment and given the non-essential role of T cells in acute DSS colitis, we were surprised to observe a dramatic increase in disease activity in mice deficient for *Gpr15l* as demonstrated by mini-endoscopy, histology, intravital imaging of reactive oxygen species, weight loss and clinical disease activity (figure 1A). We even had to decrease the DSS dose to 1% in these experiments, since we observed high mortality among *Gpr15l*^{-/-} mice in initial experiments at the usual lab standard of 1.5%. Consistently, we measured increased mRNA expression of key pro-inflammatory cytokines such as *Il1b*, *Il13* and *Tnf* in lamina propria mononuclear cells (LPMCs; figure 1B), which was mirrored by increased secretion of interleukin (IL)1β, IL13 and tumour necrosis factor (TNF) from re-stimulated LPMCs to the culture supernatant (figure 1C). This was further corroborated by a higher abundance of myeloperoxidase (MPO)⁺ and CD4⁺ cells in the colon of *Gpr15l*-deficient mice (online supplemental

figure 1A and B) along with an increase of apoptotic epithelial cells (online supplemental figure 1C). Although the phenotype was in principle compatible with the idea of reduced gut homing of regulatory T cells (Tregs), where GPR15 is predominantly expressed in mice,¹⁶ our doubts on this explanation were fostered by the finding that Foxp3⁺ cells were also increased in *Gpr15l*-deficient mice and that the ratio of Foxp3⁺/CD4⁺ cells was not affected (online supplemental figure 1D).

We next sought to confirm these observations by inducing acute DSS colitis (standard 1.5%) in mice with transgenic GPR15L overexpression and littermate controls. Indeed, GPR15L overexpression substantially reduced endoscopic, histological and clinical disease activity as well as reactive oxygen species production and weight loss (figure 1D).

Together, these data strongly suggested that *GPR15L* plays a protective role in experimental colitis.

The effects of GPR15L on experimental colitis are not mediated by lymphocytes or GPR15

We subsequently aimed to further challenge the concept that the described alterations in disease activity are driven by the role of GPR15L in T cell recruitment. To that end, we crossed *Gpr15l* knockout mice to *Rag1*-deficient mice lacking native lymphocytes and induced acute DSS colitis with *Gpr15l*-proficient and *Gpr15l*-deficient *Rag1*^{-/-} animals. We found that the previously described phenotype was fully recapitulated (figure 2A, online supplemental figure 2A) also in the absence of T cells.

Furthermore, we used the T cell transfer colitis model and transferred *Gpr15l*-deficient or *Gpr15l*-proficient CD45RB^{high} T cells into *Rag1*^{-/-} mice deficient or proficient for *Gpr15l*. Disease activity as determined by weight course, colon length, colon length-to-weight ratio and histology did not differ between *Gpr15l*-proficient mice that received *Gpr15l*^{+/+} or *Gpr15l*^{-/-} T cells. However, disease activity on transfer of *Gpr15l*^{-/-} T cells to *Rag1*^{-/-}*Gpr15l*^{-/-} mice was clearly increased as compared with transfer to *Rag1*^{-/-}*Gpr15l*^{+/+} mice (figure 2B, online supplemental figure 2B). These data indicated that the effects of GPR15L on experimental colitis are independent of GPR15 and therefore unlikely to be explained by T cell recruitment.

Host-microbiota interactions mediate the impact of GPR15L on colitis

To screen for potential mechanisms underlying our phenotypic observations, we performed bulk RNA sequencing on LPMCs as well as full colon tissue obtained from *Gpr15l*^{-/-} and WT mice.²¹ We found that only very few genes were differentially expressed in LPMCs (figure 3A), providing additional validation for the conclusion that GPR15L-dependent lymphocyte homing is non-essential for experimental colitis. To the contrary, a substantial number of genes was differentially expressed in full colon tissue (figure 3B) and these were enriched for genes associated with host-environment interactions at the intestinal barrier such as *Cldn4*, *Tlr5* or *P2rx1* (figure 3C).

However, in 2D cultures of mouse large intestinal organoids treated with or without GPR15L, we did not observe an impact of GPR15L on the expression of genes involved in barrier function and epithelial proliferation (online supplemental figure 3A), suggesting that epithelial cell signatures in the bulk RNA-seq are shaped downstream of another primary mechanism. Thus, considering a previous report suggesting that GPR15L has anti-bacterial properties

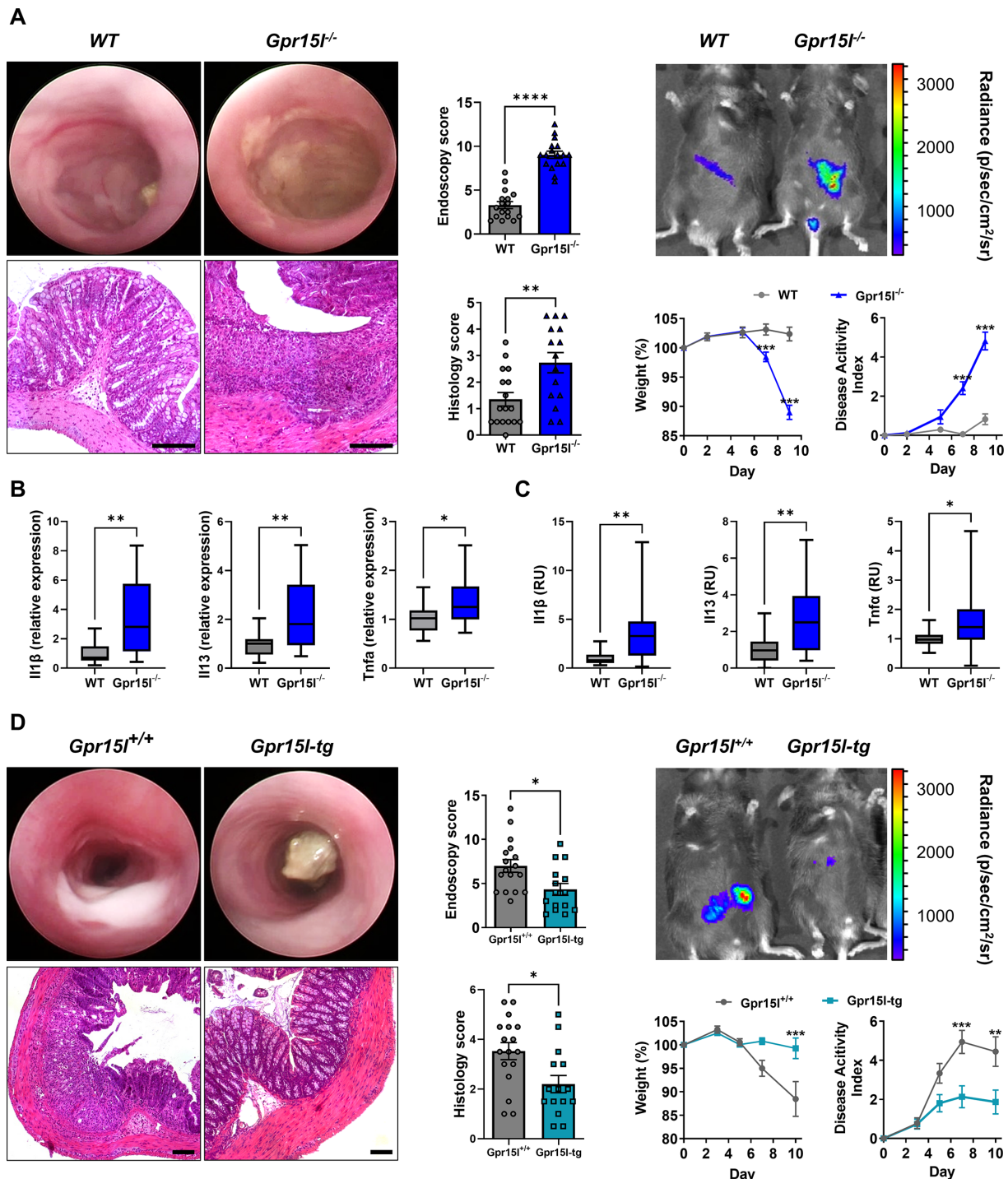
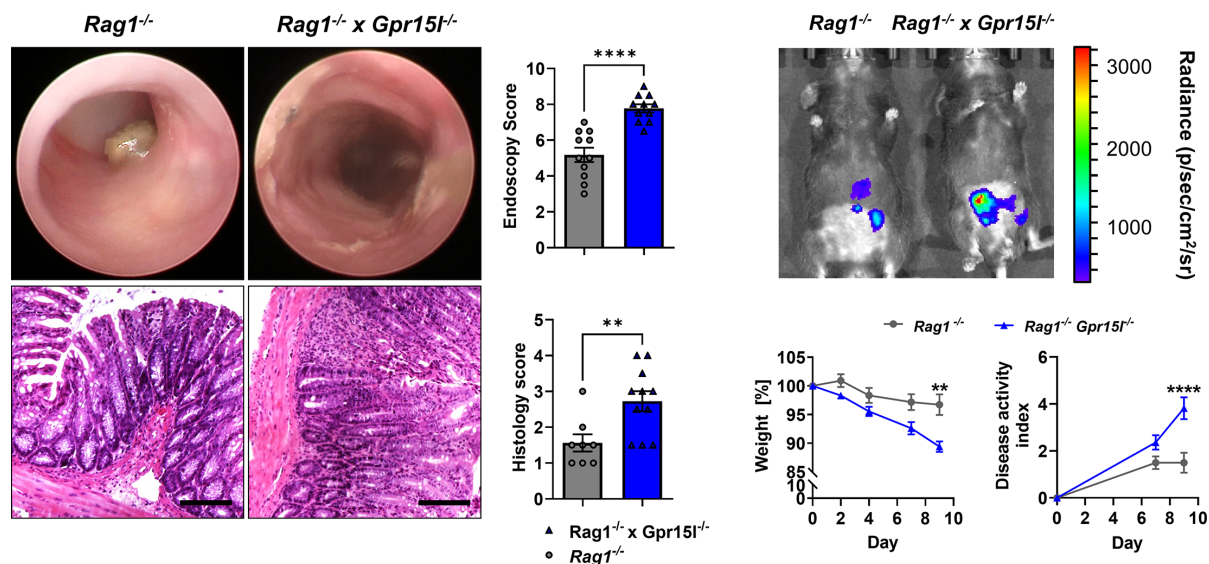


