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Gpr109a limits microbiota-induced IL-23 production to constrain ILC3-mediated colonic inflammation

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Abstract

A set of coordinated interactions between gut microbiota and the immune cells surveilling the intestine play a key role in shaping local immune responses and intestinal health. Gpr109a is a G-protein coupled receptor expressed at a very high level on innate immune cells and previously shown to play a key role in the induction of colonic Tregs. Here we show that *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice exhibit spontaneous rectal prolapse and colonic inflammation, characterized by the presence of an elevated number of IL-17-producing Rorγt^{+} innate lymphoid cells (ILC3). Genetic deletion of Rorγt ameliorated the spontaneous colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Gpr109a-deficient colonic dendritic cells produce higher amounts of IL-23, and thereby promote ILC3. Moreover, the depletion of gut microbiota by antibiotics treatment decreased IL-23 production, ILC3, and colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. The caecums of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice showed significantly increased colonization by members of *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellacea, Streptococcaceae*, *Christensenellaceae*, and *Mogibacteriaceae* as well as IBD-associated microbiota such as *Enterobacteriaceae* and *Mycoplasmataceae* than *Rag1*$^{-/-}$ mice, housed in a facility positive for *Helicobacter* and murine norovirus. Niacin, a Gpr109a agonist, suppressed both IL-23 production by colonic DCs and ILC3 number in a Gpr109a-dependent manner. Collectively, our data presents a model suggesting that

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Disclosures

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targeting Gpr109a will be potentially beneficial in the suppression of IL-23 mediated immunopathologies.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), together termed inflammatory bowel diseases (IBD), are chronic and relapsing inflammatory diseases of the gut with unknown etiology. Recent observations indicate that a complex interaction between an individual's genetics, immune system, diet, and gut microbiota plays a critical role in the development of IBD (1, 2). Genetic studies have identified more than 100 susceptibility loci linked to IBD (3). Dysregulation of adaptive immune responses is thought to be a dominant reason for the induction of IBD (4–8). However, recent findings indicate that an anomaly in innate responses also plays a critical role in the initiation and progression of IBD. Single nucleotide polymorphisms in nucleotide-binding oligomerization domain 2 (NOD2) are associated with susceptibility to Crohn's disease (9, 10). In addition, Crohn's disease is also associated with mutations in the ATG16L1 and IRGM genes (11, 12). These findings have generated considerable interest in the activation and regulation of the innate immune system at the gut mucosal surface in the pursuit of understanding the mechanisms regulating the pathogenesis of IBD.

Innate lymphoid cells or ILCs are a recently identified family of immune cells that are found at a much lower frequency than adaptive immune cells. They are mostly present at barrier surfaces such as the gut, lungs, and skin. They play an important role in the induction, regulation, and resolution of inflammatory responses. ILCs are of lymphoid origin but lack antigenic receptor expressed by B and T cells, as well as the markers associated with myeloid cells. ILCs are stimulated by microbes and cytokines present in the microenvironment to rapidly produce proinflammatory and regulatory cytokines. Based on the expression of transcription factors and related cytokines, ILCs are classified into three groups. ILC1 express T-bet and produce IFN-γ and TNF-α. ILC2 express high levels of GATA3 and produce IL-5 and IL-13. ILC3 express transcription factor Rorγt and produce IL-17 and IL-22. ILC3 play a critical role in regulating and promoting inflammation in the intestine. IL-22 producing ILC3 protect the intestine by inducing production of anti-bacterial peptides by the gut epithelium (13, 14), promoting protein fucosylation, which modifies bacterial metabolism and attenuates their virulence (15), containing intestinal bacteria (16), inducing T cell tolerance to gut bacteria antigens (17, 18), maintaining the tolerogenic potential of intestinal DCs (19), and protecting intestinal stem cells during graft versus host disease (20). In contrast, in *Helicobacter hepaticus*- or anti-CD40-driven colitis in *Rag1*$^{-/-}$ mice and in spontaneous colonic inflammation in TRUC (*Tbx21*$^{-/-}$*Rag2*$^{-/-}$) mice, ILC3 induce colonic inflammation (21–25). Similarly, in IBD, IL-22 producing ILC3 are decreased (26, 27). IL-22 produced by ILC3 promotes colon carcinogenesis (28). In the azoxymethane (AOM)/ dextran sulfate sodium (DSS) model, IL-22 production at the peak of inflammation leads to the resolution of inflammation and the inhibition of carcinogenesis. In contrast, uncontrolled IL-22 production in the same model supports tumorigenesis (29).

Gpr109a is a G protein coupled receptor that is highly expressed on innate immune cells and adipose tissue. Gpr109a is activated by niacin and butyrate, a short chain fatty acid and fermentation product of dietary fiber by gut microbiota. Selected gut microbiota such as *Lactobacillus acidophilus* produce niacin. Niacin deficiency results in intestinal inflammation and diarrhea (30, 31). We have previously shown that Gpr109a signaling plays a critical role in the homeostasis of Treg cells in the colon and in the suppression of colonic inflammation and carcinogenesis (32). However, whether Gpr109a regulates innate lymphoid cells and its relevance to colonic inflammation and carcinogenesis remain unknown. Our study identifies a critical role for Gpr109a in the inhibition of IL-23 production by colonic DCs leading to suppression of ILC3 and colonic inflammation.

Materials and Methods

Mice

Rag1$^{-/-}$ (C57BL/6 background), *Rorγt*$^{-/-}$ (C57BL/6 background) and C57BL/6 mice were originally from Jackson Laboratory (Bar Harbor, ME) and bred on-site. *Gpr109a*$^{-/-}$ mice (C57BL/6 background) have been described. *Gpr109a*$^{-/-}$ and *Rag1*$^{-/-}$ mice were interbred to generate *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Murine norovirus and *Helicobacter* were detected in our mouse colony. The Institutional Animal Care and Use Committee (IACUC), Augusta University approved all animal procedures.

Histopathology

Colons were excised and fixed in neutral buffered formalin (Thermo Fisher, Waltham, MA). Fixed colon tissues were embedded in paraffin and 5 μm thick sections were sliced and placed on glass microscope slides. Hematoxylin and eosin (H&E) staining on sections were visualized using Olympus BX43 microscope. H&E stained sections were scored for colitis based on 4 histologic parameters and leukocyte infiltration, as follows: 0 = normal histology, 1 = mild hyperplasia of epithelium, 2 = moderate hyperplasia with marked leukocyte infiltration, 3 = severe hyperplasia with leukocyte infiltration and significant decrease in goblet cells, 4 = severe hyperplasia with inflammatory cells, ulceration, crypt abscesses and severe depletion of goblet cells.

Cell Isolation and Analysis

For the isolation of intestinal lamina propria cells, colons and small intestines were opened and luminal contents were removed. Intestines were cut into ~ 1 centimeter pieces and shaken in Hanks Balanced salt solution (HBSS, Corning, Corning NY) containing 5% fetal bovine serum (GE Healthcare, Logan, Utah), 5mM EDTA, 10 mM HEPES pH 7.4 (Sigma, St. Louis, MO) twice for 20 minutes each to remove the epithelial layer. The left over intestinal pieces were digested with 0.5 mg/ml of collagenase D and DNAse 1 (Roche Diagnostics, Germany) twice for 30 minutes each. The released cells were collected by centrifugation and contaminating epithelial cells were removed by centrifugation over 40% percoll. The cell pellet was washed and used as lamina propria immune cells. Cells were stained for CD45, Rorγt, Ly6G, GATA3, CD11b, CD11c, CD103, NKP46, Sca, CD127, IL-17, IL-22 (Thermo-Fisher, Waltham, MA), Thy1.2, T-bet, Ly6G (Biolegend, San Diego, CA) and analyzed using a LSRII flow cytometer (BD Biosciences, San Jose, CA). Colonic

DCs were sorted as $CD45^+Thy1.2^-Ly6G^-CD11c^+$ population by FACS Aria Flow cytometer (BD Biosciences). In some experiments colonic DCs were sorted into $CD103^-$ and $CD103^+$ subsets.

