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Activation of Gpr109a, Receptor for Niacin and the Commensal Metabolite Butyrate, Suppresses Colonic Inflammation and Carcinogenesis

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SUMMARY

Commensal gut microflora and dietary fiber protect against colonic inflammation and colon cancer through unknown targets. Butyrate, a bacterial product from fermentation of dietary fiber in the colon, has been implicated in this process. GPR109A (encoded by *Niacr1*) is a receptor for butyrate in the colon. GPR109A is also a receptor for niacin, which is also produced by gut microbiota and suppresses intestinal inflammation. Here we showed that Gpr109a signaling promoted anti-inflammatory properties in colonic macrophages and dendritic cells and enabled them to induce differentiation of Treg cells and IL-10-producing T cells. Moreover, Gpr109a was essential for butyrate-mediated induction of IL-18 in colonic epithelium. Consequently, *Niacr1*$^{-/-}$ mice were susceptible to development of colonic inflammation and colon cancer. Niacin, a pharmacological Gpr109a agonist, suppressed colitis and colon cancer in a Gpr109a-dependent manner. Thus, Gpr10a has an essential role in mediating the beneficial effects of gut microbiota and dietary fiber in colon.

INTRODUCTION

Commensal microbiota in the gut have profound effects on human health (Bäckhed et al., 2005; Honda and Littman, 2012). Germ-free and antibiotic-treated mice are more susceptible to dextran sulfate sodium (DSS)-induced colonic inflammation (Maslowski et al., 2009; Rakoff-Nahoum et al., 2004). *Bacteroides fragilis* and *Clostridium* clusters IV and XIVa protect against trinitrobenzenesulfonic acid- or DSS-induced colitis (Atarashi et al., 2011; Mazmanian et al., 2008). Multiple intestinal neoplasia (Min, *Apc*$^{Min/+}$) mice carry a germline-truncating mutation in one copy of *Apc* and spontaneoulsy develop adenomas throughout the intestinal tract. *Lactobacillus acidophilus* and certain gut microbial metabolites such as conjugated linoleic acids decrease intestinal tumorigenesis in *Apc*$^{Min/+}$ mice (Davis and Milner, 2009; Urbanska et al., 2009). In contrast, depletion of microbiota ameliorates intestinal inflammation and cancer in mouse models of spontaneous colitis (*Il10*$^{-/-}$, *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$, or *Apc*$^{Min/+}$) (Garrett et al., 2009; Grivennikov et al., 2012; Li et al., 2012; Uronis et al., 2009). *Bacteroides fragilis* toxin (BFT) and *Bacteroides vulgatus* increases inflammation and colon cancer in *Apc*$^{Min/+}$ and *Il10*$^{-/-}$ mice, respectively (Uronis et al., 2009; Wu et al., 2009). Thus, commensal bacteria promote as well as suppress colonic inflammation and colon cancer in a context-dependent manner.

One of the mechanisms by which gut microbiota promote colonic health is through production of the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate by fermentation of dietary fiber. Among SCFAs, butyrate has received most attention for its effects on colonic health (Hamer et al., 2008). The functions of butyrate in promoting colonic health range from being energy source for colonocytes to being a key mediator of anti-inflammatory and antitumorigenic effects. Gut microbiome analysis has revealed a significant decrease in the number of butyrate-producing bacteria in colon of patients with ulcerative colitis and colon cancer (Frank et al., 2007; Wang et al., 2012). Colonic irrigation with butyrate suppresses inflammation during ulcerative colitis (Hamer et al., 2008).

IL-10 deficiency leads to spontaneous colitis (Huber et al., 2011; Izcue et al., 2009; Rubtsov et al., 2008). Polymorphisms in the genes that encode IL-10 or IL-10 receptor are linked to increased incidence of ulcerative colitis and inflammatory bowel disease (Franke et al., 2008; Glocker et al., 2009). Human monocyte-derived dendritic cells (DCs), when matured in the presence of butyrate, have increased expression of IL-10 and decreased production of IL-6 (Millard et al., 2002; Wang et al., 2008). IL-18 plays an essential role in suppression of colonic inflammation and inflammation-associated cancers (Chen et al., 2011; Dupaul-Chicoine et al., 2010; Elinav et al., 2011; Salcedo et al., 2010; Zaki et al., 2010). Moreover, an *IL-18* gene promoter polymorphism leading to decreased expression is found at higher frequency in patients with ulcerative colitis (Takagawa et al.,

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2005). Butyrate induces expression of IL-18 in colonic epithelium (Kalina et al., 2002). In addition, the G protein-coupled receptor 43 (Gpr43) mediates proliferation of colonic regulatory T (Treg) cells in response to exogenously administered SCFAs but not under steady-state conditions (Smith et al., 2013). Although these studies demonstrate that SCFAs serve as anti-inflammatory agents in the colon, the underlying molecular mechanisms remain poorly understood.