Figure 1 Beneficial role of GPR15L in experimental colitis. (A–C) Acute dextran sodium sulfate (DSS) colitis in *Gpr15l*^{-/-} and wild-type (WT) mice (1% DSS). (A) Left upper panels: representative and quantitative endoscopic disease activity. Left lower panels: representative and quantitative histologic disease activity. Right upper panel: representative *in vivo* measurement of reactive oxygen species. Right lower panels: weight course and clinical disease activity. n=15–17 per group. (B) qPCR of mRNA expression of the indicated cytokines in lamina propria mononuclear cells (LPMCs). n=13–17 per group. (C) Quantification of cytokine secretion from re-stimulated LPMCs. n=14–17 per group. (D) Acute DSS colitis in *Gpr15l*-transgenic and littermate control mice (1.5% DSS). Left upper panels: representative and quantitative endoscopic disease activity. Left lower panels: representative and quantitative histologic disease activity. Right upper panel: representative *in vivo* measurement of reactive oxygen species. Right lower panels: weight course and clinical disease activity. n=15–17 per group. Scale bars: 100 μ m. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. IL, interleukin; qPCR, quantitative PCR; mRNA, messenger RNA.

in vitro,²² we hypothesised that the intestinal microbiota might be involved in our observations. Thus, we used colon sections from the well-controlled acute DSS colitis experiments in *Gpr15l*-transgenic and littermate mice and

stained for bacteria in the mucus. We observed that *Gpr15l*-transgenic mice harboured substantially less bacteria in their mucus layer and that these did not penetrate as deep as in the littermate controls (figure 3D). Similarly, mucus penetration

A



B

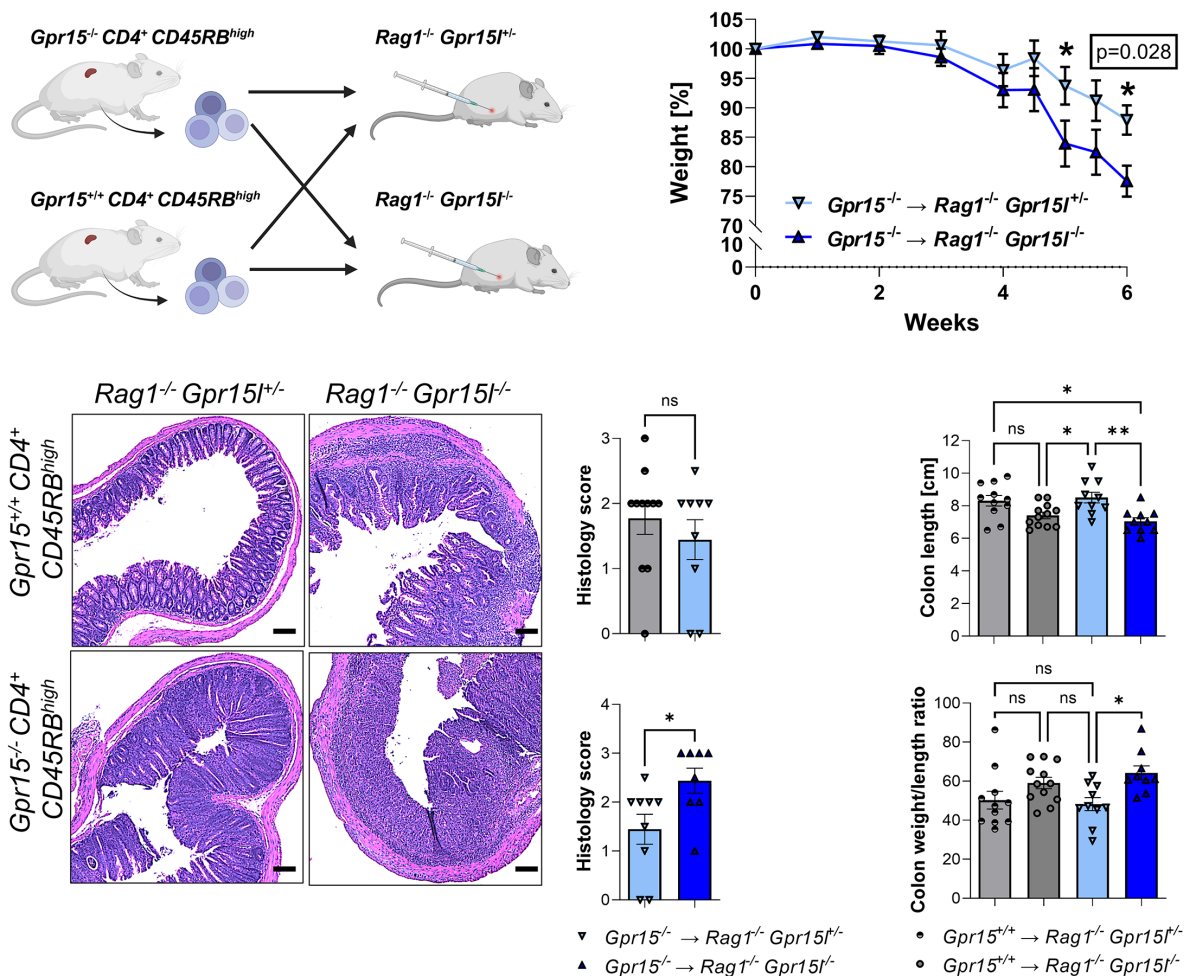


Figure 2 T cells and GPR15 are dispensable for the beneficial role of GPR15L in colitis. (A) Acute DSS colitis with *Rag1*-deficient (*Rag1*^{-/-}) and *Rag1*^{-/-} *Gpr15l*^{-/-} double knockout mice. Left upper panels: representative and quantitative endoscopic disease activity. Left lower panels: representative and quantitative histologic disease activity. Right upper panel: representative *in vivo* measurement of reactive oxygen species. Right lower panels: weight course and clinical disease activity. n=8–14 per group. (B) T cell transfer colitis model with *Gpr15*-proficient or *Gpr15*-deficient donor CD4⁺CD45RB^{high} T cells adoptively transferred to *Rag1*^{-/-}*Gpr15l*^{+/+} or *Rag1*^{-/-}*Gpr15l*^{-/-} littermate mice. Upper left panel: schematic representation of the experimental design. Upper right panel: weight course of mice transferred with *Gpr15*^{-/-} T cells. Lower left panels: representative and quantitative histologic disease activity. Right lower panels: colon length and colon length/weight ratio as indicated. n=8–12 per group. Scale bars: 100 μ m. *p<0.05; **p<0.01; ***p<0.001. DSS, dextran sodium sulfate.