Cell Culture

Isolated mesenteric lymph node and colonic or small intestinal lamina propria cells were cultured in 0.2 ml of RPMI 1640 (Corning, Corning NY) fortified with 10% fetal bovine serum (GE Healthcare, Logan, Utah), 10 mM HEPES pH 7.4 (Sigma, St. Louis, MO) and 50μM of 2-mercaptoethanol (Thermo-Fisher, Waltham, MA), 100 ng/ml of PMA (Sigma, St. Louis, MO) and 1 μM of ionomycin (EMD Millipore Billerica, MA), monensin and brefeldin A (Thermo-Fisher, Waltham, MA). Four hours later cells were fixed and intracellular staining for transcription factors and cytokines was performed. In some experiments, mesenteric lymph node $Thy1.2^+$ cells were cultured with colonic DCs in the presence of IL-2 (10 ng/ml) and IL-7 (10 ng/ml, both from Peprotech, Rocky Hill, NJ). Three days later cells were analyzed for Rorγt and IL-17 as above.

Antibiotics and niacin treatment

Mice were given a cocktail of ciprofloxacin.HCl (TCI, Portland, Oregon, 0.15 g/liter), gentamycin sulfate (0.2 g/liter), bacitracin (1g/liter) and streptomycin (2 g/liter) (Thermo-Fisher, Waltham, MA) in drinking water *ad libitum*. In some experiments, niacin (10 mg/ml) was given in drinking water *ad libitum* for the indicated period of time.

Quantitative real time PCR

Total RNA was extracted from cells using the RNeasy micro kit (Qiagen, Hilden, Germany). RNA was converted into cDNA using the Superscript III reverse transcription system (Thermo-Fisher, Waltham, MA). Quantitative polymerase chain reaction (qPCR) was performed using Sybr green PCR mix (Bio-rad, Hercules, CA) and StepOnePlus machine (Applied Biosystems). PCR primers were *Gpr109a;* forward 5'-ATGGCGAGGCATATCTGTGTAGCA-3', reverse 5'-TCCTGCCTGAGCAGAACAAGATGA-3' *Gapdh;* forward 5'AGGTCGGTGAACGGATTTG-3', reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3' *Hprt;* forward 5'-GCGTCGTGATTAGCGATGAAC-3' reverse 5'CCTCCCATCTCCTTCATGACATCT-3', *Il23P19;* forward 5'-GACCCACAAGGACTCAAGGA-3', reverse 5'-CATGGGGCTATCAGGGAGTA-3'.

Antibody treatment

Animals were intraperitoneally injected with anti-Thy1.2 (Clone 30H12) or isotype control (Clone LTF2, both from BioXcell, West Lebanon, NH) antibodies (100 μg/mouse/week). Two months later animals were sacrificed and used in experiments.

Microbiome sequencing

DNA from caecal content was isolated as described previously. Briefly ~100–200 mg of caecal content was suspended with 710 μL disruption buffer and 500 μL phenol/chloroform/isoamyl alcohol, pH 8.0, inside tubes containing Zirconium beads (0.1mm diameter,

Benchmark Scientific). Following centrifugation, DNA was precipitated from the aqueous phase using isopropanol. Sequencing (300-bp paired end) of V3–V4 regions of bacterial 16S rDNA was performed on the Illumina MiSeq platform and data was analyzed using Quantitative Insights into Microbial Ecology (QIIME).

Statistical analysis

Statistical significance was calculated using Student's t-test with two-tailed analysis.

Results

Spontaneous Colitis in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice

To test the role of Gpr109a in the absence of the adaptive immune system, we crossed *Gpr109a*$^{-/-}$ animals onto the *Rag1*$^{-/-}$ background. *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice spontaneously develop rectal prolapse. Rectal prolapse starts appearing as early as 3 months of age and approximately 50% of the animals developed rectal prolapses by 9 months of age (Fig. 1A, 1B). In contrast, *Rag1*$^{-/-}$ mice housed in the same colony and on the same rack did not show any sign of rectal prolapse (Fig. 1A, 1B). Colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice showed more mass than *Rag1*$^{-/-}$ mice (Fig. 1C). Moreover, colons and caecum of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice show signs of inflammation with infiltration of immune cells within the epithelial and sub-epithelial spaces. In addition, highly proliferative, crowded, and hyper cellular colonic crypts with elongated, hyperchromatic, and pseudostratified nuclei were present in the colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (Fig. 1D, 1F). The numbers of infiltrating neutrophils were also significantly increased in the colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$ mice (Supplemental Fig. 1A). This resulted in significantly higher colitis scores for colons and caecum of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice than *Rag1*$^{-/-}$ mice (Fig. 1E, 1G). Chronic colitis is one risk factor for the development of colon cancers. Signs of ongoing adenomatous transformation were present in the caecum of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (**arrows in** Fig. 1F). Spleens of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice were significantly enlarged and contained significantly more cells than *Rag1*$^{-/-}$ mice (Supplemental Fig. 1B–C).

Increased numbers of ILC3 in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice

To characterize the mechanisms underlying the enhanced colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, we analyzed the presence of innate lymphoid cell subsets in intestinal lamina propria and mesenteric lymph node cells. CD45^{+}Thy1.2^{+} cells were examined for expression of Rorγt and GATA3, markers for ILC3 and ILC2 respectively. Significantly higher numbers of Rorγt^{+} cells (ILC3) were present among CD45^{+}Thy1.2^{+} cells in the colonic lamina propria of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice than *Rag1*$^{-/-}$ mice (Fig. 2A, 2B). Similarly, compared to *Rag1*$^{-/-}$ mice, *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice exhibited increased numbers of ILC3 in their mesenteric lymph node and small intestines (Fig. 2A, 2B and Supplemental Fig. 2A, 2B). In sharp contrast, the frequency of CD45^{+}Thy1.2^{+} GATA3^{+} cells (ILC2) was significantly decreased in colons, mesenteric lymph node, and small intestines of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$ mice (Fig. 2A, 2B and Supplemental Fig. 2A, 2B). Next, cytokine production by ILC3 was analyzed. IL-17-producing ILC3 were present at a significantly higher percentage in the colonic lamina propria of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice (Fig. 2C, 2D). A similar trend was observed in mesenteric lymph nodes. On the other hand,

IL-22 production by ILC3 was present at a comparable frequency in colons and lymph nodes of both *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice and *Rag1*$^{-/-}$ mice (Fig. 2C, 2D). GM-CSF produced by ILC3 plays a critical role in innate immune cell driven colitis (33). Proportions of ILC3 producing GM-CSF were similar in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice (Supplemental Fig 2C).

ILC3 consists of 2 major subsets, lymphoid tissue-inducer (LTi)-like ILC3 and NKP46^{+} ILC3 (34–36). Both subsets are dependent on Rorγt and aryl hydrocarbon receptor (AHR). LTi-like ILC3 are CCR6^{+} and negative for natural killer (NK) cell markers such as NKP46. In contrast to LTi-like ILC3, NKP46^{+} ILC3 express NK cell markers and require T-bet, Notch, and the presence of gut microbiota for their development (37–41). Similar fractions of ILC3 in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice were positive for NKP46 and T-bet (Fig 2E). SCA-1^{+} and CD127 (IL-7R)$^{+}$ ILCs mediate *Helicobacter*-induced colonic inflammation on the *129SvEvRag2*$^{-/-}$ background (21, 22). The frequency of SCA-1^{+} and CD127^{+} ILC3 was comparable between ILC3 from Gpr109a$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice (Fig 2E). ILC2 produce IL-13 and regulate homeostasis at mucosal surfaces (42, 43). IL-13 production by ILC2 cells was unchanged in the colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice when compared to *Rag1*$^{-/-}$ mice (Supplemental Fig. 2D, 2E). Taken together, these data demonstrate that colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice possess an increased number of IL-17 producing ILC3.

Colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice is ILC3 dependent

To test the functional relevance of ILC3's role in inducing colitis in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, we first treated these animals with depleting antibody against Thy1.2. Anti-Thy1.2 treatment reduced the frequency of Thy1.2^{+} cells among CD45^{+} cells by >90% (Fig. 3A). Compared to ~ 27.9% ILC3 of CD45^{+} cells in the isotype control-treated animals, ~ 1.5% of the CD45^{+} cells were ILC3 in anti-Thy1.2 treated animals (Fig. 3B). Anti-Thy1.2 treatment also reduced the colon mass and spleen cellularity (Supplemental Fig. 3). Most importantly, colons of anti-Thy1.2 treated animals showed a significant reduction in colitis histopathological than control antibody treated animals (Fig. 3C, 3D). Anti-Thy1.2 antibody depletes all the ILC on *Rag1*$^{-/-}$ background. To test a direct role of ILC3 in colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, we crossed these animals on *Rorγt*$^{-/-}$ background. No sign of colonic inflammation or hyperplasia was present in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$*Rorγt*$^{-/-}$ mice (Fig. 3E). Accordingly, histopathological scores for colitis were significantly reduced in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$*Rorγt*$^{-/-}$ mice than *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice (Fig. 3F). Collectively, these findings demonstrate that ILC3 mediates colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice.

A role for Gpr109a in regulating ILC3 homeostasis

Data presented above show that in the absence of Gpr109a, ILC3 numbers are increased on a *Rag1*$^{-/-}$ background. A recent report shows that the number and distribution of ILC3 subsets are altered in *Rag1*$^{-/-}$ mice (44). Therefore, to directly assess the role of Gpr109a in influencing ILC3 population in the gut, ILC3 numbers were enumerated in WT and *Gpr109a*$^{-/-}$ animals. Fig. 4 shows that the colonic lamina propria of *Gpr109a*$^{-/-}$ mice contained a significantly higher number of Rorγt^{+} cells among Lin^{-}CD45^{+}Thy1.2^{+} cells than WT mice. Collectively, these data show that Gpr109a regulates ILC3 number in intestine.

Gpr109a signaling suppresses IL-23 production

To characterize the mechanism underlying the Gpr109a mediated suppression of the ILC3 population in the gut, the expression of Gpr109a on various cells was examined. We have shown that in the gut, innate immune cells such as macrophages and dendritic cells express very high levels of Gpr109a. On the other hand, Gpr109a is expressed at very low levels by the colonic epithelium and it is undetectable in T and B cells (32). As expected, dendritic cells (DCs) expressed Gpr109a. In contrast, Gpr109a expression was undetectable on ILCs (Fig. 5A). To understand how the deficiency of Gpr109a in DCs can influence ILC3 numbers in the gut, we focused on IL-23 production by DCs. IL-23 is a key cytokine that promotes differentiation of ILC3 and regulates colonic inflammation (21, 45–47). Two main subsets of intestinal DCs, $CD103^-$ and $CD103^+$, differ in their ability to produce IL-23. Therefore, the expression of IL-23 by colonic $CD103^-$ and $CD103^+$ DCs was analyzed. Both subsets of colonic DCs from *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice produced higher amounts of IL-23 than their counterparts from *Rag1*$^{-/-}$ mice (Fig. 5B). To test the functional relevance of overproduction of IL-23 by gut DCs from *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, we cultured sorted colonic DCs from *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice with $Thy1.2^+$ cells from *Rag1*$^{-/-}$ mice. Three days later, the development of ILC3 in these cultures was analyzed. Significantly higher numbers of ILC3 developed in cultures containing DCs from colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice than *Rag1*$^{-/-}$ mice (Fig. 5C). Moreover, higher proportions of ILC3 from cultures containing *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ colonic DCs produced IL-17 than *Rag1*$^{-/-}$ cultures (Fig. 5D). The ability of colonic DCs from *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice to promote the development of ILC3 was shown to be dependent on IL-23 production because the addition of neutralizing antibody against IL-23 significantly reduced the development of ILC3 in the cultures (Fig. 5C, 5D). Collectively, these data demonstrate that Gpr109a suppresses ILC3 by diminishing IL-23 production by intestinal DCs.

Niacin, a Gpr109a ligand, inhibits IL-23 and ILC3

Based on the data presented above, we tested whether niacin, a Gpr109a agonist, suppresses IL-23 production and ILC3 generation. *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice were administered niacin in their drinking water. One month later, the number of ILC3 was analyzed from their colonic lamina propria. Niacin significantly diminished IL-23 production by colonic DCs from *Rag1*$^{-/-}$ mice, whereas IL-23 production remained unaffected in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice by niacin treatment (Fig. 6A). In accordance with this, niacin treatment significantly reduced the numbers of ILC3 in *Rag1*$^{-/-}$ mice (Fig. 6B, 6C). On the other hand, under the same treatment conditions, niacin failed to affect the ILC3 in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Collectively, these data suggest that Gpr109a activation decreased IL-23 production by DCs, leading to the suppression of ILC3.

Colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice is dependent on gut microbiota

Trillions of bacteria, collectively called gut microbiota, reside in the gut and impact inflammation. To test the role of gut microbiota in the colonic inflammation of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, the animals were given a cocktail of antibiotics at 3 months of age. Colonic inflammation and hyperplasia of the epithelial layer were significantly reduced in antibody treated animals and lead to a significant decrease in histopathological score for colitis (Fig.

7A, 7B). Consistent with this, overall ILC3 number and the frequency of IL-17 producing ILC3 were significantly decreased in the colons of antibiotics treated mice compared to control untreated mice (Fig. 7C, 7D, 7E). Moreover, antibiotics treatment significantly inhibited IL-23 production by colonic DCs (Fig. 7F). Taken together, these data suggest that Gpr109a limits microbiota induced IL-23 production and ILC3 to regulate colonic homeostasis.

Altered gut microbiota in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice

Since antibiotics treatment ameliorated spontaneous colonic inflammation in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, the composition of the gut microbiota between *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice was compared. An average of 158160 ± 29409 sequences per mouse were analyzed. The relative abundance of *Bacteroidetes* belonging to family *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, and *Firmicutes* belonging to family *Streptococcaceae*, *Christensenellaceae*, and *Mogibacteriaceae* among all bacteria was significantly higher in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice than *Rag1*$^{-/-}$ mice (Fig. 8A, 8B). On the other hand, *Bacteroidetes* belonging to family *S24-7*, *Paraprevotellaceae*, *Firmicute*s belonging to family *Peptococcaceae*, and *Proteobacteria* belonging to family *Desulfovibrionaceae* were less abundant in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$ mice (Fig. 8A, 8B). Microbiota of the family *Helicobacteraceae* have been shown to cause colitis in mouse models, while in humans the results have been inconclusive and even indicated a potential protective effect against IBD (21, 46, 48–50). Furthermore, *Enterobacteriaceae* and *Mycoplasmataceae* in the gut have been associated with IBD (51–54). Bacteria of the family *Enterobacteriaceae* were present in the *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice (7 out of 9 mice), whereas they were undetectable in *Rag1*$^{-/-}$ mice (Fig. 8B). Similarly, *Mycoplasmataceae* were found to be present in the gut of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, while not in the gut of *Rag1*$^{-/-}$ mice (Fig. 8B). *Helicobacteraceae*, on the other hand, were present in comparable numbers between *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice (Fig. 8B). Collectively, these data indicate that the gut of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice show an increased colonization by some microbiota associated with IBD than *Rag1*$^{-/-}$ mice.

