


Research Article

Microbial metabolites, short-chain fatty acids, restrain tissue bacterial load, chronic inflammation, and associated cancer in the colon of mice

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The intestinal immune system is regulated by microbes and their metabolites. The roles of gut microbial metabolites in regulating intestinal inflammation and tumorigenesis are incompletely understood. We systematically studied the roles of short-chain fatty acids (SCFAs) and their receptors (GPR43 or GPR41) in regulating tissue bacterial load, acute versus chronic inflammatory responses, and intestinal cancer development. SCFA receptor-, particularly GPR43-, deficient mice were defective in mounting appropriate acute immune responses to promote barrier immunity, and developed uncontrolled chronic inflammatory responses following epithelial damage. Further, intestinal carcinogenesis was increased in GPR43-deficient mice. Dietary fiber and SCFA administration suppressed intestinal inflammation and cancer in both GPR43-dependent and independent manners. The beneficial effect of GPR43 was not mediated by altered microbiota but by host tissue cells and hematopoietic cells to a lesser degree. We found that inability to suppress commensal bacterial invasion into the colonic tissue is associated with the increased chronic Th17-driven inflammation and carcinogenesis in the intestine of GPR43-deficient mice. In sum, our results reveal the beneficial function of the SCFA-GPR43 axis in suppressing bacterial invasion and associated chronic inflammation and carcinogenesis in the colon.

Keywords: Colon cancer · Dietary fiber · Inflammation · Short-chain fatty acids



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Introduction

The intestine harbors a myriad of commensal microorganisms, which benefit the host in many ways [1, 2]. Digestion resistant-

dietary materials reach the colon for microbial fermentation. A major group of dietary materials processed by microbial fermentation includes dietary fibers (DF) and resistant starch. They are fermented to produce short-chain fatty acids (SCFAs), which play diverse roles from fueling colonocytes to regulating cells of the immune system, energy storage/metabolism, and gut barrier function [3–14].

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SCFAs exert their functions in the host through both SCFA receptor-dependent and independent mechanisms [3]. SCFA receptors include G-protein-coupled receptors (GPCRs) such as GPR43, GPR41, GPR109A and Olfr78. GPR43 and GPR41 are activated by all major SCFAs such as acetate (C2), propionate (C3), and butyrate (C4) [15, 16]. GPR109A is activated by C4 along with a non-SCFA ligand, niacin [17]. Olfr78 is activated by C2 and C3, but not C4 [18]. Triggering these receptors induce activation of heterotrimeric G proteins and many downstream signaling molecules, including MAPK/ERK proteins [13, 16, 19, 20]. GPR43, GPR41 and GPR109A are differentially expressed by many cell types, including gut epithelial cells, macrophages, and dendritic cells [13, 15, 16]. Olfr78 is expressed in kidney endothelial cells [18].

Inflammation is a major risk factor for cancer development in the digestive tract [21–24]. Colon cancer is increased in patients with inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease. Similarly, celiac disease and Crohn's disease are risk factors for small intestinal cancer. It has been documented that high SCFA levels and DF intake decrease colon cancer development [25]. GPR43 expression is decreased in tumor cells and this receptor functions to suppress colon cancer development [26–28]. Employing acute or chronic dextran sodium sulfate (DSS)-induced colitis models, two groups found that GPR43 has heterogeneous effects on DSS-induced inflammatory responses [29, 30]. GPR43 and GPR41 are required for mounting acute inflammatory responses following gut barrier breach [13, 29], which would be important for barrier repair and tissue bacterial clearance. GPR43 functions to increase beneficial *Bifidobacterium* species but suppress inflammatory *Helicobacter hepaticus* and *Prevotellaceae* [27], and cohousing of *Gpr43*^{-/-} mice with wild-type (WT) littermates suppressed acute 2% DSS-induced colitis [28]. However, it is also reported that DF suppress inflammation-associated colon cancer development in a manner independent of the microbiota change but dependent on another SCFA receptor GPR109A [31]. In this regard, DF or SCFA-receptor-associated microbiota effect on inflammation-associated colon cancer development is unknown. Moreover, the differential effect of the SCFA-SCFA receptor axis on acute versus chronic responses should be studied to better understand the working mechanisms of DF, SCFAs and their receptors in suppressing colon cancer development.

In this study, we addressed two critical issues regarding the role of the SCFA-SCFA receptor axis in regulating inflammation and cancer. First, we systematically determined the impact of SCFAs and their receptors, with the focus on GPR43, on tissue bacterial load and acute versus chronic inflammatory responses and how this relates to colon cancer development. Second, we interrogated whether the function of GPR43 is dependent on microbiota difference between WT and *Gpr43*^{-/-} mice employing cohoused mice. Our work demonstrates the importance of the SCFA receptor-dependent barrier immunity in regulating bacterial invasion, subsequent inflammatory responses and colon cancer development in a microbiota change-independent manner.

Results

SCFA receptors promote acute but suppress chronic inflammatory responses

To systematically understand the roles of SCFA receptors in regulating inflammation, we compared the inflammatory responses of WT and SCFA receptor (*Gpr43* and *Gpr41*)-deficient mice induced with DSS administered in acute and chronic inflammatory regimens. In response to an acute inflammation-inducing DSS treatment regimen (3.5% of DSS for 5 days and euthanized at day 9), SCFA receptor-deficient mice were hypo-responsive in mounting inflammatory responses at this early time point. Compared to WT mice, they lost less weight, had longer colons, failed to increase the frequencies of gut effector T cells (Th1 and Th17) and neutrophils, and did not up-regulate the mRNA expression of inflammatory cytokines and chemokines such as *Il18*, *Il22*, *Il6*, and *Cxcl1* (Fig. 1; Supporting Information Figs. 1 and 2A and B). Increased frequencies of Tregs were detected in *Gpr43*^{-/-} mice. In general, *Gpr43*^{-/-} mice were more defective in mounting the acute inflammatory response than *Gpr41*^{-/-} mice.

In response to a chronic inflammation-inducing DSS treatment regimen (3 cycles of 1.5% DSS for 6 weeks), *Gpr43*^{-/-} mice suffered less initially but more in the late phase based on weight change and stool score (Fig. 2A). However, *Gpr41*^{-/-} mice were not different from WT mice in the late phase. At termination following the chronic DSS treatment, *Gpr43*^{-/-} mice had signs of more severe inflammation based on colon length and frequencies of inflammatory T cells (Th1 and Th17 cells) and neutrophils (Fig. 2B–D, Supporting Information Fig 2C and D). In general, the expression of inflammatory cytokines, such as *Il18*, *Il22* and *Il6*, was higher in SCFA receptor-deficient mice (Fig. 2E). Overall, these results indicate that the SCFA receptor GPR43 suppresses chronic inflammatory responses.

GPR43 functions to suppress Th17-driven inflammatory response and intestinal carcinogenesis

Inflammation induces production of cytokines and genotoxic reactive oxygen and nitrogen species, which cause mutations and epithelial damages. These events compromise gut barrier function and cause bacterial invasion, further increasing inflammatory responses leading to epithelial transformation [32–35]. Thus, we determined the impact of SCFA receptor deficiency on azoxymethane (AOM)/DSS-induced carcinogenesis. We found that *Gpr43*^{-/-} mice initially suffered less from AOM/DSS-induced weight loss, and diarrhea the first 3–4 weeks but more in the late phase (Fig. 3A, Supporting Information Fig. 3). The effect of GPR41 deficiency on cancer development was not as clear as in GPR43 deficiency, which mirrors the weak effect of GPR41 deficiency on chronic inflammation (Supporting Information Fig. 3 and Fig. 2). Because of its clear function in suppressing DSS-induced chronic inflammation and AOM/DSS-induced cancer,