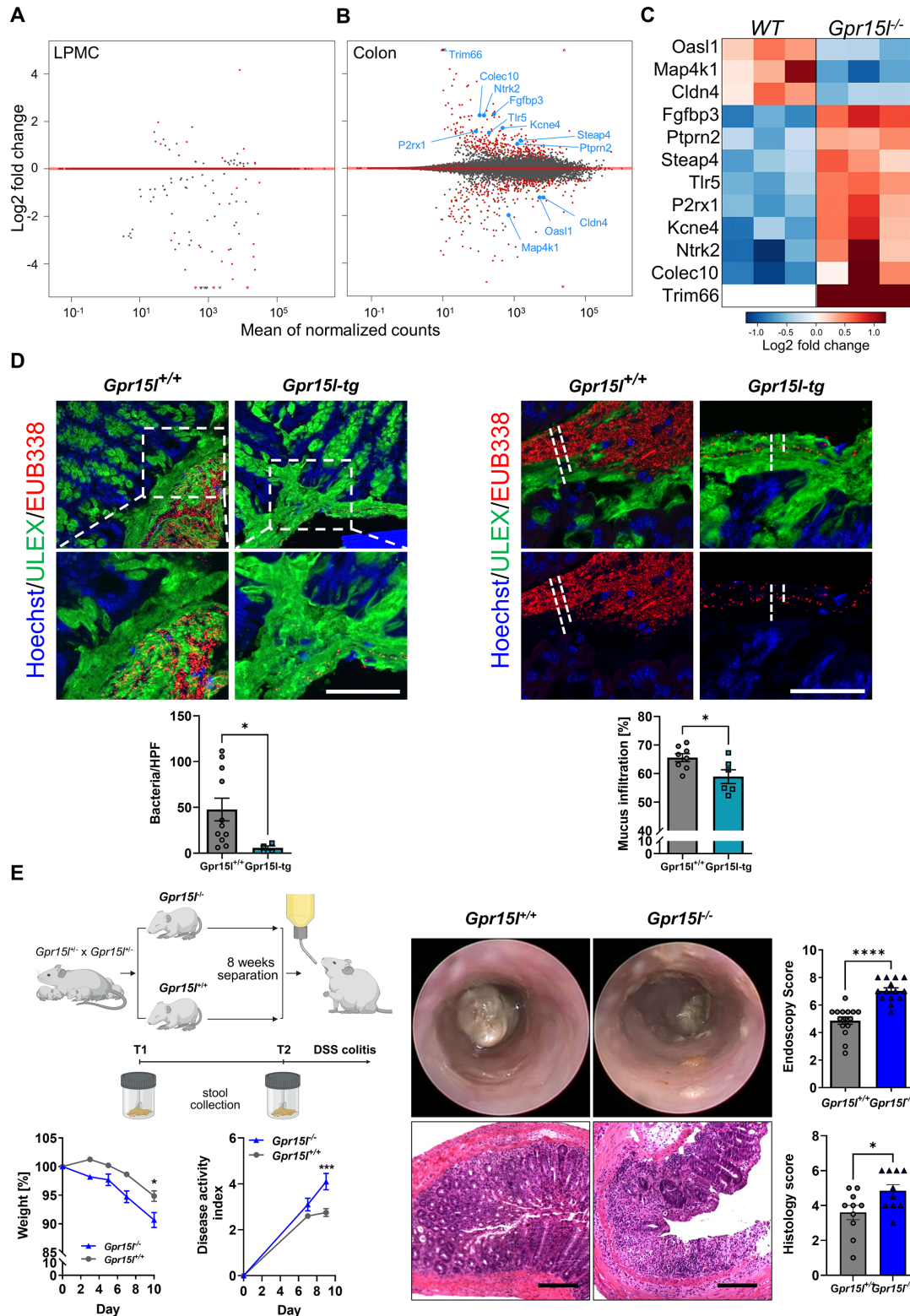


Figure 3 GPR15L shifts host-microbiota interaction in experimental colitis. (A–C) Bulk RNA sequencing of lamina propria mononuclear cells (LPMCs), (A) and whole colon tissue (B,C) from *Gpr15l*^{-/-} and wild-type mice with acute DSS colitis. (A,B) MA-plots of differential gene expression. Selected genes are highlighted. (C) Heatmap of differential expression of selected genes associated with host-microbiota crosstalk and barrier function. n=3 per group. (D) Representative (upper panels) and quantitative (lower panels) fluorescence in situ hybridisation (FISH) of colon tissue from *Gpr15l*^{-/-} and littermate control mice with acute DSS colitis. Staining for nuclei (Hoechst, blue), mucus (ULEX, green) and bacterial rRNA (EUB388, red). Quantitative analysis of bacterial abundance and relative depth of bacterial mucus layer infiltration. n=4–11 per group. (E) Acute DSS colitis with littermate *Gpr15l*^{-/-} and *Gpr15l*^{+/+} mice separated after weaning for 8 weeks. Left upper panel: schematic representation of the experimental design. Left lower panels: weight course and clinical disease activity. Right upper panels: representative and quantitative endoscopic disease activity. Right lower panels: representative and quantitative histologic disease activity. n=10–15 per group. Scale bars: 50 µm (FISH staining, (D)), 200 µm (histology, (E)). *p<0.05; ***p<0.001; ****p<0.0001. DSS, dextran sodium sulfate; HPF, high power field; WT, wild type.

of intestinal bacteria in *Gpr15l*^{-/-} mice was higher than that in WT controls (online supplemental figure 3B).

Since this supported the idea of host-microbiota interactions driving differential colitis activity, we next aimed to understand the impact of GPR15L on microbial communities in knockout mice in adequately controlled experiments. First, we co-housed *Gpr15l*^{-/-} and WT mice for 5 weeks directly after weaning and subsequently re-separated them for 6 weeks to prevent further microbiota or GPR15L exchange by coprophagy.^{23–25} Stool was obtained before and after co-housing as well as after the re-separation period and 16S rRNA gene sequencing was performed (online supplemental figure 4).²¹ After weaning, knockout and WT mice had a clearly different beta-diversity. As expected, this assimilated after the co-housing period. However, differences in beta-diversity re-emerged after the re-separation period, consistent with GPR15L-driven trajectories in microbial communities. Very interestingly, the phenotype of DSS colitis was clearly associated with these observations in the beta-diversity. When DSS was administered to co-housed animals during continued co-housing, there were no differences in colitis activity (online supplemental figure 5A). When co-housed animals were re-separated after co-housing and DSS was administered directly afterwards, there was a trend towards increased disease activity in *Gpr15l*^{-/-} mice (online supplemental figure 5B). However, when co-housed mice were re-separated for 6 weeks and DSS was applied then, increased colitis activity in *Gpr15l*^{-/-} mice was fully re-established (online supplemental figure 5C).