Discussion

In the current study, we showed that Gpr109a signaling suppresses IL-23 production by dendritic cells. Gpr109a-deficient colonic DCs promote ILC3 via a mechanism dependent on IL-23. Opposing roles of ILC3 in intestinal inflammation have been reported. IL-23 and IL-1β stimulate ILC3 to produce IL-22 and/or IL-17. IL-22 acts on gut epithelial cells and promotes their proliferation, wound healing, and barrier function (55, 56). Moreover, IL-22 also induces expression of anti-microbial peptides by the gut epithelia and thus protects against pathogenic bacteria (13). Thus, the actions of IL-22 improve intestinal health. IL-17 favors the release of chemoattractant factors and chemokines, which recruit pro-inflammatory neutrophils and leads to intestinal inflammation. Our data shows that in contrast to IL-22, IL-17 production is significantly increased by ILC3 in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice than *Rag1*$^{-/-}$ mice. These findings are consistent with an inflammatory role for ILC3 in colons of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Although IL-23 stimulates production of both IL-17 and IL-22 by ILC3, the conditions favoring preferential IL-17 production remain poorly

defined and identification of such mechanisms will be key in understanding the regulation of ILC3 homeostasis by Gpr109a.

Dendritic cells play a key role in the homeostasis of ILC1 and ILC3 through the production of IL-12 and IL-23 (45). IL-23 favors the differentiation of ILCs to ILC3, whereas IL-12 promotes differentiation to ILC1. Data obtained in animals as well as humans unequivocally demonstrate a pathogenic role for IL-23 in the induction of inflammatory bowel disease (IBD). Whole genome association and transmission disequilibrium studies have identified that the point mutations Arg381Gln and R381Q in the IL-23 receptor (IL-23R) confer protection against Crohn's disease (57, 58). Elevated levels of IL-23 mRNA are present in the mucosa of individuals with IBD and its expression level is correlated with disease severity (59, 60). Myeloid DCs from the mesenteric lymph nodes of Crohn's disease subjects produce higher amounts of IL-23 and lower levels of IL-10 than the healthy controls. However, what factors regulate increased IL-23 and decreased IL-10 expression in IBD individuals remains unknown. In the current study, we have identified Gpr109a as one the determinants that suppresses IL-23 production. We have previously published that Gpr109a-deficient colonic DCs produce lower amounts of IL-10 and higher amounts of IL-6 (32). These findings establish a critical role for Gpr109a in the regulation of multiple cytokines relevant to the etiology of IBD. Future studies aimed at understanding the molecular mechanisms underlying Gpr109a-mediated regulation of IL-23 and IL-10 will be important in understanding the dysregulation of these cytokines in IBD.

Gut microbiota play a critical role in intestinal homeostasis. The engagement of pathogen-associated molecular patterns, or PAMPs, derived from gut microbiota to toll-like receptors (TLRs) or NOD expressed by innate immune cells induces production of inflammatory molecules. Therefore, the presence of trillions of bacteria in the gut poses a threat of inflammatory responses. However, the presence of overlapping immunoregulatory mechanisms arising from both the host and microbiota prevent the undesirable induction of inflammatory responses in the gut (2). Several studies show that a breakdown in these immunoregulatory mechanisms results in IBD. In mice lacking IL-10 or TGF-β and IL-10, the presence of commensals like *Helicobacter hepaticus* or *Bacteroides* is enough to induce colonic inflammation (50). Similarly, the *Helicobacter hepaticus* strain 51449 induces colitis in *129SvEvRag2*$^{-/-}$ mice and *Helicobacter* infection is associated with rectal prolapses in mice (21, 61). Murine norovirus infection has previously been linked to the induction of colon inflammation and Crohn's Disease-like symptoms in mice expressing hypomorphic *Atg16L1* and also accelerates *Helicobacter bilis*-induced colitis in *Mdr1a*$^{-/-}$ mice (62, 63). In this study, *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice (both on a C57BL/6 background) were housed in a colony that is positive for *Helicobacter* and murine norovirus. Gut microbiota analysis using 16S rDNA sequencing of caecum bacteria confirmed the presence of a comparable number of *Helicobacter* in *Rag1*$^{-/-}$ mice and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Further investigation is required to determine the impact *Helicobacter* and murine norovirus may have on the observed differences in bacterial colonization of the gut between *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ and *Rag1*$^{-/-}$ mice as well as the spontaneous colitis of the former. The preferential presence of *Enterobacteriaceae*, which is associated with IBD, in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice is interesting and future studies should be aimed at investigating its role in inducing colonic inflammation, IL-23 production, and the promotion of ILC3 in *Gpr109a*

$^{-/-}Rag1^{-/-}$ mice. Niacin suppresses LPS mediated production of IL-6, TNF-α, IL-12p40, and IL-1β in a Gpr109a dependent manner (64). Therefore, it is possible that Gpr109a signaling inhibits microbiota-induced production of several inflammatory cytokines, including IL-23 in the colon, leading to suppression of inflammation. Identification of the signaling mechanisms underlying Gpr109a mediated suppression of the production of inflammatory cytokines will be very important in understanding the beneficial role of this receptor in the intestine and will facilitate the design of new therapeutics aimed at ameliorating colonic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

DC	dendritic cell
Gpr109a	G protein coupled receptor 109a
H&E	hematoxylin and eosin
IBD	inflammatory bowel disease
ILC	innate lymphoid cell
PMA	phorbol 12-myristate-13-acetate
Treg	regulatory T cell

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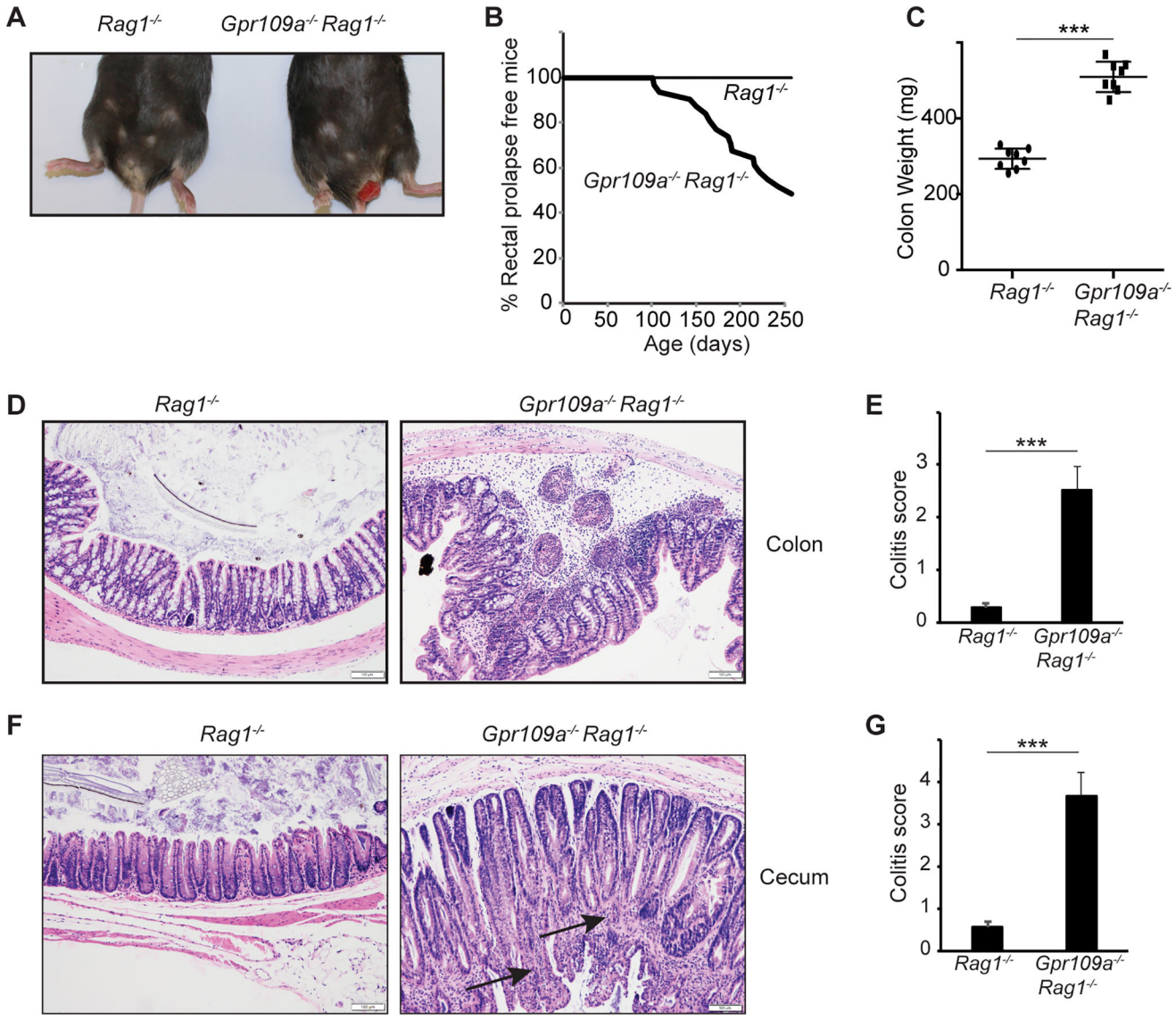