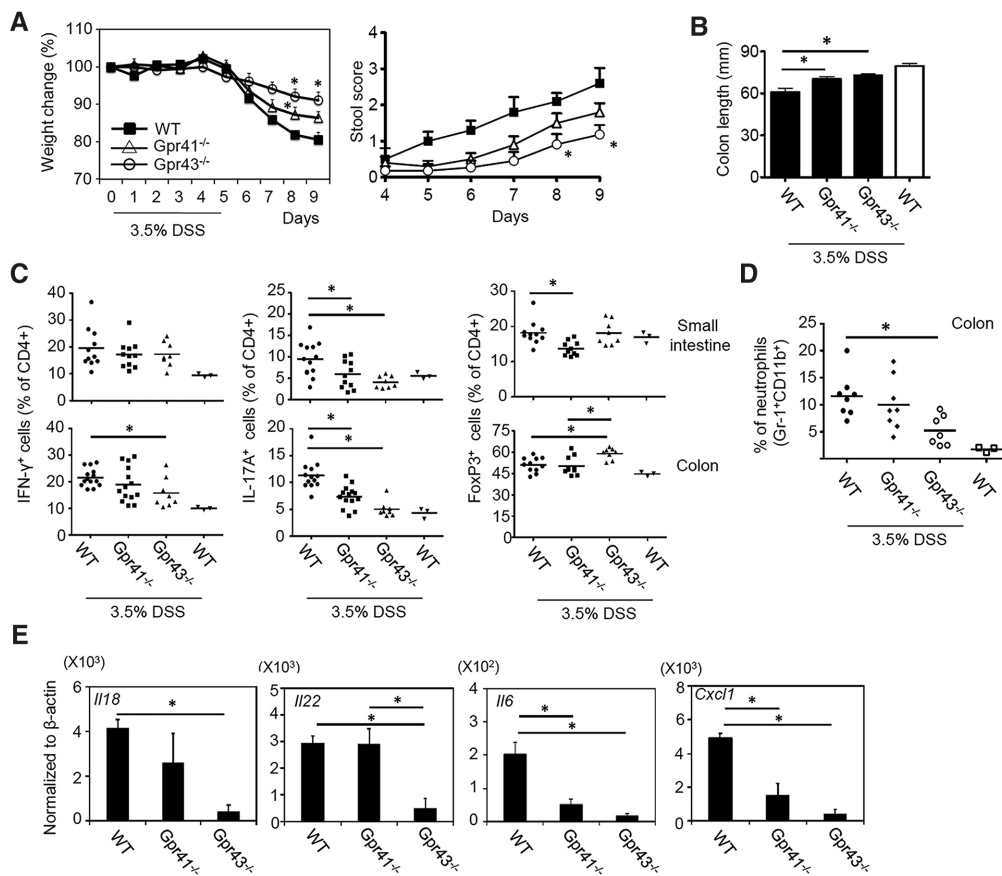


Figure 1. Acute DSS-induced inflammatory responses in WT and SCFA receptor-deficient mice. (A) Body weight change (%) and stool score during DSS-induced acute inflammation. (B) Colon length of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice after 3.5% DSS treatment. (C) Frequency of IFN- γ ⁺, IL-17A⁺, and FoxP3⁺ cells among intestinal lamina propria (LP) CD4⁺ T cells of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice. (D) Frequency of Gr-1⁺CD11b⁺ cells in colonic LP cells of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice. (E) Expression of inflammatory cytokine and chemokine genes in colonic tissues of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice. mRNA expression was measured by qRT-PCR and normalized to β -actin (*Actb*). Mice received 3.5% DSS in drinking water for 5 days before they were fed regular water prior to sacrifice at day 9. Representative and pooled data obtained (mean \pm SEM, $n = 8$ –10) from two independent experiments are shown. For panel A, once significant group effects are established, pairwise differences between WT and *Gpr41*^{-/-} or *Gpr43*^{-/-} on days 8 and 9 were separately examined. Group effects were significant for weight change ($p = 0.0009$) and for stool score ($p = 0.02$) with repeated measures ANOVA. * Significant differences from WT with two-sample t-tests with Bonferroni's adjustments for four comparisons (significance at 0.0125 for each). For panel B–E, one-way ANOVA followed by Tukey's multiple comparisons test was used to determine significance between all pairs of groups. * Significant differences between indicated pairs ($p < 0.05$).

we focused the remaining study on GPR43. At 10 weeks post-AOM treatment, *Gpr43*^{-/-} mice had greater numbers of polyps and advanced (bigger) adenomas often with complete loss of crypts, medullary inflammatory cells (I) and necrotic abscesses (N) (Fig. 3B and C). Further, they had enlarged spleen and mesenteric lymph nodes (MLN, Fig. 3D), which indicate active systemic and intestinal inflammatory responses respectively.

It has been documented that GPR43-deficient mice have altered microbiota [27, 28] and, therefore, it is possible that the protective effect of GPR43 is mediated by the altered microbiota. To determine this, we induced AOM/DSS-induced colon cancer in co-housed mice and mice with shared bedding, both of which were effective in creating the same shared microbiota profile based on 16S rRNA gene quantitative (q)-PCR of selected bacterial groups such as Firmicutes (Lachnospiraceae), Firmicutes (Lactobacillus), Firmicutes (SFB), Bacteroidetes (Porphy-

romonadaceae), Bacteroidetes (*B. fragilis*), and Proteobacteria (Enterobacteriales) (Supporting Information Fig. 4). *Gpr43*^{-/-} mice maintained the same increased susceptibility to AOM/DSS-induced colon cancer even after the microbiota-equalization procedures (Fig. 4A and B). In the colon, the frequencies of Th17 cells and neutrophils were increased whereas there was no change in those of Th1 and FoxP3⁺ Tregs (Fig. 4C). In addition, increased organ weight and frequencies of Th1, Th17 and Treg cells were detected in the spleen and MLN, indicating elevated systemic and intestinal inflammation (Fig. 4D and E). We also examined the immune cell profile at an early stage (16–17 days after AOM injection) in co-housed mice. We observed longer colon, somewhat decreased numbers of CD4⁺ T cells and increased frequencies of Th17 cells in the colon of *Gpr43*^{-/-} mice at this early stage of carcinogenesis (Supporting Information Fig. 5). Thus, sustained high Th17-driven inflammatory response is the characteristic