While strongly encouraging the idea that GPR15L shapes microbial communities, this required further confirmation in a littermate setting. Thus, we crossed *Gpr15l*^{+/-} mice and compared *Gpr15l*^{-/-} and *Gpr15l*^{+/+} mice in further experiments. After weaning, such mice were either co-housed or separately housed before they received DSS. Indeed, co-housed mice had very similar disease activity (online supplemental figure 6A), while separately housed *Gpr15l*^{-/-} mice suffered from clearly more severe disease (figure 3E) along with increased infiltration of MPO⁺, CD4⁺ and CD11b⁺ cells to the colon and increased epithelial apoptosis (online supplemental figure 6B).

Taken together, these data showed that the intrinsic genetic alterations in the host alone were not sufficient to drive differential phenotypes in experimental colitis, but that host-microbiota interactions are required to explain the beneficial role of GPR15L for experimental colitis.

GPR15L acts as an antimicrobial peptide to shape microbial communities

To further understand the impact of GPR15L on microbial communities, we performed 16S rRNA gene sequencing on stool samples from *Gpr15l*-transgenic mice separately housed from their littermate controls after weaning and from *Gpr15l*^{-/-} and *Gpr15l*^{+/+} littermate mice co-housed or separately housed after weaning.²¹ While the principal component analyses of the beta-diversities in these mice showed some overlap after weaning (T1; figure 4A; online supplemental figure 7A), a clear divergence appeared after separation (T2; figure 4A; online supplemental figure 7A), whereas there even occurred an assimilation in co-housed mice (online supplemental figure 7B). Alpha-diversity measures were overall not significantly different, but trended towards higher richness in the knockout mice (figure 4B). However, taxonomic analyses further suggested that GPR15L is associated with clearly distinct communities. The abundance of commensal taxa such as *Prevotellaceae* and *Alistipes*, which have previously been linked with anti-inflammatory potential,^{26–29}

was reduced and *Lachnospiraceae*, which has been associated with CD,³⁰ was enriched in *Gpr15l*^{-/-} compared with *Gpr15l*^{+/+} mice (figure 4C; online supplemental figure 8A). Interestingly, functional *in silico* prediction with PICRUSt2 (figure 4D) showed that *Gpr15l* proficiency is associated with the upregulation of a number of metabolic pathways that are considered beneficial in the context of chronic colitis such as increased mixed acid fermentation, which can lead to higher amounts of protective short-chain fatty acids.³¹ Similarly, among others, this was the case for serotonin and dopamine degradation as well as flavin biosynthesis, which are necessary for within-community metabolic interchange and for bacterial cross-feeding protecting against intestinal inflammation and associated with clinical remission in IBD.^{32–33}

To further trace the trajectories induced by GPR15L, we performed shotgun metagenomic sequencing from the stool of *Gpr15l*^{-/-} and *Gpr15l*^{+/+} littermates after weaning and after 8 weeks of separation.²¹ Indeed, clear differences in the evolution of the communities became apparent (figure 4E). More precisely, consistent with the 16S data, we observed an increase in beneficial genera such as *Lachnospiraceae* and *Alistipes* in *Gpr15l*-proficient mice (figure 4F). To the contrary, the abundance of these genera as well as of *Roseburia* and *Rikenella*, which are also considered protective in IBD,^{34–36} decreased in *Gpr15l*-deficient mice. At the same time, *Bacteroides*, which have been ascribed roles as opportunistic pathogens in the context of IBD,^{37–40} decreased in *Gpr15l*^{+/+}, but increased in *Gpr15l*^{-/-} mice. Collectively, these data consistently associated GPR15L with a more homeostatic composition of microbial communities *in vivo*.

To investigate whether this is a direct mechanism, we performed radial diffusion assays under anaerobic conditions as prevailing in the colon and incubated a set of pathogenic and commensal bacteria with GPR15L. While we observed a clear inhibitory role on the growth of pathogenic species such as *Escherichia coli*, *Salmonella enterica* sv. Typhimurium or *Enterococcus faecalis*, bacteria that were previously linked with anti-inflammatory potential such as *Parabacteroides faecis*, *distasonis* and *goldsteini*^{34–41} were not affected (figure 4G, online supplemental figure 8B). In a next step, we aimed to further characterise the anti-microbial properties of GPR15L. To this end, we explored the growth of *S. enterica* sv Typhimurium on treatment with various concentrations of GPR15L (using polymyxin B as a reference) under anaerobic conditions. We observed a dose-dependent anti-microbial activity of GPR15L with a minimum inhibitory concentration (MIC) of 14.7 µM and an inhibitory concentration corresponding to 50% growth reduction (IC₅₀) of 11.3 µM (figure 5A,B). Since GPR15L is a highly cationic peptide, we aimed to investigate the possibility that it exerts its anti-microbial activity through interaction with the bacterial cell wall.²² Thus, bacteria were treated with 32 µM GPR15L together with various concentrations of NaCl to disrupt ionic interactions.^{42–43} Indeed, NaCl dose-dependently counteracted the anti-microbial activity of GPR15L with an MIC of 26 mM (figure 5C). *Vice versa*, the growth of bacteria treated with 100 mM NaCl was not affected by GPR15L along the full range of concentrations tested (figure 5D).

Moreover, consistent with the concept of luminal anti-microbial activity of GPR15L, we were able to detect substantial amounts of the peptide in stool samples from healthy human donors (figure 5E).

Finally, to provide further evidence for the relevance of GPR15L for shaping intestinal microbial communities to modulate susceptibility to experimental colitis, we treated WT mice with a broad-spectrum antibiotic cocktail to reduce the native