FIGURE 1.

Spontaneous colitis in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (**A**) Photograph showing rectal prolapse in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (**B**) Incidence of rectal prolapse in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice and *Rag1*$^{-/-}$ mice (n = 22 mice of each genotype). (**C**) Colon mass of *Rag1*$^{-/-}$ and *Gpr109a* $^{-/-}$*Rag1*$^{-/-}$ mice. Each circle represents an individual mouse, lines represent the mean (n = 9 mice of each genotype). A representative photomicrograph of H&E stained cross sections of colons (**D**) and caecum (**F**) of indicated mice at 6 months of age. (**E**, **G**) Histopathological score of colons and caecum of mice indicated in D and F, respectively. Error bars represent standard deviation of the mean (n = 4–5 mice/group). *** P<0.0005

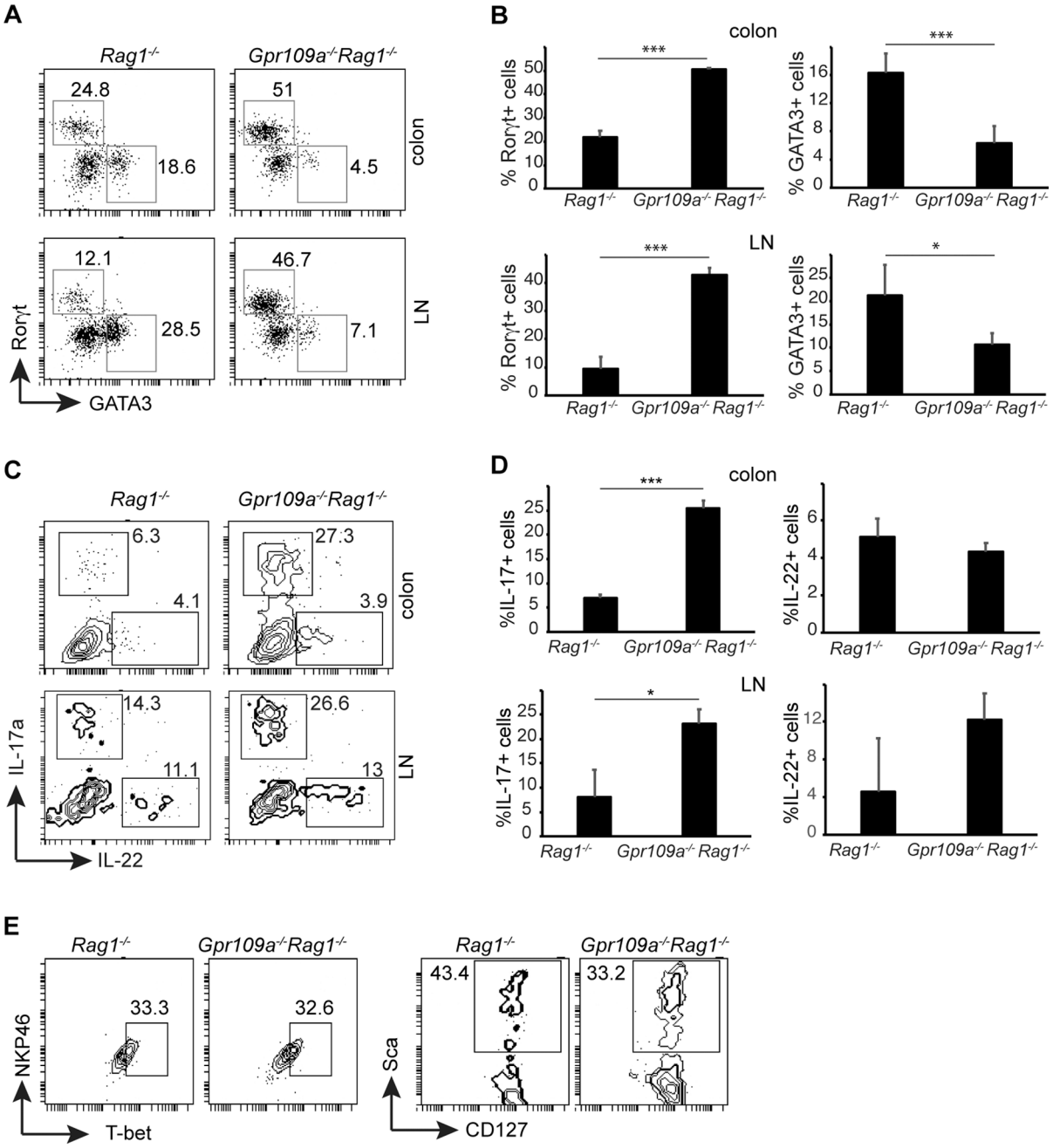

FIGURE 2.

Increased number of ILC3 in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (**A**) Frequency of Rorγt^{+} and GATA3^{+} cells among CD45^{+}Thy1.2^{+} cells in colon and mesenteric lymph node cells of indicated mice at 6 months of age. (**B**) Number of Rorγt^{+} and GATA3^{+} cells in colons and mesenteric lymph node of *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Error bars represent standard deviation of mean (n = 5 mice/group). (**C**) Frequency of IL-17 and IL-22 producing cells following PMA and ionomycin stimulation among CD45^{+}Thy1.2^{+}Rorγt^{+} cells in colons and mesenteric lymph node cells of indicated mice. (**D**). Summary of IL-17 and IL-22 producing cells in indicated organs of *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Error

bars represent standard deviation of mean (n = 3 mice/group). * P<0.05, *** P<0.0005. (**E**) Phenotype of colonic $CD45^+Thy1.2^+Ror\gamma t^+$ cells in *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. A representative or pooled data of at least two experiments is shown.