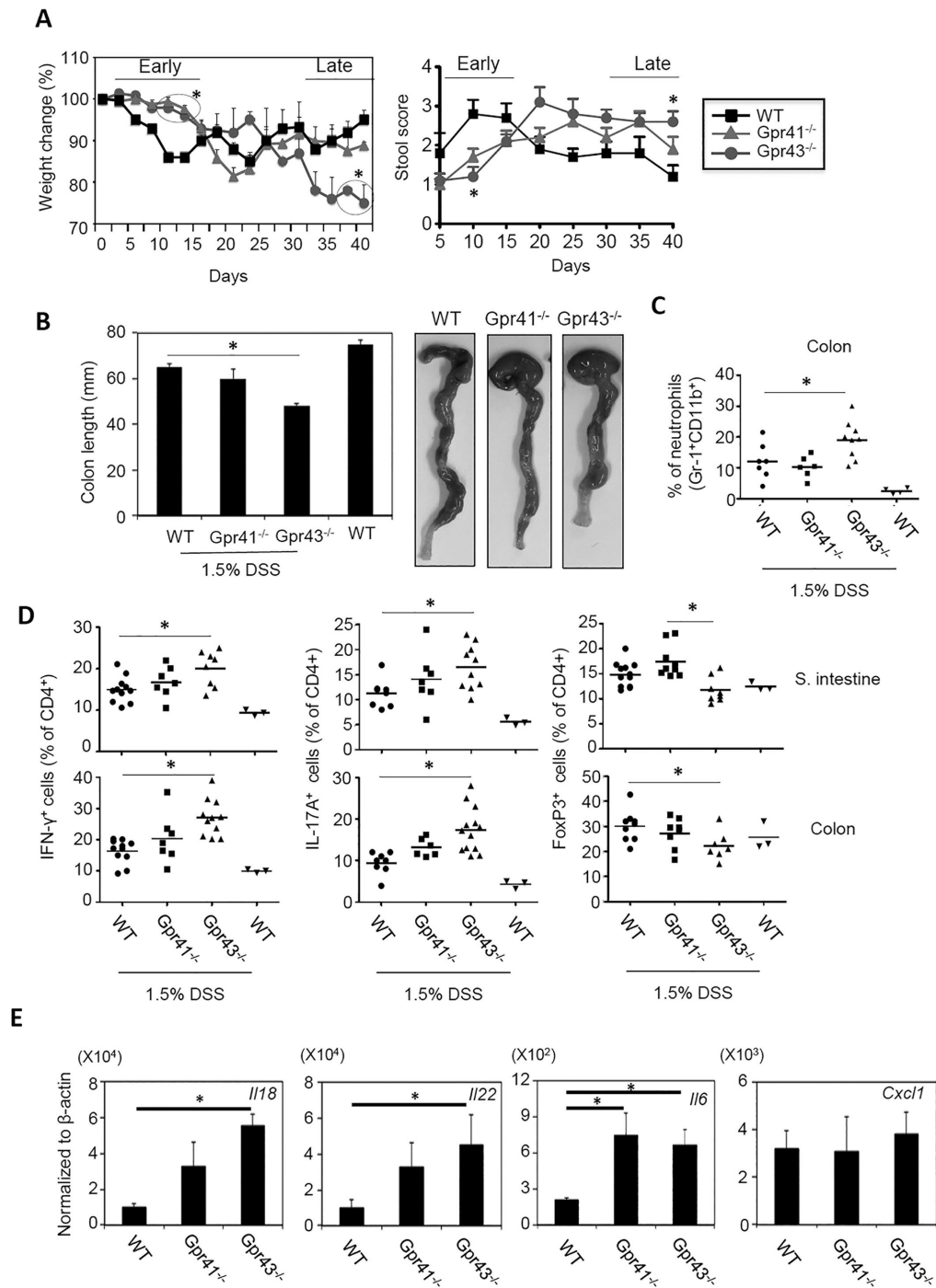


Figure 2. Chronic DSS-induced inflammatory responses in WT and SCFA receptor-deficient mice. (A) Body weight change (%) and stool score during DSS-induced chronic inflammation. (B) Colon length of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice after 3 cycles of 1.5% DSS treatment. (C) Frequency of Gr-1⁺CD11b⁺ cells in the colonic LP cells of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice. (D) Frequency of IFN- γ ⁺, IL-17A⁺, and FoxP3⁺ cells among LP CD4⁺ T cells of WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice. (E) Expression of inflammatory cytokine and chemokine genes in colon tissues isolated from WT, *Gpr41*^{-/-} and *Gpr43*^{-/-} mice. mRNA expression was measured. Mice received 3 cycles of 1.5% DSS for 7 days followed by regular water for 7 days. Representative and pooled data (mean \pm SEM, $n = 8-10$) from two independent experiments are shown. For panel A, once significant group effects for early and late phases are established, differences between WT and *Gpr41*^{-/-} or *Gpr43*^{-/-} on specific days were separately examined by phases. Significant group effects were seen in early phase ($p = 0.034$ for weight change and $p = 0.026$ for stool score) and in late phase ($p < 0.001$ for weight loss, and $p = 0.010$ for stool score) with repeated measures ANOVA. Differences from WT were further examined with multiple t-tests on day 11 and 14 of early and day 38 and 40 of late phase for weight change and day 10 and 15 for early and 35 and 40 for late phase for stool score. * Significant difference from WT with Bonferroni's adjustments for four comparisons on each day within each phase (significance at 0.0125 for each). For panel B–E, one-way ANOVA followed by Tukey's multiple comparisons test was used to determine significance between all pairs of groups. * Significant differences between indicated pairs.

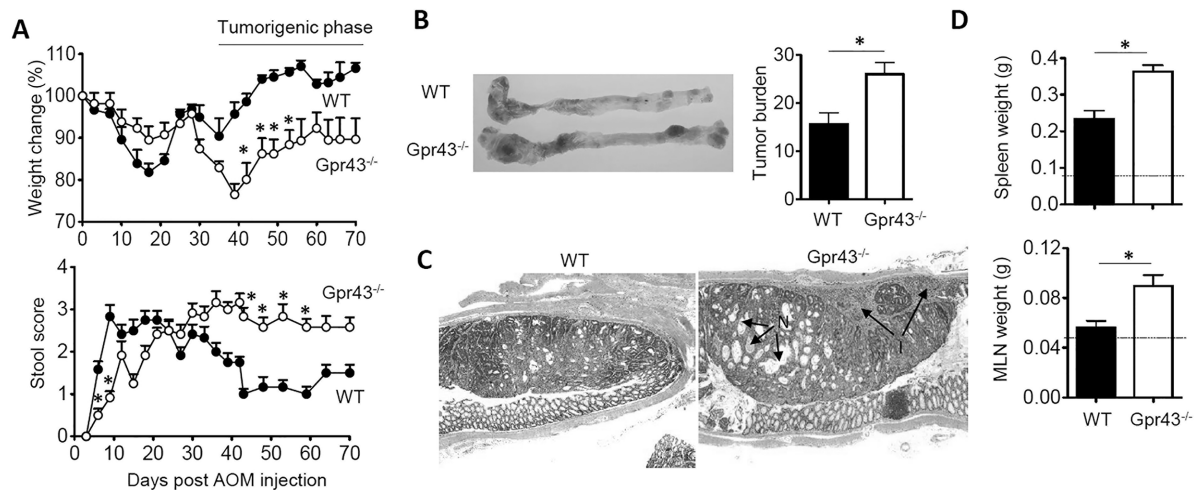


Figure 3. AOM/DSS-induced colon cancer development in WT versus GPR43-deficient mice. (A) Changes in body weight (%), and stool scores of WT and *Gpr43*^{-/-} mice during AOM/DSS treatment. (B) Tumor burden (adenoma number) in the colon of WT and *Gpr43*^{-/-} mice. (C) Histological assessment of colon tissues of WT and *Gpr43*^{-/-} mice. Paraffin-embedded tissue sections (6 μ m) were stained with hematoxylin and eosin. Medullary inflammatory cells (I) and necrotic abscesses (N) are marked in the picture. (D) Enlargement of spleen and MLN following AOM/DSS treatment. The broken line indicates average weight of the indicated organs of untreated age-matching control WT mice. Mice were treated with AOM (10 mg/Kg of body weight, i.p.) and three cycles of 1% DSS in drinking water and sacrificed at day 70 post AOM injection. Representative and pooled data (mean \pm SEM, $n = 8$ –12) from three independent experiments are shown. For panel A, significant differences between *Gpr43*^{-/-} and WT were separately examined for early and late phases, followed by pairwise comparisons on specific days with Bonferroni adjustments for multiple comparisons. Differences from WT were further examined using multiple t-tests with Bonferroni's adjustments for 8 comparisons on day 42, 46, 49, and 53 for weight change (significance at $p < 0.0063$) and for 4 comparisons on day 3 and 9 in early phase and for 8 comparisons day 43, 48, 53, and 59 in late phase for stool score (significance for each at $p < 0.0125$ and $p < 0.0063$). * Significant differences from WT obtained with t-tests. For panel B, D, Mann–Whitney U test was performed with * indicating for significant differences ($p < 0.05$).

phenotype of *Gpr43*^{-/-} mice during the colon cancer development. Importantly, this phenotype was detected in the absence of microbial changes due to GPR43 deficiency.

Next, we employed another intestinal cancer model (*APC*^{Min/+} mice) to verify the protective role of GPR43. This model develops spontaneous adenomas in both the small intestine and colon. *Gpr43*^{-/-} *APC*^{Min/+} mice developed more tumors in the small intestine and colon than *APC*^{Min/+} mice, with greater numbers of adenomas (Fig. 5A and C). Histological analysis revealed disordered epithelial cancer continuous from intestinal crypts in the intestine of *Gpr43*^{-/-} *APC*^{Min/+} mice (Fig. 5B). The spleen and MLN of *Gpr43*^{-/-} *APC*^{Min/+} mice were abnormally enlarged (Fig. 5D).