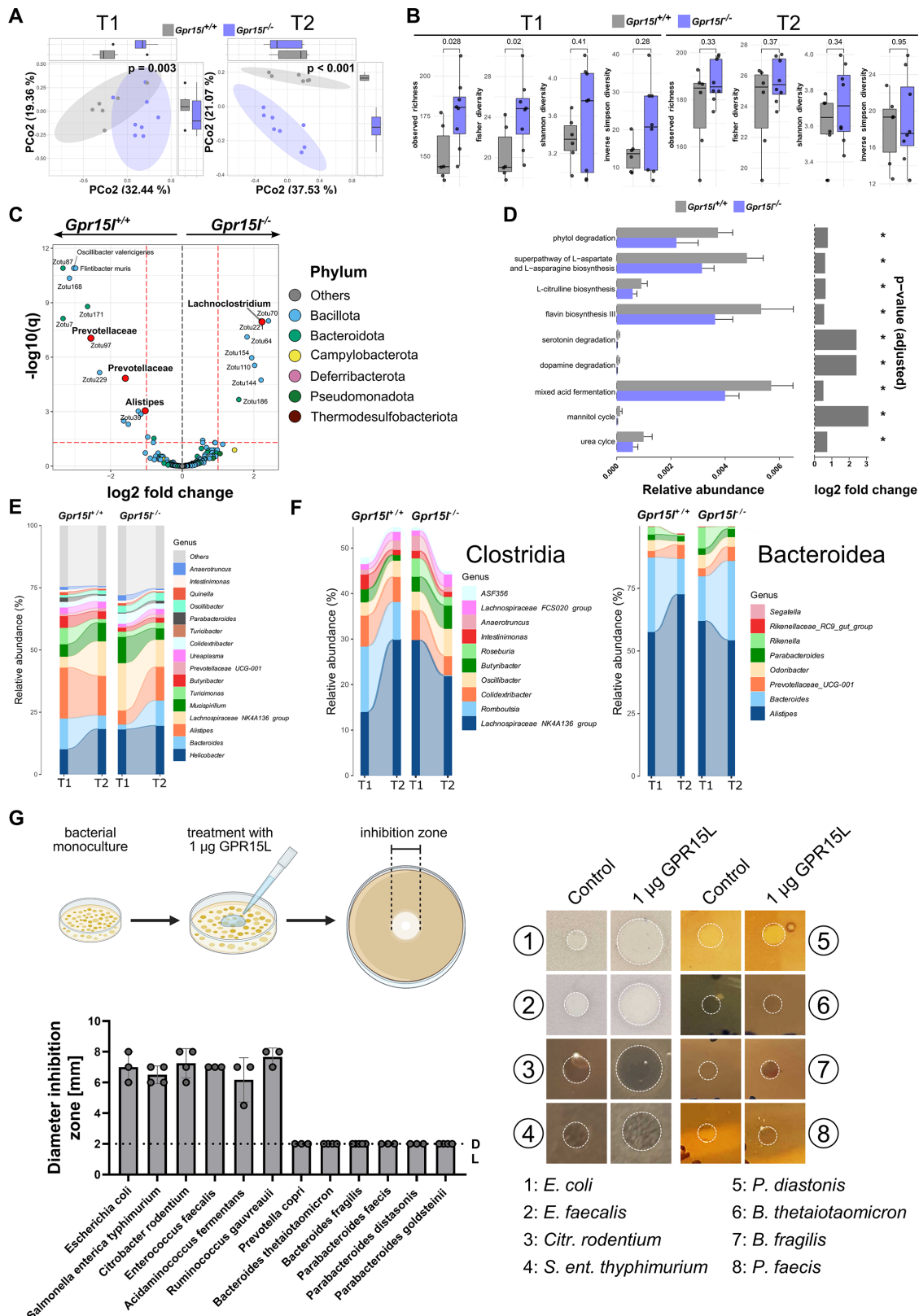


Figure 4 *GPR15L* drives profound alterations of the gut microbiome *in vivo* and has antimicrobial effects on intestinal bacteria *in vitro*. Analysis of the microbiome in faecal samples from littermate *Gpr15l^{-/-}* and *Gpr15l^{+/+}* mice before (T1) and after separation (T2; cf. figure 3E) with 16S rRNA sequencing (A–D) and shotgun metagenomics (E, F). (A) Beta diversity (Bray-Curtis dissimilarity). (B) Alpha diversity (observed richness, Fisher diversity, Shannon diversity and inverse Simpson diversity). (C) Differential abundance (Limma (voom)) of bacterial taxa in *Gpr15l^{-/-}* vs *Gpr15l^{+/+}* mice. (D) Upregulated pathways in *Gpr15l^{+/+}* mice identified by prediction of metagenome functions with PICRUSt2. (E, F) Relative abundance and trajectories of abundance of the bacterial genera (E) with separate analysis of the phyla *Clostridia* and *Bacteroides* (F). (G) Radial diffusion assays with selected intestinal bacteria and with or without GPR15L treatment under anaerobic conditions. Left upper panel: schematic representation of the experimental design. Representative (right panels) and quantitative (left lower panel) analysis of antimicrobial activity of GPR15L.

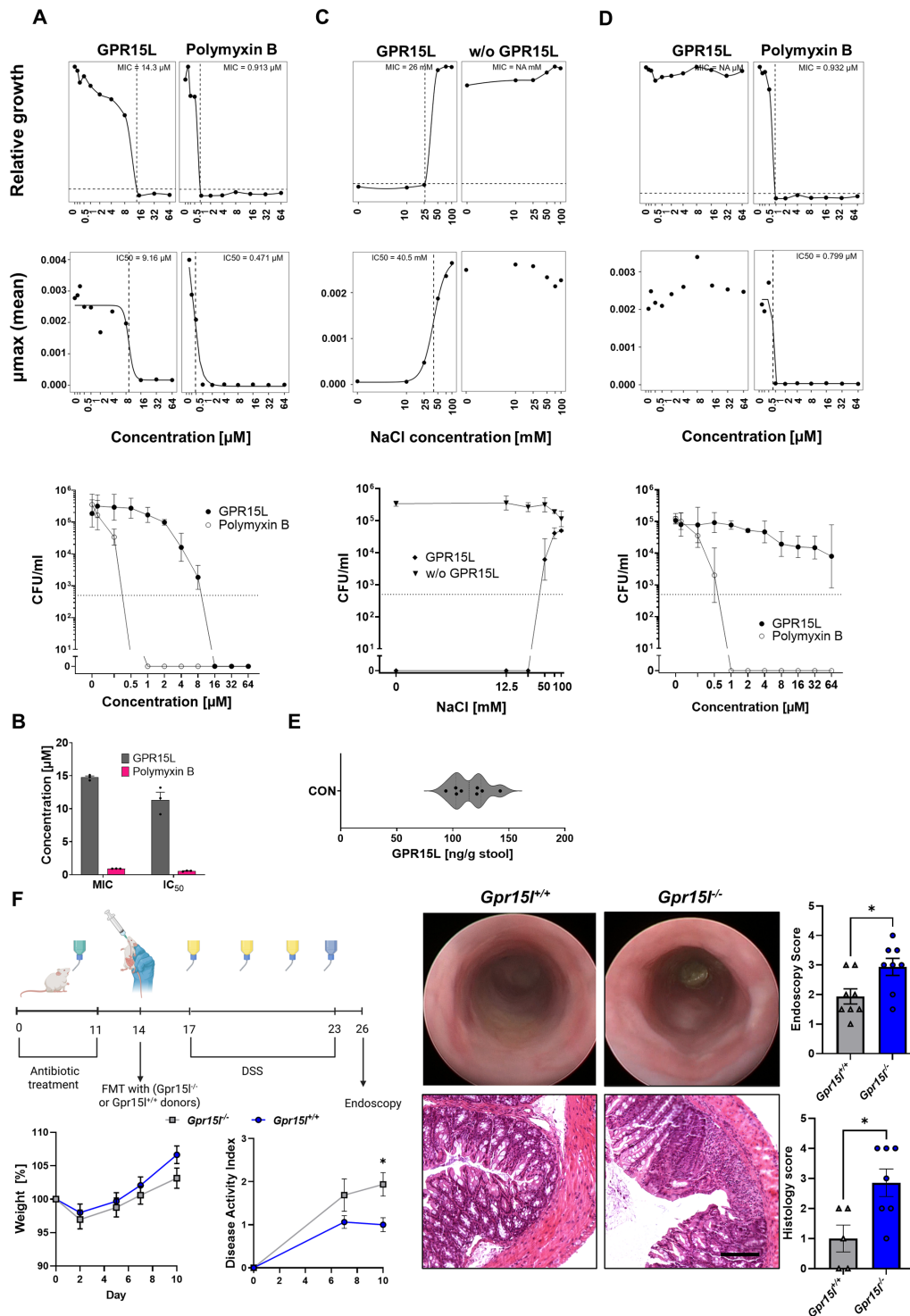


Figure 5 Characterisation of GPR15L anti-microbial properties and dextran sodium sulfate (DSS) colitis after faecal microbiota transfer (A–D) Growth of *Salmonella enterica* sv. Typhimurium with (A), (C) and (D) showing representative relative growth based on absorbance from an end-point assay with the derived minimum inhibitory concentrations (MIC) (upper row), inhibitory concentrations corresponding to 50% growth reduction (IC₅₀) deduced from maximum growth rates (μ max) of growth kinetics (middle row) as well as impact on viability based on colony forming units (CFU, lower row), at various concentrations of anti-bacterial compounds (A, D) or NaCl (C). All graphs are representative for three individual experiments. (A) Growth on treatment with GPR15L or polymyxin B as indicated. (B) Mean $\pm$ SEM of the MIC and IC₅₀ for GPR15L and polymyxin B. (C) Bacterial growth on treatment with 32 μ M GPR15L together with NaCl at the indicated concentrations. (D) Microbial growth on treatment with 100 mM NaCl together with GPR15L or polymyxin B at the indicated concentrations. (E) Quantification of GPR15L in stool samples from healthy human donors (CON) with ELISA. (F) Acute DSS colitis with littermate WT mice treated with 2% DSS after broad-spectrum antibiotic treatment for microbial depletion and subsequent faecal microbiota transfer (FMT) from *Gpr15l*^{-/-} or *Gpr15l*^{+/+} mice. Left upper panel: Schematic representation of the experimental design. Left lower panels: Weight course and clinical disease activity. Right upper panels: Representative and quantitative endoscopic disease activity. Right lower panels: Representative and quantitative histologic disease activity. n=5–8 per group. Scale bar: 100 μ m. * p<0.05. IC₅₀, inhibitory concentration corresponding to 50% growth reduction; MIC, minimum inhibitory concentration.

flora and subsequently performed faecal microbial transfer (FMT) from *Gpr15l*-deficient or *Gpr15l*-proficient mice before inducing DSS colitis. Interestingly, the phenotype of the donor mice was reproduced in the FMT recipients with increased disease activity (figure 5F) along with higher infiltration of pro-inflammatory immune cells and more epithelial cell death (online supplemental figure 9) in mice that had received FMT from *Gpr15l*^{-/-} mice.