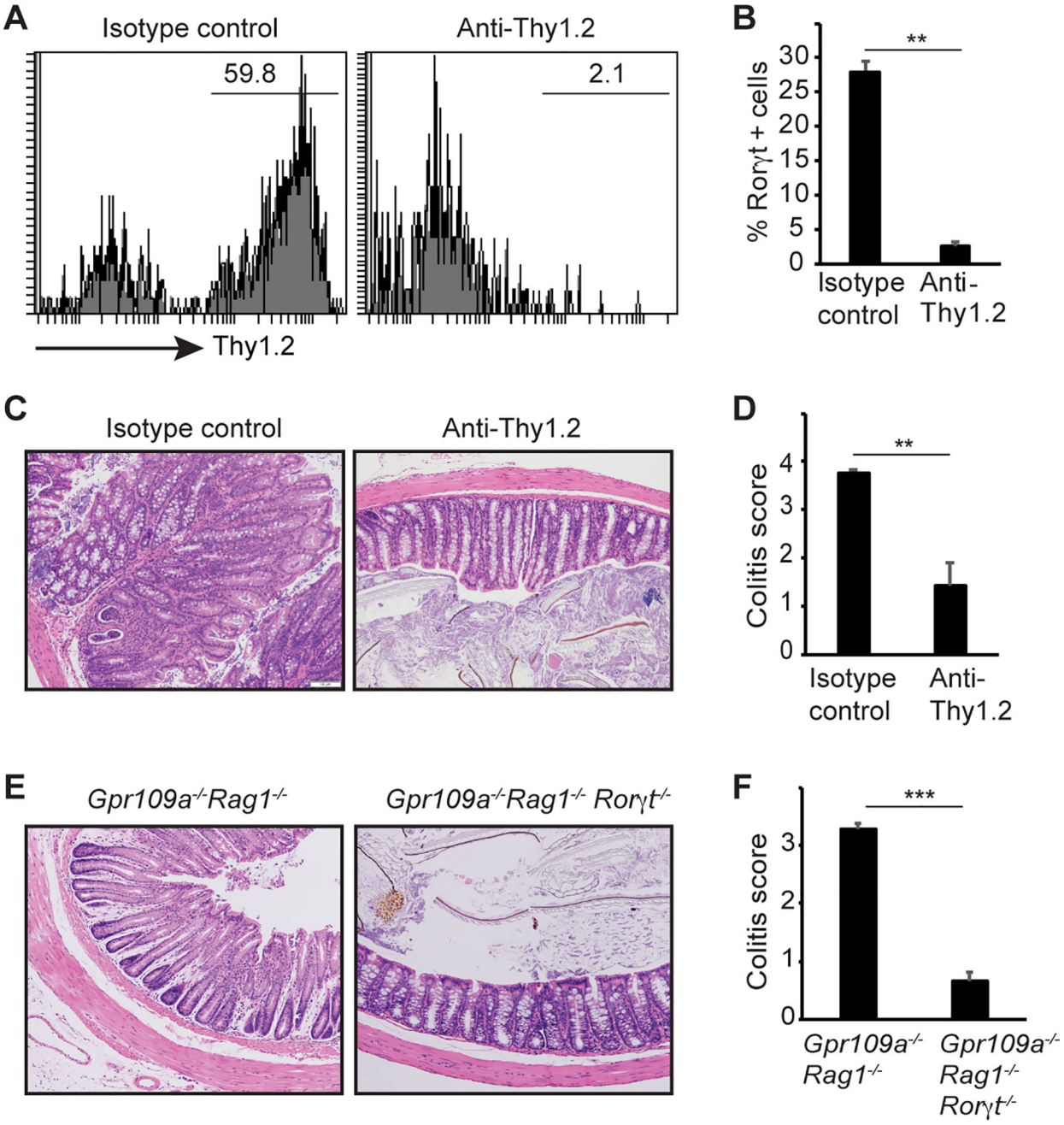

FIGURE 3.

ILC3 mediate spontaneous colitis in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (**A**). Thy1.2 staining on CD45^{+} cells in *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice treated with isotype control or anti-Thy1.2 antibody. (**B**) Frequency of Rorγt^{+} cells among CD45^{+} cells in colonic lamina propria of mice treated as in A (n = 3 mice/group). (**C**) Photomicrographs of H&E stained cross sections of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice indicated (original magnification X200). (**D**) Histopathological colitis score of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice treated as in C (n = 5 mice/group). Photomicrographs of H&E stained cross sections (**E**) and histopathological colitis score (**F**) of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$*Rorγt*$^{-/-}$ mice. Error bars represent

standard deviation of mean (n = 5 mice/group). ** P<0.005. A representative or pooled data of at least two experiments is shown.

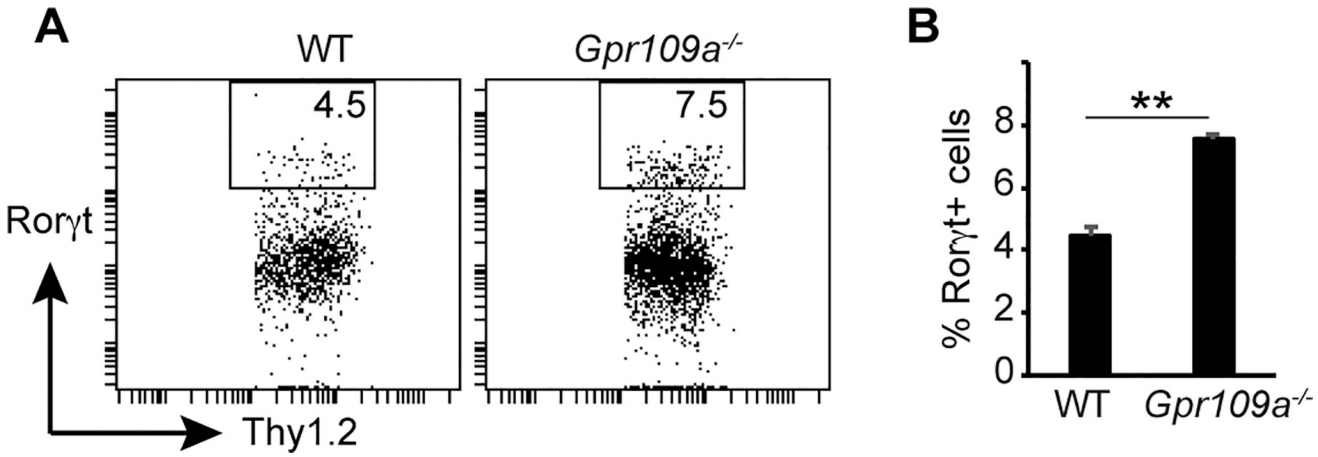

FIGURE 4.

A critical role for Gpr109a in ILC3 homeostasis. (**A**) A representative flow cytometric staining showing Rorγt and GATA3 expression by colonic $CD45^{+}Lin^{-}Thy1.2^{+}$ cells from WT and *Gpr109a*$^{-/-}$ mice. (**B**) Quantification of Rorγt and GATA3 cells among colonic $CD45^{+}Lin^{-}Thy1.2^{+}$ cells from indicated mice. Error bars represent standard deviation of mean (n = 3 mice of each genotype). A representative of two experiments is shown.