Gpr43 is expressed by both tissue cells and hematopoietic cells, including gut epithelial cells, enteroendocrine L cells, adipocytes, eosinophils, basophils, neutrophils, monocytes, dendritic cells, mucosal mast cells, and myometrium endothelium [3]. To determine the relative importance of tissue versus hematopoietic cells in mediating the protective function of GPR43 on colon cancer, we performed a bone marrow (BM) transplantation study. We induced colon cancer with AOM/DSS in these mice and measured weight change, tumor burden, and lymphoid tissue enlargement. While differences between groups in not all the readouts were statistically significant, there was a trend that the “*Gpr43*^{-/-} (BM donor) \rightarrow *Gpr43*^{-/-} (recipient)” group was most susceptible, followed by the “WT \rightarrow *Gpr43*^{-/-}” group, and then by the “*Gpr43*^{-/-} \rightarrow WT” group (Fig. 6). Thus, both cell types may mediate the protective effect of GPR43, but it was the non-hematopoietic tissue cells that play a major role.

GPR43 partially mediates the protective effect of DF and SCFAs

DF and SCFAs have SCFA receptor-independent functions. For example, SCFAs function as histone deacetylase (HDAC) inhibitors or metabolic regulators [36], which do not require SCFA receptors. To determine if the protective effect of DF on AOM/DSS-induced colon cancer is GPR43-dependent, WT and *Gpr43*^{-/-} mice were fed low-fiber diet (LFD, 0% soluble DF: 1:1 = pectin and inulin), medium-fiber diet (MFD, 5% soluble DF), or high-fiber diet (HFD, 15% soluble DF) and were examined for their susceptibility to AOM/DSS-induced carcinogenesis. As demonstrated in the literature, DF suppresses colon cancer development in WT mice in a dose-dependent manner (Fig. 7). DF also suppressed carcinogenesis in *Gpr43*^{-/-} mice but this effect was smaller than that in WT. This indicates that DF can suppress carcinogenesis in both *Gpr43*-dependent and independent manners.

We next studied if GPR43 mediates the protective effect of C3. C3 was chosen because it is a high affinity ligand for GPR43 and an efficient inhibitor of HDACs [15, 16, 37]. The increased AOM/DSS-induced cancer development, along with spleen enlargement, in LFD-fed WT mice was suppressed by C3 administered in drinking water (Fig. 8A and B). C3 decreased cancer formation and spleen enlargement in WT mice and in *Gpr43*^{-/-} mice to a lesser degree (Fig. 8C and E). Interestingly, C3 and DF (i.e. MFD) had additive suppressive effects on cancer development and spleen enlargement (Fig. 8C–E). Overall these results indicate that DF and C3 suppress cancer development in *Gpr43*-dependent and independent manners.

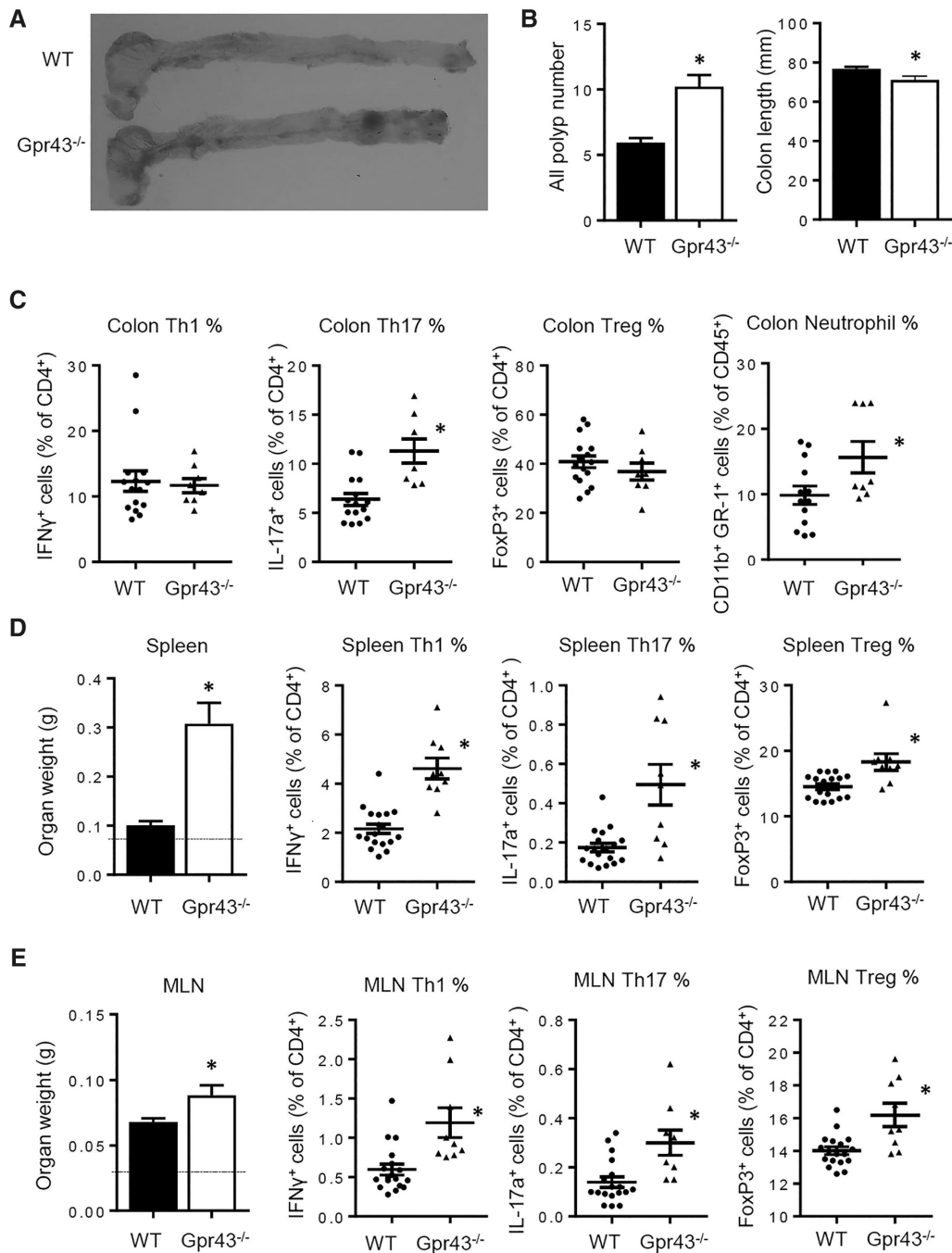


Figure 4. AOM/DSS-induced colon cancer development and T cell responses in co-housed GPR43-deficient mice. (A) Gross colon images of co-housed WT and *Gpr43*^{-/-} mice. (B) Tumor formation (polyp number) in the colon and colon length (mm) of co-housed WT and *Gpr43*^{-/-} mice. (C) Effector T cells, Tregs and neutrophils in the colon of co-housed mice. (D) Spleen weight and T helper cell subset frequencies in the spleen of co-housed mice. (E) MLN weight and effector T cells and Tregs in the MLN of co-housed mice. In panel D and E, the broken lines indicate average weight of the indicated organs of untreated age-matching WT mice. To equalize microbiota between WT and *Gpr43*^{-/-} mice, female mice were co-housed at least one week prior to and until termination of the experiments. Alternatively, male mice were housed on mixed bedding (20% of used bedding equally from WT and *Gpr43*^{-/-} mice and 80% new bedding; changed twice a week). The data from these two experiments were similar and all data were combined. Mice were treated with AOM (10 mg/Kg of body weight, i.p.) and 3 cycles of 1% DSS in drinking water and sacrificed at day 62–63 post AOM injection. Representative and pooled data (mean \pm SEM, $n = 8$ –12) from three independent experiments are shown. * Significant differences ($p < 0.05$, Mann–Whitney U test) from AOM/DSS-treated WT mice.