Taken together, these data strongly suggested that GPR15L acts as a selective anti-microbial peptide to promote homeostasis and to counteract colitis in the gut.

Treatment with GPR15L alleviates experimental colitis and beneficially shapes microbial communities from the human gut

Given these observations, we wondered whether it is possible to therapeutically exploit the antimicrobial properties of GPR15L in experimental colitis. To this end, we induced acute DSS colitis in *Gpr15l*^{-/-} mice and rectally supplemented recombinant GPR15L once daily from day 3 to day 9 at a concentration close to the MIC identified above. As compared with placebo, such treatment clearly decreased clinical, endoscopic and histological disease activity (figure 6A). Consistently, the abundance of MPO⁺, CD11b⁺ and CD4⁺ cells in the colon as well as epithelial cell death decreased (online supplemental figure 10). Moreover, treatment with GPR15L induced clear shifts in the intestinal microbiome of these mice, since 16S rRNA sequencing from stool obtained before and at the end of the experiments demonstrated that the initially similar alpha and beta diversities were different after treatment (figure 6B,C).

To model which effects such treatment might induce in patients, we obtained suspensions of stool from human donors and treated them ex vivo under anaerobic conditions with GPR15L or placebo control prior to shotgun metagenomic sequencing (figure 6D; online supplemental table 1).²¹ As expected, this resulted in clearly different alpha and beta diversities (figure 6E). At the genus level, this corresponded to distinct microbial profiles, most strikingly with *Escherichia* being enriched under control treatment and *Bifidobacteria* under GPR15L treatment (figure 6F). At the species level, consistent with the radial-diffusion assay data, pathogenic bacteria such as *E. coli* were clearly decreased on GPR15L treatment. Similarly, consistent with the data obtained in mice, *Lachnospiridia* and other bacteria that have been specifically linked to IBD such as *Ruminococcus torques* or *Blautia wexlerae*^{44 45} were downregulated (figure 6G). On the other hand, commensal bacteria associated with protection from IBD such as *Bifidobacterium longum* or several *Prevotella* species^{46 47} were increased after GPR15L treatment. Metabolic function inference with HUMAnN4 revealed an upregulation of pathways such as fatty acid beta oxidation and fructan biosynthesis (figure 6H), which have previously been linked with mucosal homeostasis and cross-feeding capacity.^{48 49}

Collectively, these data showed that rectal supplementation of GPR15L mitigates experimental colitis and is associated with shifts towards a potentially protective composition in microbial communities from the human large intestine.

GPR15L is decreased during active IBD and correlates to microbial richness and flare-free survival

To further evaluate the translational relevance of GPR15L for IBD, we studied its expression in patients with UC, CD and non-IBD controls. Initially, we performed quantitative PCR on mRNA

from biopsies available in the biobank of the University Hospital Erlangen. As expected, expression in the colon was higher than that in the ileum (online supplemental figure 11A). Moreover, focusing on colon biopsies, we observed a substantial decrease of GPR15L expression in patients with UC, but not in those with CD (figure 7A; online supplemental table 2). In CD, there was no association of GPR15L expression with disease extent (online supplemental figure 11B). In UC, the expression showed a clear association with endoscopic disease activity as categorised by the Mayo score (figure 7B). To corroborate these findings at the protein level, we stained colon sections from patients with IBD and non-IBD controls for GPR15L and Epithelial Cell Adhesion Molecule (EpCAM). To correct for the epithelial damage associated with severe inflammation, we normalised GPR15L to EpCAM intensity. Yet, we observed a strong reduction of GPR15L expression both in UC and CD (figure 7C; online supplemental table 3).

We further studied the mRNA expression of GPR15L in colon biopsies from a subset of a previously reported cohort of patients with UC from Switzerland.¹² GPR15L was clearly decreased in pancolitis patients with endoscopic disease activity as compared with patients in endoscopic remission (figure 7D; online supplemental table 4). Moreover, consistent with the data on GPR15L treatment shown above (figure 6C,E), GPR15L expression was negatively correlated with various measures of the microbial alpha-diversity observed in tissue samples from the same donors (figure 7E).

Furthermore, we studied the expression of GPR15L in patients with UC in the IBDome database pooling transcriptomic and clinical data from a German multi-centre cohort.⁵⁰ Again, GPR15L expression was negatively associated with clinical and endoscopic disease activity (figure 7F; online supplemental table 5), complementing the evidence for its downregulation in active IBD across three independent cohorts.

Finally, we prospectively monitored the further course of UC in patients in endoscopic remission and determined the expression of GPR15L mRNA in colon biopsies at baseline. In an analysis that was not adjusted for potential confounders, we observed that high GPR15L expression (above the median of the whole cohort) was linked to a clearly better flare-free survival as compared with low GPR15L expression (figure 7G; online supplemental table 6).

Together, these data demonstrated a downregulation of GPR15L in active UC and supported the notion that it modulates the intestinal microbiome to counteract IBD.

DISCUSSION

Despite the previous implication of GPR15 in controlling lymphocyte homing to the large intestine,^{16 20 51} the exact role of its endogenous ligand GPR15L in the context of chronic intestinal inflammation has been unclear to date. Here, we report for the first time that GPR15L is indeed involved in experimental colitis and IBD, but surprisingly rather via a mechanism as an antimicrobial peptide than by driving the recruitment of T cells to the inflamed gut.

Pleiotropic functions of GPR15L had previously been described. Prior to its nomination as the so far only ligand of GPR15,^{18 19} antimicrobial properties²² and cell cycle modulation via the receptor SUSD2 inhibiting the proliferation of colorectal cancer cells⁵² had been described in vitro. However, the impact on T cell homing had dominated the discussion in the field of IBD⁵³ and the relative contribution of these different functions had not been explored. Using two complementary experimental models of colitis, our data clearly show that neither T cells nor GPR15 are required for the beneficial role of GPR15L. Moreover, we provide comprehensive evidence for