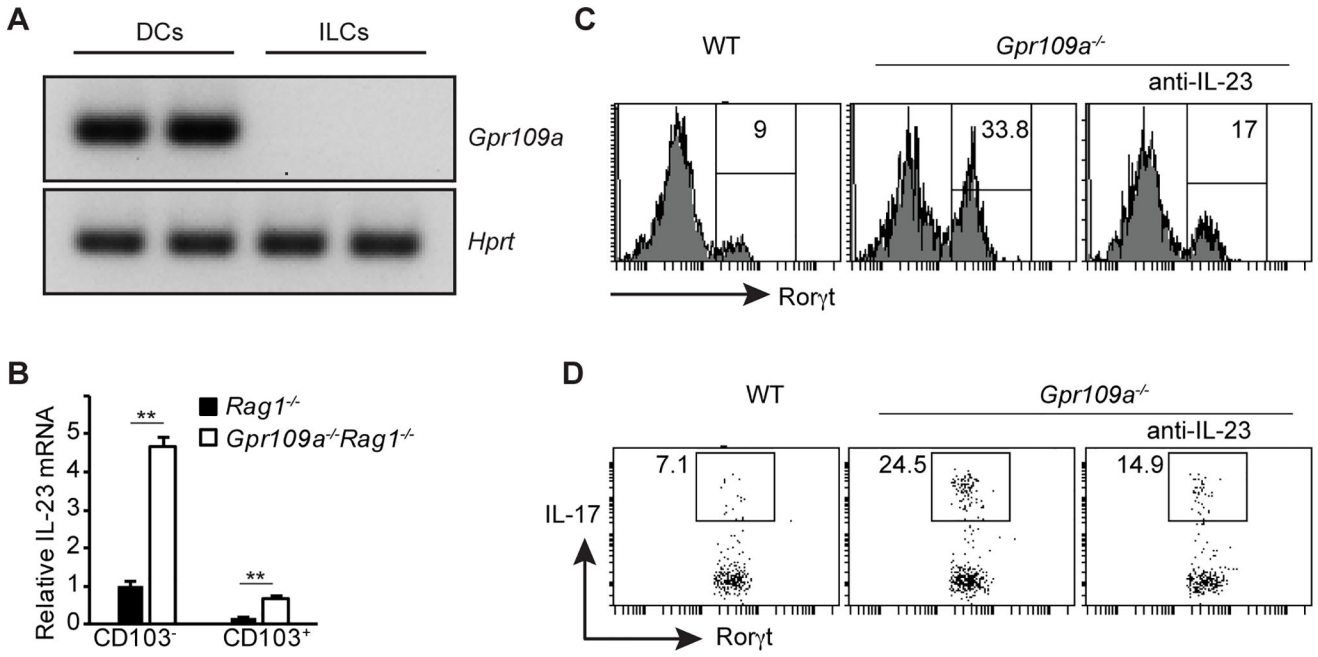

FIGURE 5.

Role of IL-23 in Gpr109a mediated promotion of ILC3. (**A**) Expression of *Gpr109a* and *Hprt* by CD11c$^+$ and ILC (CD45$^+$Thy1.2$^+$) was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). (**B**) IL-23 expression by colonic CD103$^-$ and CD103$^+$ DCs from *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice was analyzed by quantitative RT-PCR (qRT-PCR) Error bars represent standard deviation of triplicates. (**C**) Rorγt expression by Thy1.2$^+$ cells cultured with colonic DCs in the presence or absence of anti-IL-23 from indicated mice. (**D**) IL-17 production by Rorγt cells from C following PMA and ionomycin. Error bars represent standard deviation of triplicates (n = 3 mice/group). A representative of two experiments is shown.

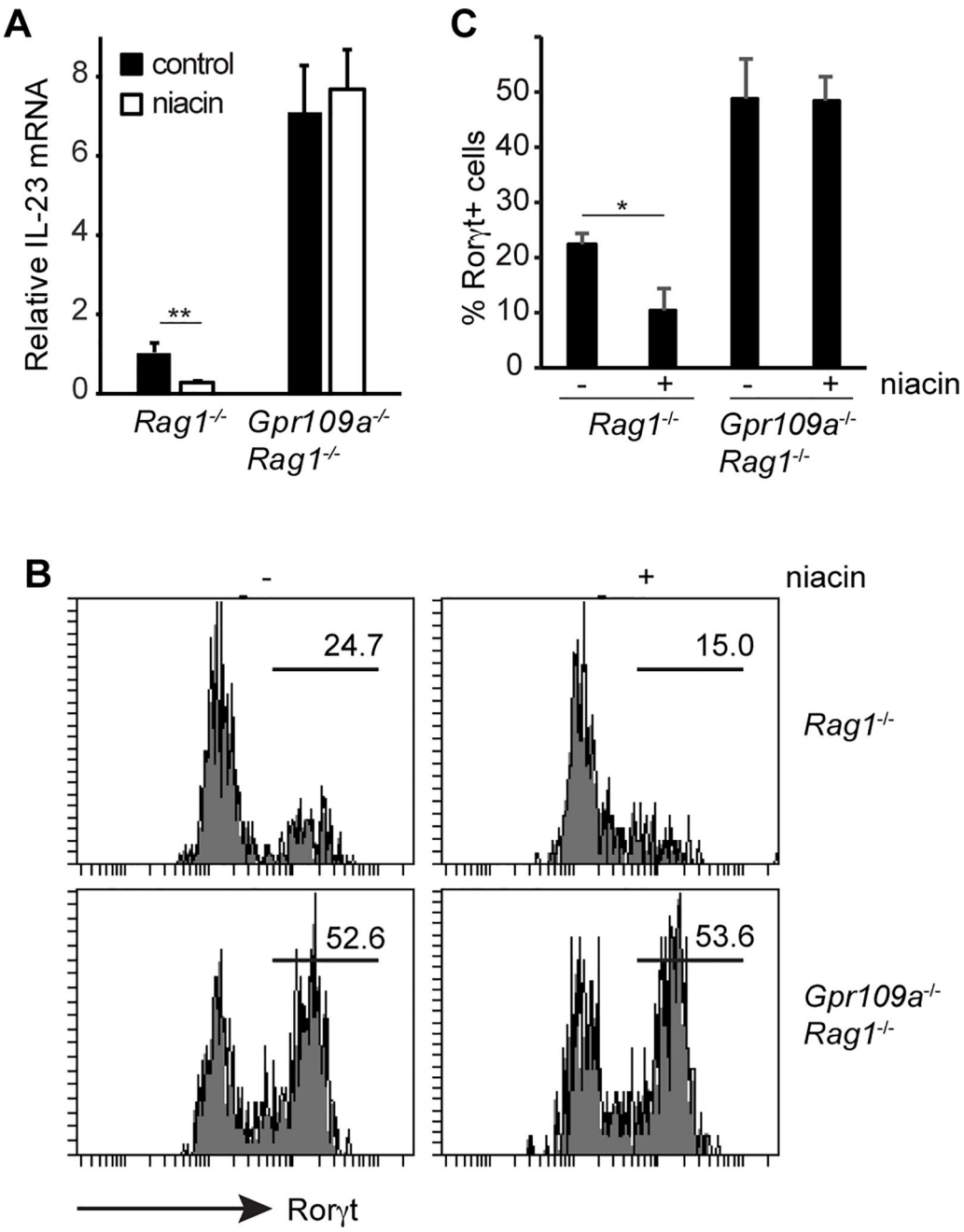

FIGURE 6.

Gpr109a agonist suppresses IL-23 and ILC3. (**A**) *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice were treated with niacin in drinking water. One month later, IL-23 expression by colonic DCs was analyzed. Error bars represent standard deviation of triplicates. (**B**) Rorγt expression among colonic CD45^{+}Thy1.2^{+} cells from mice treated as in (A). (**C**) Rorγt^{+} cell frequency among colonic CD45^{+}Thy1.2^{+} cells from mice treated as in A. (n = 3 mice/group). A representative of two experiments is shown.