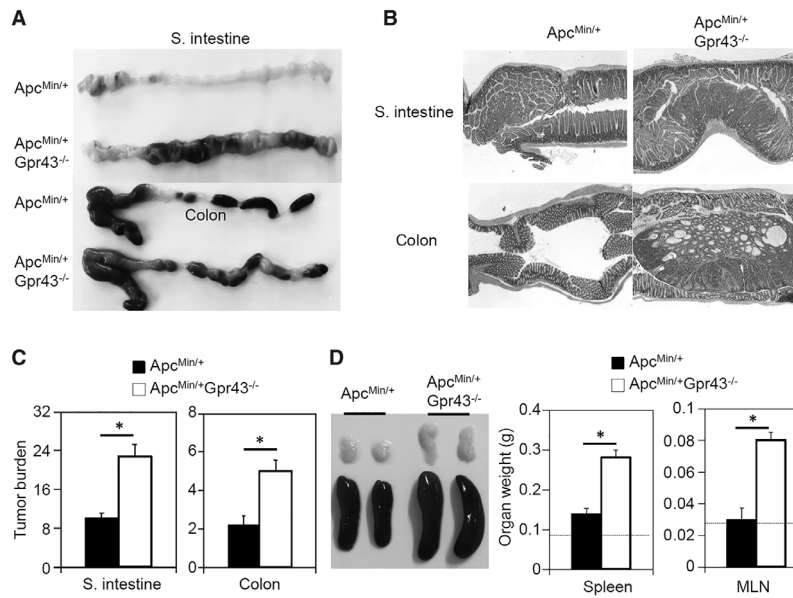


Figure 5. Intestinal tumor formation in WT versus *Gpr43^{-/-} Apc^{Min/+}* mice. (A and B) Images and histological assessment of intestine tissues. Tumor formation in the small intestine and colon *Apc^{Min/+}* and *Apc^{Min/+}Gpr43^{-/-}* mice. (C) Histological assessment of intestine tissues. (D) Enlargement of spleen and MLN in *Apc^{Min/+}* and *Apc^{Min/+}Gpr43^{-/-}* mice. *Apc^{Min/+}* and *Apc^{Min/+}Gpr43^{-/-}* mice were sacrificed at 4 months of age. In panel D, the broken lines indicate average weight of the indicated organs of untreated age-matching WT mice. Representative and pooled data (mean ± SEM; n = 6) from two experiments are shown. * Significant differences from *Apc^{Min/+}* mice ($p < 0.05$, Mann–Whitney U-test).

Increased tissue bacterial load contributes to intestinal carcinogenesis in *Gpr43^{-/-}* mice

Defective regulation of gut permeability in inflammatory conditions has been reported for *Gpr43^{-/-}* mice [13]. We found that the expression of *ocln* (the gene for Occludin) at mRNA level was greatly decreased in the colon of *Gpr43^{-/-}* mice after 3 cycles of 1.5% DSS treatment (Fig. 9A). C3 increased the expression of *ocln* and *tjp1* (the gene for ZO-1) in the colon of WT mice but

failed to increase their expression in *Gpr43^{-/-}* mice. Consistently, gut permeability was higher in *Gpr43^{-/-}* than WT mice following the chronic DSS treatment (Fig. 9B). We performed fluorescent in situ hybridization (FISH) for eubacterial 16S RNA gene and q-PCR analysis to detect bacteria in colon tissues. The abnormal gut permeability in *Gpr43^{-/-}* mice was supported by increased levels of eubacteria in colon tissues (Fig. 9C). We found that many more bacteria were detected within the colon tissues of *Gpr43^{-/-}* compared to WT mice during chronic inflammation. Importantly,

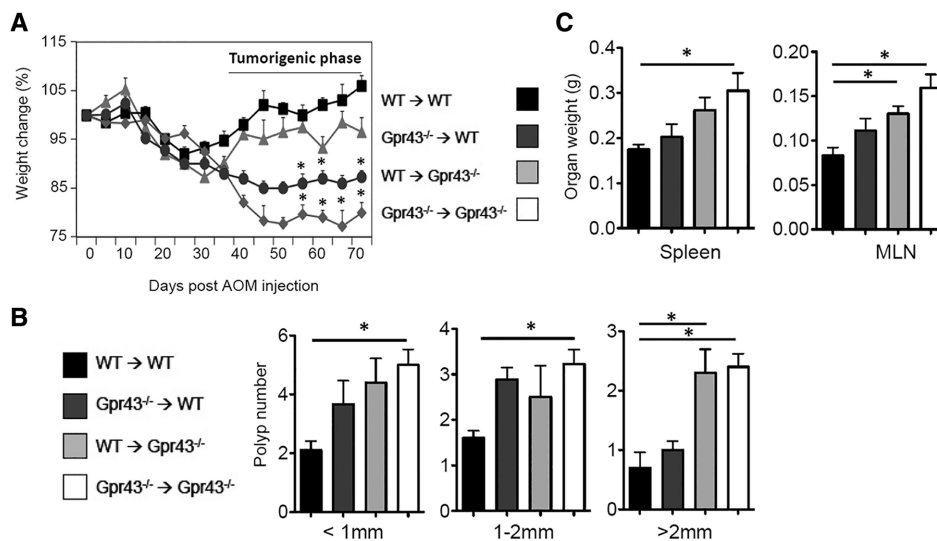


Figure 6. Comparison of non-hematopoietic and hematopoietic GPR43 in suppressing colon cancer. (A) Weight change (%) of mice during AOM/DSS treatment. (B) Tumor formation (number and size) in the colon. (C) Enlargement of spleen and MLN. BM from WT or *Gpr43^{-/-}* mice (10 million cells) were transferred into lethally irradiated WT or *Gpr43^{-/-}* mice, and the chimera mice were treated with AOM/DSS for induction of colon cancer. Chimeras were treated with AOM (10 mg/Kg of body weight, i.p.) and 3 cycles of 1% DSS in drinking water and sacrificed at day 70 post-AOM injection. Representative and pooled data (mean ± SEM, n = 7–9) from two independent experiments are shown. For panel A, group effect was examined for late phase (days 35–70), and upon establishing significant group effect, pairwise comparisons on specific days (days 55, 60, 65, and 70) were done with Bonferroni adjustments for multiple comparisons. Significant group effect was seen in weight change ($p < 0.0001$) using repeated measures ANOVA during late phase. * Significant differences from WT → WT group on days 55–70, obtained with multiple t-tests with Bonferroni’s adjustments for eight multiple pairwise comparisons (significance at $p < 0.0063$). For panel B–C, one-way ANOVA followed by Tukey’s multiple comparisons test was used to make all pairwise comparisons. * Significant differences between indicated pairs.