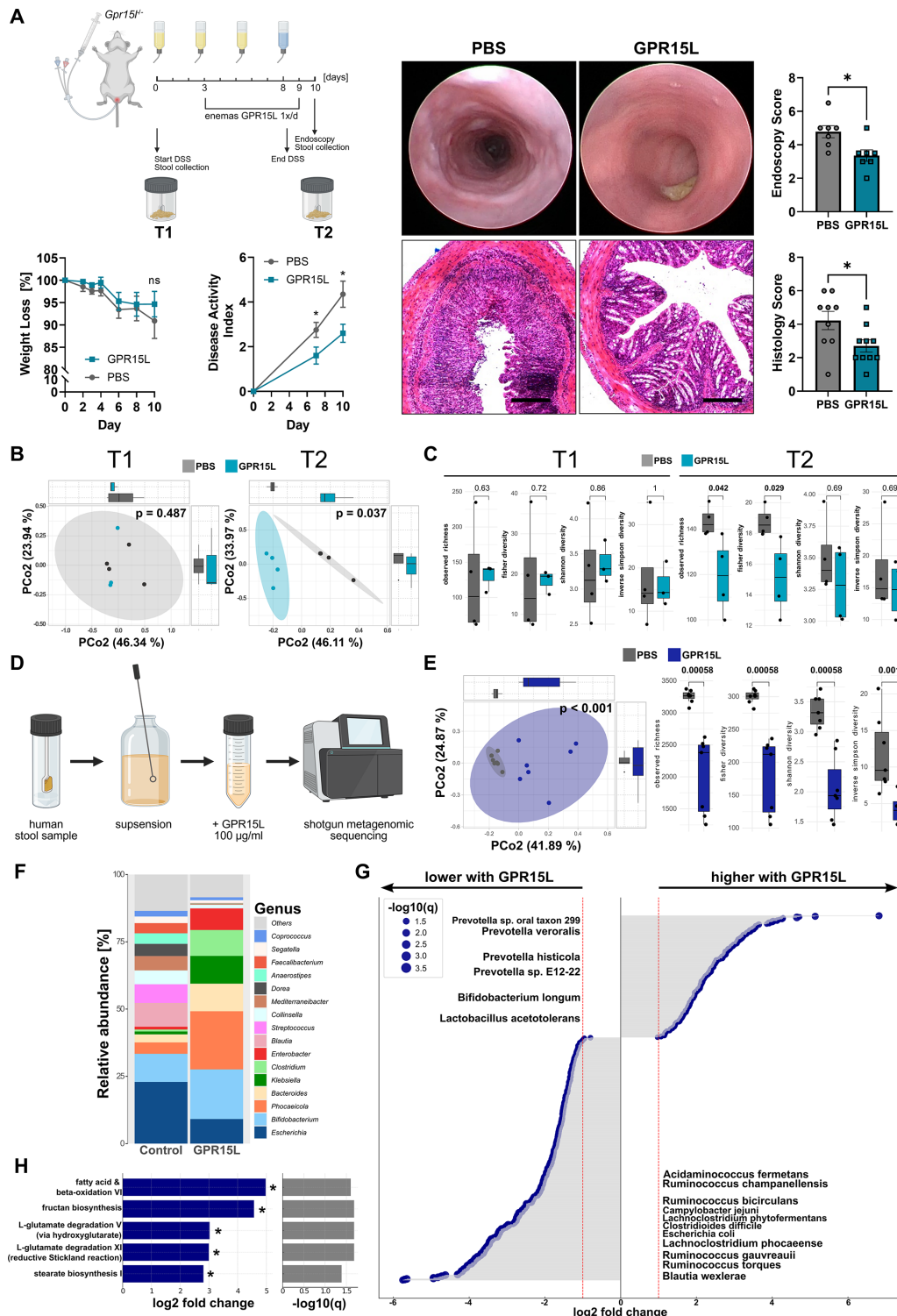


Figure 6 Treatment with GPR15L mitigates colitis and promotes a homeostatic gut microbiome. (A-C) Acute dextran sodium sulfate (DSS) colitis with littermate *Gpr15l*^{-/-} mice treated with rectal GPR15L or placebo (PBS). (A) Left upper panel: schematic representation of the experimental design. Left lower panels: weight course and clinical disease activity. Right upper panels: representative and quantitative endoscopic disease activity. Right lower panels: representative and quantitative histologic disease activity. n=7–10 per group. Scale bars: 100 µm. (B) Beta diversity (Bray-Curtis dissimilarity) of faecal samples collected before (T1) and after (T2) DSS colitis. (C) Alpha diversity of faecal samples obtained at T1 and T2, indicated by observed richness, Fisher diversity, Shannon diversity and inverse Simpson diversity. (D-H) Treatment of human faecal samples with GPR15L *ex vivo*. (D) Schematic representation of the experimental design. Faecal suspensions were incubated with GPR15L or control solution for 16 hours under anaerobic conditions. Samples were analysed by shotgun metagenomic sequencing (E-H). (E) Relative abundance of the bacterial species and genera. (F) Beta diversity (Bray-Curtis dissimilarity) and alpha diversity (observed richness, Fisher diversity, Shannon diversity and inverse Simpson diversity). (G) Upregulated metabolic pathways in GPR15L-treated samples as predicted by HUMAnN4 analysis. (H) Differential abundance analysis with bias correction of 664 significantly regulated microbial species in GPR15L-treated versus GPR15L-untreated samples. Selected taxa are highlighted. n=7. *p<0.05. PBS, phosphate-buffered saline.