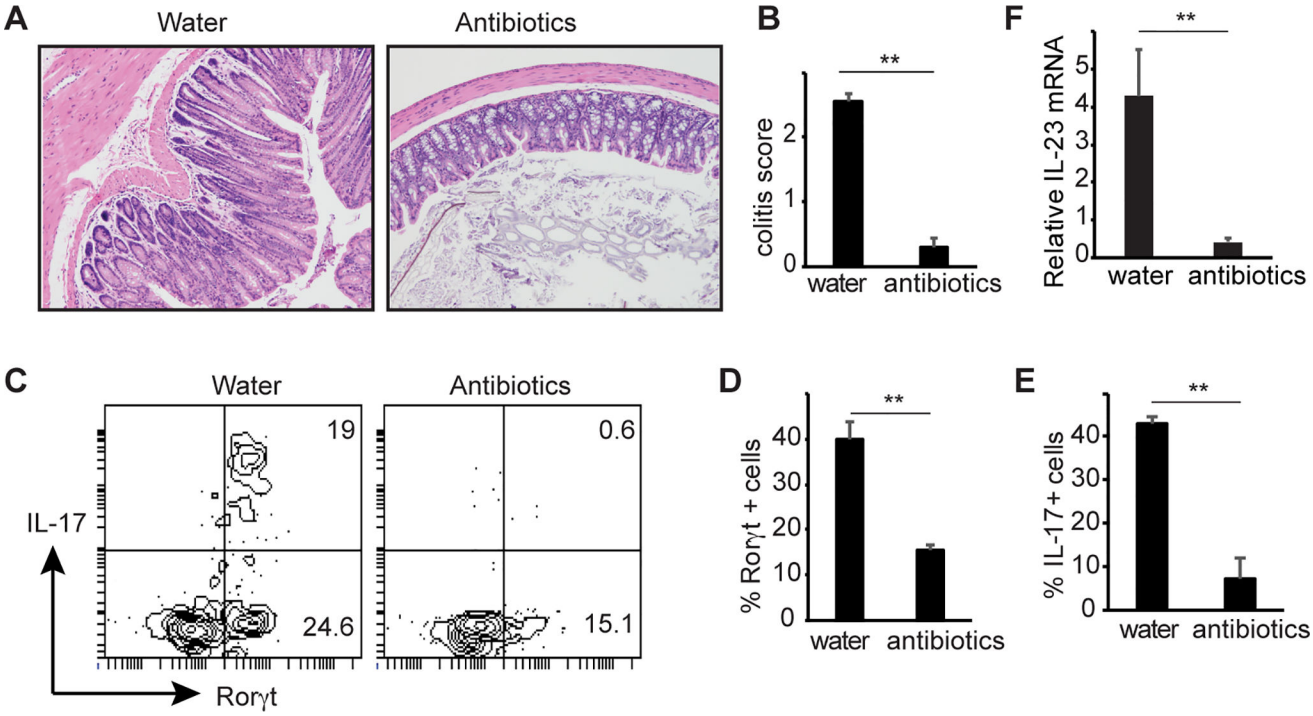

FIGURE 7.

Role of microbiota in spontaneous colitis of *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. (**A**) *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice were given plain water or water containing a cocktail of antibiotics. Two months later colons of mice were analyzed for colitis. A representative photomicrograph of H&E stained cross section of colon. (**B**) Histopathological score of colitis. n = 4 (water) and 5 (antibiotics) mice. (**C**) A representative flow cytometric plot showing expression of Rorγt and IL-17 by CD45^{+}Thy1.2^{+} cells. (**D**) and (**E**) Enumeration of frequency of Rorγt and IL-17 positive cells among CD45^{+}Thy1.2^{+} cells (n = 3 mice/group). (**F**) IL-23 expression by colonic DCs from mice treated as in A. Error bars represent standard deviation of triplicates. A representative or pooled data of two experiments is shown.

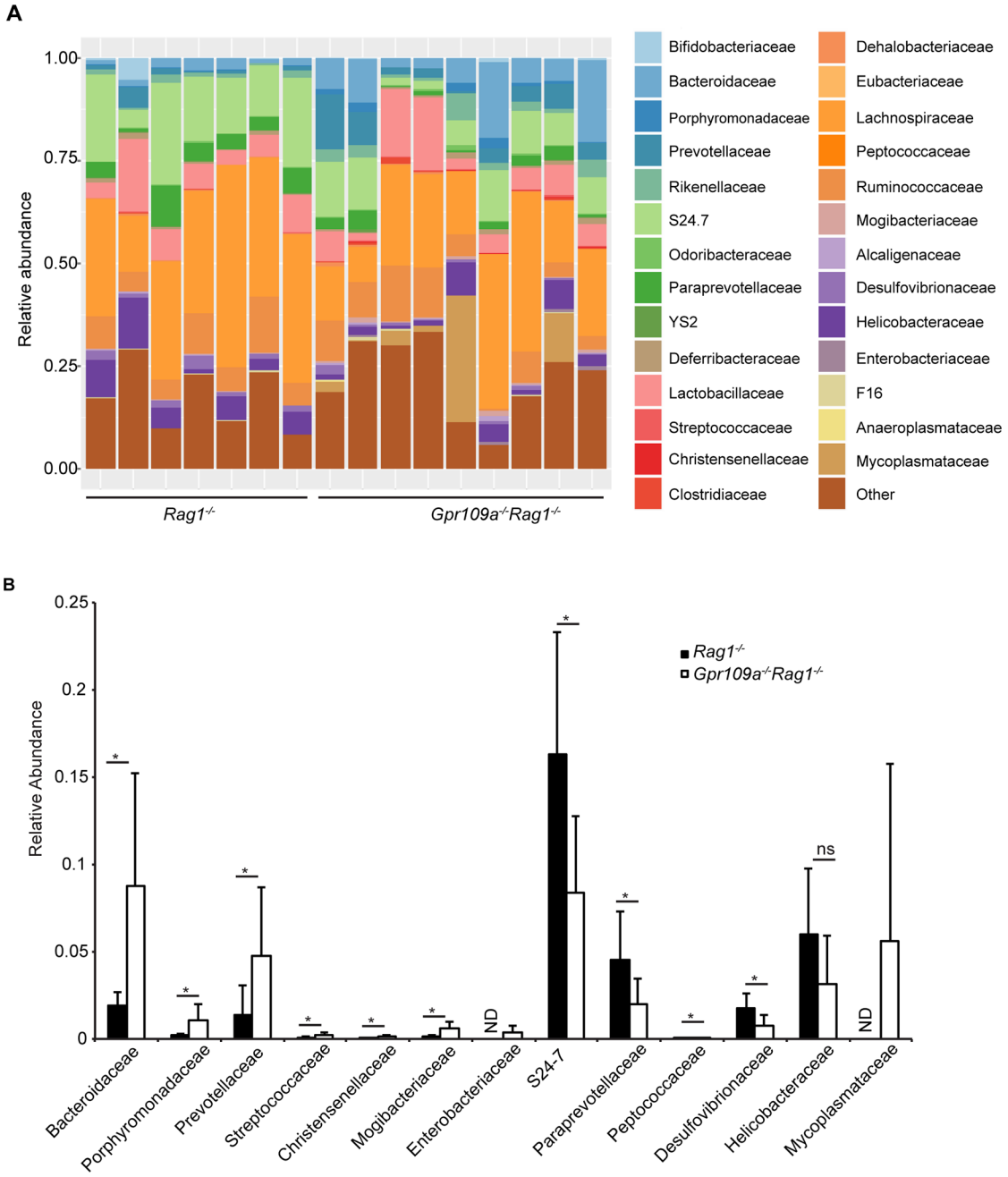

FIGURE 8.

Microbial communities in the gut of *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice. Sequencing of V3–V4 regions of bacterial 16S rDNA was performed on DNA isolated from caecum content. Shown is the (**A**) relative abundance of family-level phylotypes present within total bacterial taxonomic units sequenced in the indicated mice. Each bar in A represents an individual mouse. (B) Data from A is replotted for bacterial families showing significant differences between *Rag1*$^{-/-}$ and *Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ mice, as well as *Helicobacteraceae*. n

= 7 and 9 mice of *Rag1*$^{-/-}$ *and Gpr109a*$^{-/-}$*Rag1*$^{-/-}$ genotype respectively. * P<0.05, ND = not detected.