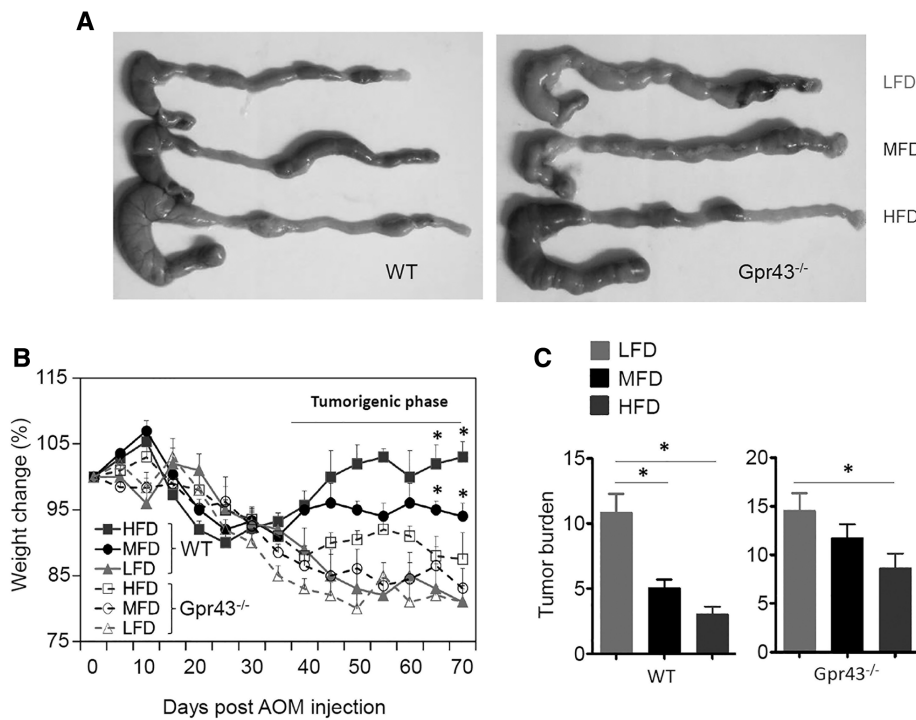


Figure 7. Effect of DF on AOM/DSS-induced colon cancer development in WT and *Gpr43*^{-/-} mice. (A) Colon tissues of WT or *Gpr43*^{-/-} mice fed different levels of DF (LFD, MFD, and HFD) and treated with AOM/DSS. (B) Weight change (%) of WT and *Gpr43*^{-/-} mice during AOM/DSS treatment. (C) Tumor formation in the colon of WT and *Gpr43*^{-/-} mice. Mice were treated by AOM (10 mg/Kg of body weight, i.p.) and 3 cycles of 1% DSS in drinking water and sacrificed at day 70 post AOM injection. Representative and pooled data (mean ± SEM, n = 8–10) from two independent experiments are shown. For panel B, within each of WT and *Gpr43*^{-/-} type, diet effect was examined for late phase (days 35–70), and when significant effect was established, pairwise comparisons on specific days (65 and 70) were done with Bonferroni adjustments for multiple comparisons. Significant diet effects for late phase ($p < 0.0001$) was seen using repeated measures ANOVA in WT mice. * Significant differences from LFD mice on day 65 and 70 with t-tests (significance at $p < 0.0125$). For panel C, one-way ANOVA followed by Tukey’s multiple comparison test was used to make all pairwise comparisons. * Significant differences between indicated pairs.

C3 feeding decreased tissue bacteria (Fig. 9C). We also examined tissue bacteria during AOM/DSS-induced carcinogenesis in the colon. Tissue bacterial load was not different in co-housed WT and *Gpr43*^{-/-} mice at an early stage but later it was decreased in WT but remained elevated in *Gpr43*^{-/-} mice (Fig. 9D), indicating

defective control of tissue bacterial load at the chronic stage in GPR43 deficiency.

To understand the role of the increased tissue bacteria in *Gpr43*^{-/-} mice, mice were treated with antibiotics during intestinal carcinogenesis. Antibiotics suppressed AOM/DSS-induced

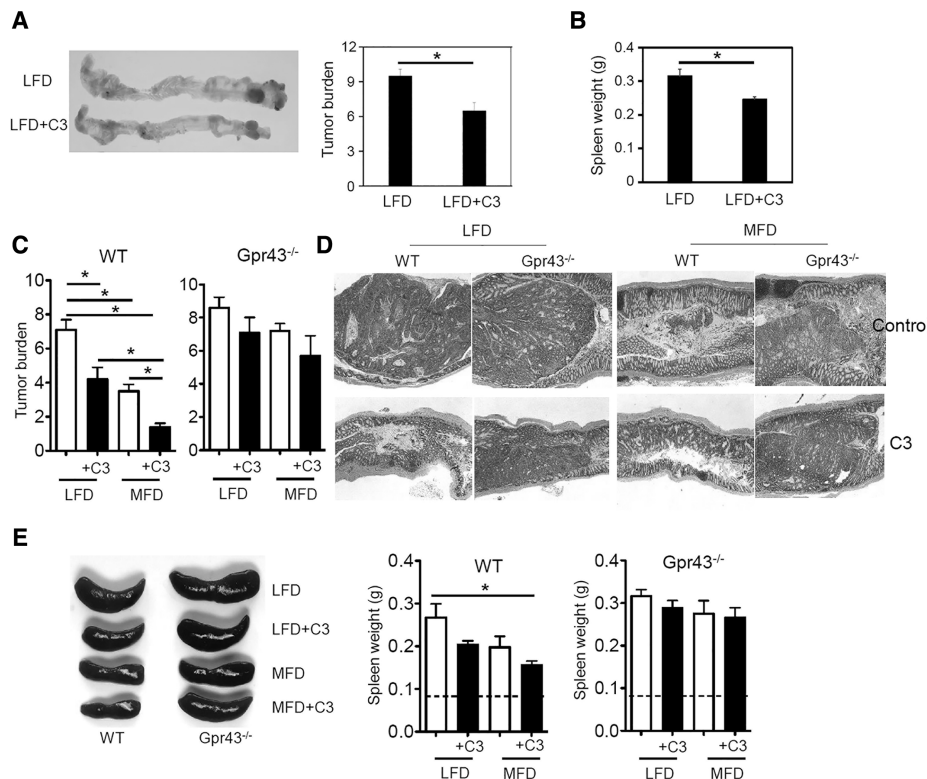


Figure 8. Effect of propionate on AOM/DSS-induced colon cancer development in WT and *Gpr43*^{-/-} mice. (A) Tumor formation in the colon of WT mice fed LFD alone or LFD and C3 (80 mM). (B) Enlargement of spleen following AOM/DSS treatment. (C) Tumor formation in the colon of mice fed indicated diet and drinking water. (D) Histological assessment of colon tissues. Paraffin-embedded tissue sections (6 μm) were stained with hematoxylin and eosin. (E) Spleen enlargement. Mice were fed indicated diet and drinking water and treated with AOM (10 mg/Kg of body weight, i.p.) and 3 cycles of 1% DSS in drinking water and sacrificed at day 70 post AOM injection. Representative and pooled data (mean ± SEM, n = 8–12) from three independent experiments are shown. For panel A and B, Mann–Whitney U test was used for significant differences between two groups *. For panel C and E, in both WT mice and *Gpr43*^{-/-} mice, no interaction of C3 by MFD was found with two-way ANOVA. For both panel C and E, we found significant overall C3 effect ($p < 0.0001$ for panel C, $p = 0.0212$ for panel E) and MFD effect ($p < 0.0001$ for panel C, and $p = 0.0108$ for panel E).