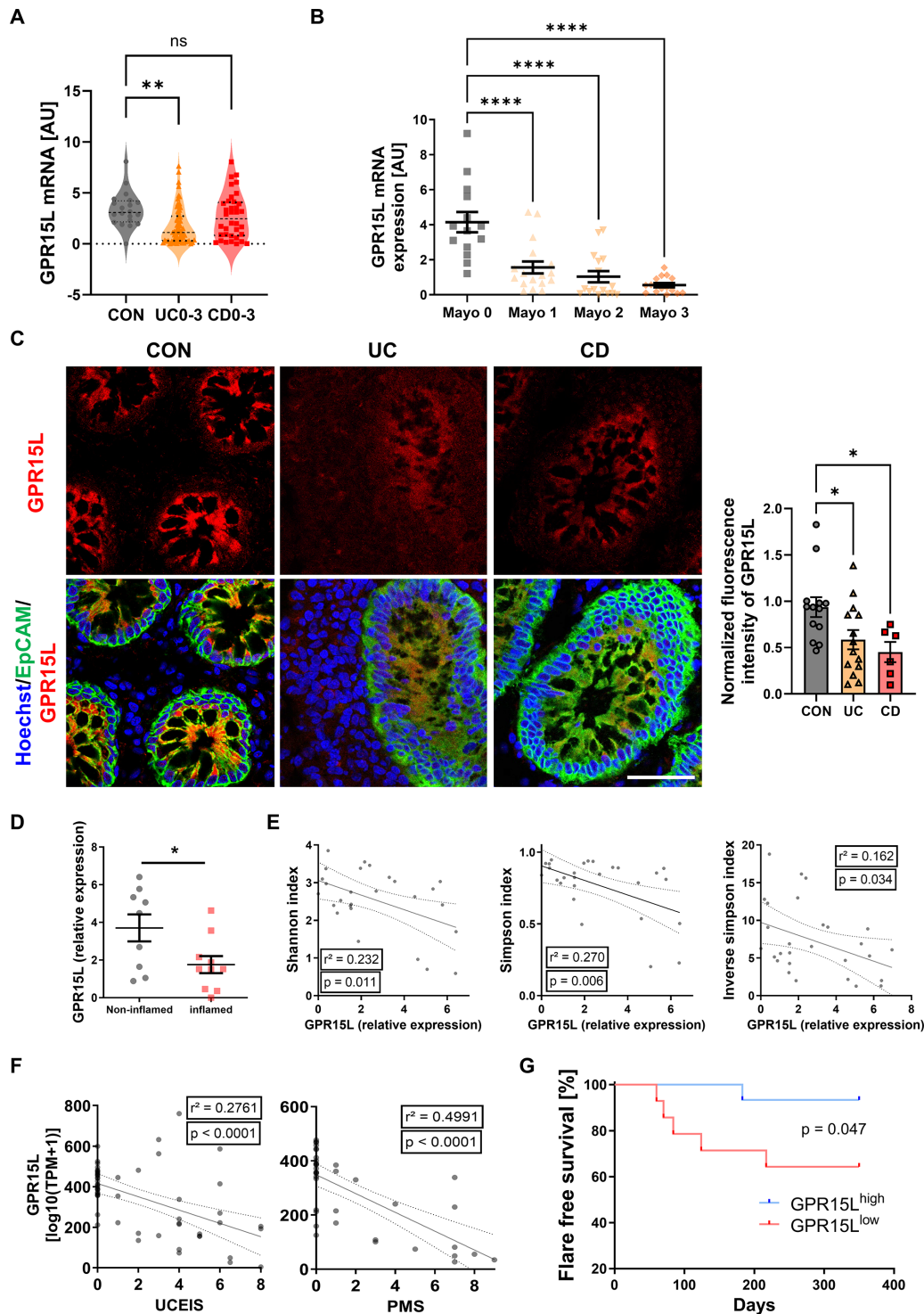


Figure 7 GPR15L is downregulated in active human IBD and correlates with flare-free survival. (A–C) GPR15L in a German cohort of patients with IBD. (A, B) qPCR analysis of *GPR15L* mRNA expression in colon biopsies from healthy controls and patients with IBD (A) and stratified in the UC subgroup according to endoscopic Mayo scores. $n=20$ (control), 36 (CD), 64 (UC). (C) Representative (left panels) and quantitative (right panel) immunofluorescence analysis of epithelial GPR15L expression in healthy controls ($n=13$) and patients with UC ($n=14$) or CD ($n=7$). The epithelium was defined by EpCAM expression and the mean fluorescence intensity (MFI) of the GPR15L signal within this region normalised to the MFI of EpCAM. (D, E) GPR15L in a Swiss cohort of patients with UC. (D) Quantitative qPCR analysis of *GPR15L* mRNA expression in colon biopsies from patients with ulcerative pancolitis stratified by the presence of endoscopic disease activity. $n=9$ –10 per group. (E) Correlation of *GPR15L* expression with microbial alpha diversity as indicated by Shannon diversity, Simpson diversity and inverse Simpson diversity. (F) GPR15L in the IBDome cohort. Correlation of colonic *GPR15L* mRNA expression as determined by bulk RNA sequencing with endoscopic (left) and clinical (right) disease activity. $n=61$. (G) Kaplan-Meier curve of flare-free survival in patients with UC in endoscopic remission from the Erlangen cohort stratified according to *GPR15L* expression above ('high', blue) or below ('low', red) the median of these patients. $n=29$. AU, arbitrary units; CON, healthy control; CD, Crohn's disease; EpCAM, epithelial cell adhesion molecule; PMS, Partial Mayo score; UCEIS, Ulcerative Colitis Endoscopic Index of Severity. Scale bar: 50 μ m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

the crucial function that GPR15L exerts as a selective antimicrobial peptide to shape homeostatic microbial communities and demonstrate that this is closely mirrored by colitis phenotypes. Our findings on increased epithelial cell death in *Gpr15L*^{-/-} mice together with the increased inflammation observed in these mice clearly argue against a functional relevance for a potential delay in epithelial proliferation.⁵² Thus, we conclude that the antimicrobial activity is probably the essential mechanism to explain the impact of GPR15L on experimental colitis.

This seems to contrast a recent report suggesting that *Gpr15L* promotes intestinal inflammation⁵⁴: O'Connor and colleagues compared *Gpr15L*^{-/-}*Gpr15L*^{-/-} with *Gpr15L*^{-/-} mice in an acute DSS colitis model and observed increased disease activity in *Gpr15L*-proficient mice. Importantly, however, only modest differences were observed, when *Gpr15L* knockout and WT mice without *Gpr15* deficiency were compared in the acute DSS colitis model and a trend towards increased disease activity was found with *Gpr15L* knockout mice in the acute trinitrobenzene sulfonic acid colitis model. Moreover, no littermate controls were used in these experiments and mice were co-housed throughout, which is associated with coprophagy-mediated microbiota exchange^{23,24} and resulted in comparable beta-diversity in our experiments. Thus, consistent with our data, this report highlights that GPR15-independent functions of GPR15L are important in the context of chronic colitis. However, while underscoring the need for further investigations into the interplay of GPR15L with GPR15, for which pleiotropic functions have also been described⁵⁵ and might contribute to the observations, the experiments were not designed to address the antimicrobial potential of GPR15L and can therefore not be seen as a contradiction to our findings.

Given the overall functional profile demonstrated by our and other data,^{20,22} GPR15L should be regarded as a host defence peptide similar to defensins or cathelicidins such as Leucine-Leucine-37 (LL-37),⁵⁶ for which similar MIC and IC₅₀ have been reported.^{57–59} Functionally, these peptides similarly possess antimicrobial properties as well as important roles for controlling immune responses including immune cell recruitment.⁶⁰ GPR15L shares a highly cationic structure with these peptides, which therefore suggests that it might possess a similar mechanism of antimicrobial activity based on interaction with the negatively charged wall of bacteria finally leading to pore formation and membrane disintegration (Shai-Matsuzaki-Huang model).^{9,57} This might also explain the selective activity of GPR15L, since commensal gut microbiota have developed strategies to decrease the potential for such interactions.⁶¹ While the identification of the exact anti-bacterial mechanism of GPR15L was not within the scope of our study, this speculation is supported by our data demonstrating salt-sensitivity of the anti-microbial activity of GPR15L.^{42,43} Moreover, in our functional prediction analyses, the stearate biosynthesis pathway was upregulated in response to GPR15L, which might indicate a membrane attacking antimicrobial effect of GPR15L leading to membrane stress and consecutive modulation of phospholipid biosynthesis, as studied for other membrane active antibiotics.⁶² Further studies are therefore warranted to investigate the precise mechanism of action of GPR15L, for instance by using super-resolution microscopy to visualise GPR15L-bacterial interactions or by treating genetically modified bacteria with GPR15L to explore its impact on specific bacterial metabolic pathways.

Beyond these mechanistic considerations, GPR15L was associated with clear homeostatic footprints in microbial consortia across different datasets as well as in antimicrobial assays at the species level. Specifically, we consistently observed that commensal bacteria that have been associated with protective functions for mucosal homeostasis such as *Prevotellaceae* or *Alistipes*^{28,29,47} were enriched in the presence of GPR15L, while pathogenic bacteria such as *E. coli* or *E.*

faecalis^{63,64} decreased. On the other hand, in the absence of GPR15L, *Bacteroides* and taxa such as *Lachnospirillum* that have been associated with IBD^{30,37,39} were enriched and key commensals such as *Roseburia* or *Rikenella*^{34–36} decreased in their abundance. Although clear cause-effect relationships between complex intestinal microbial consortia and mucosal homeostasis or even chronic intestinal inflammation still need to be established, the overall available evidence clearly supports the interpretation that these GPR15L-dependent trajectories in microbiome composition promote homeostatic host-microbiota crosstalk. Additional research is necessary to further formalise these conclusions that are partly still based on correlations rather than on causal evidence. Most importantly, this should include FMT experiments on germ-free mice, not only to explore the susceptibility to experimental colitis dependent on microbial communities shaped in the presence or absence of GPR15L, but also to longitudinally investigate the trajectories in microbial composition in *Gpr15L*-competent hosts receiving FMT from *Gpr15L*-deficient donors and *vice versa*.

Our observations on the effects of GPR15L on microbial consortia from human stool and on its expression in patients with IBD including a downregulation of GPR15L in active UC across various cohorts, its co-regulation with microbial alpha-diversity and its association with flare-free survival strongly suggest that the antimicrobial properties and the beneficial role against colitis observed in mice are conserved in human patients. Thus, also based on the therapeutic potential demonstrated in experimental colitis, this suggests that GPR15L might serve as a future therapeutic approach in IBD. In particular, topical delivery to the large intestine might counteract disease driving dysbiosis and re-establish homeostatic host-environment communication.⁶⁵ Eventually, this might even hold promise in prophylactic settings.

Thus, collectively, our data not only uncover a previously unknown role of GPR15L in modulating intestinal inflammation but also open avenues towards novel strategies to mitigate IBD based on selectively interfering with initial pathogenetic steps of host-microbiota crosstalk complementing the established targets in downstream pathways of immune system activation.

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MFN and SZ provided clinical samples, protocols, reagents or designed experiments. SW, RGG, MD and AE performed *in silico* analyses. ML, SS, LD, BO, FPB, SHM, TMM, BOS, AM, ECB, MFN and SZ analysed and interpreted the data. The manuscript was written by SZ with the help of ML, SS, TMM and MFN. All authors critically revised the manuscript for important intellectual content. Guarantor: SZ.

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