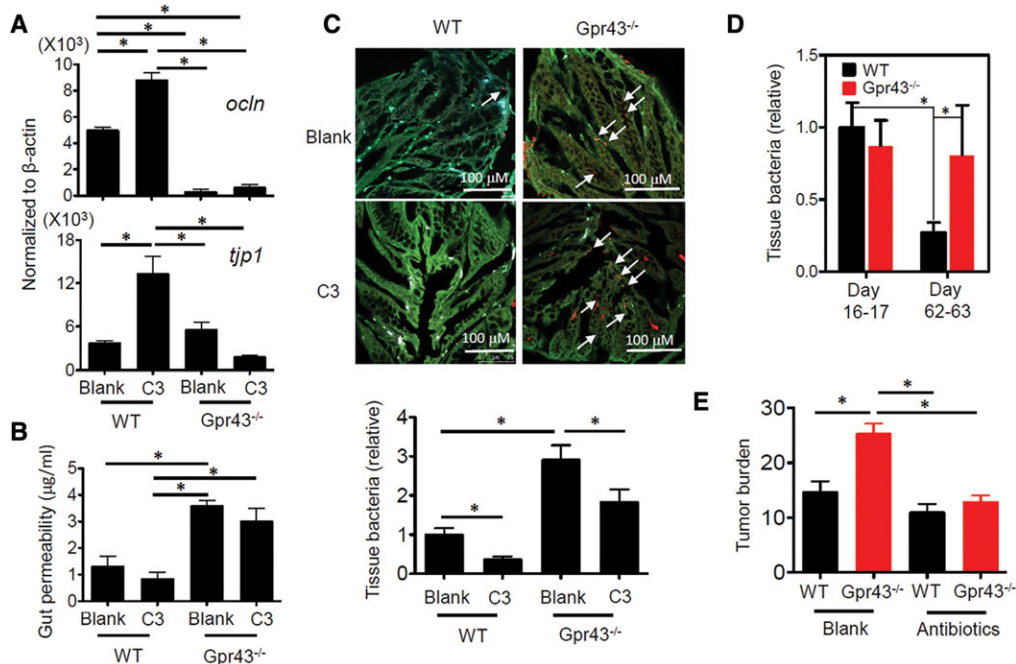


Figure 9. Effects of propionate and GPR43 on gut barrier function and tissue bacterial load during inflammation and colon cancer development in WT versus *Gpr43*^{-/-} mice. In panel A–C, WT and *Gpr43*^{-/-} mice were fed regular rodent chow and C3 (80 mM) in drinking water and treated with 3 cycles of 1.5% DSS and regular water. (A) Expression of tight junction protein genes (*ocln* and *tjp1*) at mRNA level in colon tissues of WT and *Gpr43*^{-/-} mice after DSS treatment. qRT-PCR was performed. (B) Gut permeability in WT and *Gpr43*^{-/-} mice after DSS treatment. Shown is FITC-dextran concentration in blood. (C) Tissue-associated bacteria in the colon following DSS treatment. Confocal microscopy and q-PCR was performed. For q-PCR, DNA isolated from colon tissues was PCR-amplified with primers for total eubacteria and the mouse β -actin gene. Relative levels of eubacterial levels normalized by β -actin gene signals and then by control WT levels are shown. (D) Tissue bacteria load in co-housed mice following AOM/DSS-induced colon cancer. Mice were treated with AOM and 3 cycles of 1% DSS in drinking water and sacrificed at day 16–17 or 62–63 post AOM injection. (E) Tumor formation in the colon from WT and *Gpr43*^{-/-} mice with or without antibiotics treatment. Representative and pooled data (mean \pm SEM, $n = 7$ –9 mice) from two experiments are shown. For all panels, two-way ANOVA with an interaction for the two factors was used, and for each panel A, D, and E, a significant interaction term (each $p < 0.001$) was found. Follow-up multiple 2-sample t-tests were done for pair-wise comparisons with Bonferroni adjustment for six pairwise comparisons for each panel. * Significant t-tests at $p < 0.008$. For panel B and C, interaction of C3 by *Gpr43* was not significant for both gut permeability and for tissue bacteria. Two-way ANOVA with main factors only found significant *Gpr43* ($p < 0.0001$) and C3 ($p = 0.042$) effect for gut permeability (B) and significant *Gpr43* ($p < 0.0001$) and C3 ($p = 0.0022$) effects for tissue bacteria (C).

colon cancer in *Gpr43*^{-/-} mice (Fig. 9E). Antibiotics suppressed the numbers of tumors in *Gpr43*^{-/-} mice than that not in WT mice. Antibiotics also significantly decreased the enlargement of spleen in *Gpr43*^{-/-} mice (not shown). Overall, these results indicate that increased colon cancer formation in *Gpr43*^{-/-} mice is due in part to high levels of tissue bacteria.

Discussion

We studied, in depth, the impact of DF, SCFAs, and SCFA receptors on tissue bacterial load, intestinal inflammation and carcinogenesis. Based on early *in vitro* data with cell lines, it has been proposed that SCFAs inhibit HDACs to suppress cancer cells [38–40]. However, this mechanism alone does not fully explain the SCFA effect on carcinogenesis *in vivo*. A mounting body of evidence, including the data presented in this report, suggests that the protective effect of SCFAs is, in part, mediated by SCFA receptors [27, 31], which function in a largely HDAC-independent manner. We found that SCFAs and SCFA receptors promote barrier immunity and suppress bacterial invasion, which can induce chronic inflammatory

responses. Importantly, SCFAs, exemplified by C3, and GPR43 have suppressive effects on chronic inflammatory responses and intestinal carcinogenesis.

We studied both GPR43 and GPR41 but focused our efforts on GPR43 because the anti-cancer effect of GPR43 was greater than that of GPR41. The two GPCRs are receptors for C2, C3 and C4 but their affinity to these SCFAs are different [16]. While both receptors are expressed by intestinal epithelial cells, the distribution of the two in the body are very different [3, 37]. For example, GPR43 is expressed by neutrophils, whereas GPR41 is expressed by adipocytes. Also, the two may be expressed by different cell types in the intestine. These differences in receptor expression are likely to account for the observed differences in SCFA receptor functions. Our BM transplantation experiment revealed that non-BM-derived tissue cells play a major role in mediating the GPR43-dependent suppression of intestinal inflammation and carcinogenesis, while hematopoietic cells play a relatively minor role. The most likely cells to mediate the GPR43 function are intestinal epithelial cells, which highly express GPR43 and are effectively stimulated by SCFAs for barrier immunity [13]. This finding is in line with a previous report that non-hematopoietic cells are the

major mediator of the GPR109A effect [31]. However, a minor but significant role of hematopoietic cells was still observed in both studies.

SCFAs promote the expression of intestinal tight junction proteins in the steady state and boost anti-microbial responses during infection or following gut barrier breach [14, 41]. T cell responses in the intestine reflect the inflammatory status regulated by SCFAs and their receptors. Particularly, Th17 cells, which contribute to barrier immunity, undergo expansion during an acute phase of immune responses but contraction as inflammatory responses subside. Numbers of Th17 cells remain increased in *Gpr43*^{-/-} mice along with chronic inflammation. This effect somewhat mirrors that of GPR109A [31]. These results identify important roles of SCFAs and their receptors in mounting innate and adaptive immune responses to suppress bacterial invasion across the barrier. The increased Th17 cell response in GPR43 deficiency appears to be a bystander response as the consequence of inflammatory responses due to bacterial invasion but we cannot rule out the possibility that it may involve antigen-specific responses. This finding clarifies the rather unexpected attenuated inflammation observed by Sina et al. at an acute phase of DSS-induced inflammation [29]. It has been recently reported that GPR43 and GPR109A promote epithelial inflammasome responses [28]. Our results together with these reports emphasize the defective acute inflammatory response and associated-gut permeability change in *Gpr43*^{-/-} mice following DSS or AOM/DSS treatment, observed in this study.

Sustained high bacterial burden in colon tissues can cause chronic inflammatory responses. For example, inflammatory cytokines and chemokines, such as IL-18, IL-22, IL-6 and CXCL1, which promote effective barrier immunity to ward off invading bacteria [42–44], were not normally induced at the early phase in *Gpr43*^{-/-} mice. During prolonged treatments with DSS or AOM/DSS, *Gpr43*^{-/-} mice suffer less than WT mice in the first few weeks but become more susceptible afterward. This indicates that SCFA receptors function to coordinately regulate acute and chronic inflammatory responses. The function of SCFA receptors in regulating chronic inflammation is closely related to their function in suppressing intestinal cancer. In *APC*^{min/+} mice, tumors are formed more in the small, than large, intestine, and thus this model provides additional insights into SCFA-receptor-regulated cancer outside the colon. The phenotype of this model indicates that the SCFA-GPR43 axis has anti-cancer effects in both the small and large intestines.

Inflammation is frequently linked to carcinogenesis. Our results indicate that DF and SCFAs, as represented by C3 in this study, suppress intestinal cancer in GPR43-dependent and independent manners. Our findings are in line with others' findings that DF, SCFAs and SCFA receptors exert suppressive effects on colon cancer [27, 31, 45–48] and provide a potential mechanism for the suppressive function. Our findings underscore the important role of the SCFA-SCFA receptor axis in suppressing bacterial invasion, subsequent chronic inflammation and inflammation-associated intestinal carcinogenesis. Colon cancer development is closely associated with gut microbiota changes [49–51]. DF changes the

gut microbiota. For example, DF enriches *Bacteroidaceae* and *Bifidobacteriaceae* and potentially decrease pathogenic bacteria [52]. *Firmicutes*, particularly those of the *Erysipelotrichaceae* family, are increased in DF deficiency. Moreover, GPR43 functions to increase beneficial bacterial species [27]. In this study, co-housing with WT-littermates failed to suppress the elevated chronic Th17 response, bacterial invasion and associated colon cancer development in *Gpr43*^{-/-} mice in response to AOM/DSS-treatment. This indicates that control of the microbiota by GPR43 does not have a dominant role on colon cancer development. Rather, the data underline the importance of host cells in mediating the protective effect of GPR43. We conclude that the SCFA-GPR43 axis functions to boost barrier immune responses against microbiota and this response limits persistent bacterial invasion, chronic inflammatory responses and colon cancer development.

Materials and methods

Mice, diet, and SCFA administration

All animal experiments were approved by the Purdue Animal Care and Use Committee (PACUC). CD45.1 C57BL/6 mice and *Apc*^{Min/+} mice were from the Jackson Laboratory (Bar Harbor, ME). *Gpr43*^{-/-} mice were purchased from Deltagen (San Mateo, CA), and *Gpr41*^{-/-} mice were obtained from Dr. M Yanagisawa (UT Southwestern Medical Center at Dallas). All mice were on C57BL/6 background. *Gpr43*^{-/-} mice were crossed with *Apc*^{Min/+} mice to derive *Apc*^{Min/+}*Gpr43*^{-/-} mice. The mice were kept at the Purdue Life Science Animal Facility for at least 12 months prior to the study. Mice were fed regular rodent chow (Harlan 2018S Global 18% Protein Rodent Diet) or special DF diets. The special diets containing different amounts of DF (0% of pectin and inulin at 1:1 ratio for LFD, 5% for MFD, and 15% for HFD) were custom-ordered from Envigo. Mice were fed special diets from 3 weeks of age and/or drinking water containing C3 (80 mM, pH 7.4). Mice were treated with DSS or AOM/DSS from 5 or 6 weeks of age for most mice or 11–12 weeks of age for BM chimera mice. When indicated, mice were treated with antibiotics (ampicillin, neomycin, and metronidazole at 1 g/L and vancomycin at 0.5 g/L). When indicated (Figs. 4 and 9D, and Supporting Information Fig. 2), female mice were co-housed for 1–2 weeks before and during the induction of AOM/DSS-induced colon cancer. Alternatively, WT and *Gpr43*^{-/-} mice were housed in cages inoculated with mixed soiled bedding from WT and *Gpr43*^{-/-} mice (20% old mixed and 80% new bedding; changed every 3–4 days). The data obtained with the two methods were similar and therefore were combined.

DSS-induced acute and chronic intestinal inflammation

For acute intestinal inflammation, mice were on 3.5% DSS in drinking water for 5 days and then on plain water before they

were euthanized on day 9. For chronic intestinal inflammation, mice were treated with 1.5% DSS in drinking water for 7 days and then with regular water for 7 days. This cycle was repeated three times. Intestinal tissues were examined by flow cytometry for T cells and neutrophils and by qRT-PCR for expression of inflammatory cytokines (IL-6, IL-18, IL-22, and CXCL-1) as described previously [13, 53]. Primers used for this study were previously described [13].

Tight junction proteins, tissue bacterial burden, and cecal bacterial 16S rRNA gene PCR analysis

Mice were assessed for expression of tight junction protein genes (*ocln* and *tjp1*), gut permeability, and tissue bacteria 42 days after the start of DSS treatment. The expression of *ocln* and *tjp1* was determined by qRT-PCR with the primers: ACC ACT ATG AAA CAG ACT ACA CG (Ocln-F), GGA GAA CTA CAC GCT ATT AAA CG (Ocln-R), GAA GAT GAA GAT GAG GAT GGT CA (*tjp1*-F), ACC AGT GAT TAG TCC TGT CTT TGT (*tjp1*-R). Tissue bacterial levels were determined by q-PCR of a common eubacterial 16S rRNA gene sequences and normalized by genomic β -actin levels as previously described [13]. Cecal bacterial 16S rRNA gene PCR analysis for cohoused animals was performed as described previously [13, 54].

Gut permeability

For assessing gut permeability, mice were fasted overnight and orally gavaged with fluorescein isothiocyanate (FITC)-conjugated dextran (0.2 mg/g of body weight, mean molecular weight of 3000–5000; Sigma- Aldrich, St Louis, MO) using a round-tip feeding needle. Mice were sacrificed 3 h later, and the FITC-dextran concentration in the plasma was determined with a fluorescent microplate reader (excitation at 485 nm and emission at 535 nm; Synergy HT; BioTek, Winooski, VT).

AOM/DSS and APC^{Min/+}-induced models of intestinal cancer

Mice were treated i.p. with azoxymethane (AOM, 10 mg/kg of body weight) and rested for 7 days. The mice were then administered with 1% DSS (36–50 kDa) in drinking water for 7 days and rested on regular water for 7 days. This cycle was repeated 3 times. Mice were monitored daily for changes in body weight, signs of illness, and stool score. Mice were sacrificed 62–70 days post-AOM injection and were assessed for polyp size and number and organ (spleen and MLN) weight. Colon tissues were paraffin-embedded and tissue sections were stained with hematoxylin and eosin. *Apc*^{Min/+} mice and *Apc*^{Min/+}*Gpr43*^{-/-} mice were similarly assessed for tumors in the small and large bowels and organ weight at 4 months of age.

Cell isolation and flow cytometry

For isolation of intestinal LP cells, intestinal tissues were harvested into cold PBS and trimmed to remove mesenteries, fat, and Peyer's patches. Intestinal tissues were cut into ~2 cm pieces. The epithelial layer was extracted five times with 10 mM EDTA in HBSS buffer at room temperature for 15 min, and then the remaining tissues were digested twice at 37°C for 1 h with 1.5 mg/mL collagenase (type 3, Worthington). Collected cells were stained with antibodies to Gr-1 (RB6-8C5), CD11b (M1/70) and CD45 (30-F11) for neutrophils or to CD4 (RM4-5) for T cells. For intracellular staining of IL-17a and IFN- γ , cells were activated in RPMI 1640 (10% fetal bovine serum) with phorbol myristate acetate (50 ng/mL), ionomycin (1 mM), and monensin (2mM; Sigma- Aldrich) for 4 h. Cells were then fixed, permeabilized, and stained with antibodies to mIL-17A (TC11-18H10.1) or IFN γ (XMG1.2). For FoxP3 staining, cells were fixed, permeabilized, and then stained with FoxP3 (FJK-16S) according to the manufacturer's protocol (eBioscience). Detailed gating strategies are found in Supporting Information Fig. 1.

Bone marrow transplantation

To prepare host mice, WT or *Gpr43*^{-/-} mice were irradiated at 500 rads, and this was repeated after 4 h. Host mice were injected i.v. with 1×10^7 BM cells from WT or *Gpr43*^{-/-} mice. The CD45.1 and CD45.2 congenic system was used to distinguish donor and recipient cells. Six weeks later, mice were checked for hematopoietic reconstitution and were treated with AOM and DSS. The marrow reconstitution efficiency in these animals was >95% (spleen).

Confocal microscopy and histological analysis

Colon tissues, harvested from WT or *Gpr43*^{-/-} mice subjected to chronic DSS treatment, were frozen in Tissue-Tek OCT compound (Sakura) and were cut into 8- μ m sections. The sections were fixed in cold acetone and stained with CY3-labeled EU338 (5'-GCT GCC TCC CGT AGG AGT-3, Eurofins) and Pan-cytokeratin. Confocal images were collected with a SP5 II laser scanning microscopic system (Leica). For histological analysis, sections (6 μ m) of paraffin-embedded intestinal tissues were stained with hematoxylin and eosin. Histological images were taken at 50 \times magnification.

Statistical analysis

For weight change and stool score, repeated measures ANOVA was used to determine group effects (Fig. 1A, 2A, 3A, 6A, and 7B) for indicated time periods, and after finding a significant group effect, pairwise comparisons of interest on specific days were made using two-sample *t*-tests with Bonferroni's adjustments to account for multiple comparisons. For analyzing Fig. 8C, 8E, and 9, two-way ANOVA was used with an interaction of the two factors, and upon a

significant interaction, multiple pairwise comparisons were made using t-tests with Bonferroni adjustments. When no interaction is evident, results from two-way ANOVA with main effects only are presented. For comparing multiple groups in Fig. 1B-E, 2B-E, 6B, 6C and 7C, one-way ANOVA followed by Tukey's multiple comparisons test was used. Additionally, Mann-Whitney U-test (two-sided with statistical significance set at 0.05) was used to compare two groups of interest (Fig. 3B, 3D, 4 and 5C, 5D, 8A, and 8B). All error bars indicate SEM. Prism (Graphpad) and SAS statistical software were used.

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Abbreviations: Abx: antibiotics · AOM: azoxymethane · BM: bone marrow · C2: acetate · C3: propionate · C4: butyrate · DF: dietary fiber · DSS: dextran sodium sulfate · GPCR: G-protein-coupled receptors · HFD: high fiber diet · LFD: low fiber diet · LP: lamina propria · MFD: medium fiber diet · q-PCR: quantitative PCR · SCFAs: short-chain fatty acids

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