The most widely studied function of butyrate is its ability to inhibit histone deacetylases. However, cell surface receptors have been identified for butyrate; these receptors, GPR43 and GPR109A (also known as hydroxycarboxylic acid receptor 2 or HCA2), are G protein coupled and are expressed in colonic epithelium, adipose tissue, and immune cells (Blad et al., 2012; Ganapathy et al., 2013). GPR43-deficient mice undergo severe colonic inflammation and colitis in DSS-induced colitis model and the GPR43 agonist acetate protects germ-free mice from DSS-induced colitis (Maslowski et al., 2009). Although GPR43 is activated by all three SCFAs, GPR109A (encoded by *Niacr1*) is activated only by butyrate (Blad et al., 2012; Taggart et al., 2005). GPR109A is also activated by niacin (vitamin B3) (Blad et al., 2012; Ganapathy et al., 2013). In colonic lumen, butyrate is generated at high concentrations (10–20 mM) by gut microbiota and serves as an endogenous agonist for GPR109A (Thangaraju et al., 2009). We have shown that Gpr109a expression in colon is induced by gut microbiota and is downregulated in colon cancer (Cresci et al., 2010; Thangaraju et al., 2009). Gpr109a in immune cells plays a nonredundant function in niacin-mediated suppression of inflammation and atherosclerosis (Lukasova et al., 2011). Gut microbiota also produce niacin. Niacin deficiency in humans results in pellagra, characterized by intestinal inflammation, diarrhea, dermatitis, and dementia (Hegyi et al., 2004). It is of great clinical relevance that lower abundance of GPR109A ligands niacin and butyrate in gut is associated with colonic inflammation.

Here we demonstrate an anti-inflammatory and anticancer function for Gpr109a in colon. Gpr109a signaling imposed anti-inflammatory properties in colonic antigen-presenting cells, which in turn induced differentiation of Treg cells and IL-10-producing T cells. Gpr109a was also required for the expression of IL-18. *Niacr1*$^{-/-}$ mice showed enhanced susceptibility to colitis and colon cancer. Depletion of gut microbiota or dietary fiber increased the risk for colitis and cancer, which is effectively suppressed by niacin in a Gpr109a-dependent manner.

RESULTS

Gpr109a Signaling Regulates Treg and IL-10-Producing CD4$^+$ T Cell Frequency in the Colon

We hypothesized that GPR109A has an anti-inflammatory role in the colon. Because Treg cells are potent anti-inflammatory cells, we studied the status of Treg cells in colons of mice lacking Gpr109a. Colonic lamina propria (LP) of *Niacr1*$^{-/-}$ mice harbor significantly reduced (~40%) frequency and number of Foxp3$^+$ (Treg) cells among CD4$^+$ cells than do LP of WT mice (Figures 1A and 1B; Figure S1A available online). In contrast, a similar frequency of Treg cells was present among splenic and small intestinal CD4$^+$ T cells from both WT and *Niacr1*$^{-/-}$ mice, suggesting that reduction in Treg cells is specific to colon in *Niacr1*$^{-/-}$ mice (Figures 1A, 1B, S1B, and S1C). CD4$^+$ T cells producing IL-10 were also significantly reduced in colonic LP of *Niacr1*$^{-/-}$ mice compared to those from WT mice (Figures 1C and 1D). In contrast, the frequency and number of CD4$^+$ T cells producing inflammatory cytokine IL-17 were elevated in colonic LP of *Niacr1*$^{-/-}$ mice compared to that of WT mice (Figures 1E, 1F, and S1D). Similar fractions of splenic CD4$^+$ T cells produced IL-17 or IL-10 in both WT and *Niacr1*$^{-/-}$ mice, suggesting that the proinflammatory phenotype of CD4$^+$ T cells in *Niacr1*$^{-/-}$ mice was specific to colonic LP.

Colonic DCs and Macrophages from *Niacr1*$^{-/-}$ Mice Are Defective in Inducing Differentiation of Treg and IL-10-Producing CD4$^+$ T Cells

To gain insight into the mechanism of reduced Treg and IL-10-producing CD4$^+$ T cells in colons of *Niacr1*$^{-/-}$ mice, first we evaluated the expression of Gpr109a by various immune cells. *Niacr1* mRNA is expressed by adipocytes and innate immune cells (Gille et al., 2008). To determine its expression at the protein level, human peripheral blood mononuclear cells were stained with an antibody specific to human GPR109A that was expressed at highest levels in monocytes (Figure S2A). Human blood DCs expressed lower but substantial amounts of GPR109A. In contrast, T, B, and NK cells did not exhibit detectable amounts of GPR109A. qPCR revealed similar pattern of *Niacr1* expression on mouse splenic T cells, B cells, DCs, and macrophages (Figure S2B). Similarly, colonic DCs, macrophages, and epithelium also expressed Gpr109a (Figure S2B).

Because macrophages and DCs expressed Gpr109a and because colonic DCs are critical in maintaining the balance between Treg cells and IL-10- and IL-17-producing CD4$^+$ T cells (Coombes et al., 2007; Manicassamy et al., 2010; Sun et al., 2007), we reasoned that the proinflammatory phenotype of colonic CD4$^+$ T cells in *Niacr1*$^{-/-}$ mice is dependent on colonic DCs and macrophages. Therefore, we tested the ability of macrophages (CD45$^+$I-A^{b+}CD11b$^+$) and DCs (CD45$^+$I-A^{b+}CD11c$^+$) isolated from colons of WT and *Niacr1*$^{-/-}$ mice to promote differentiation of naive CD4$^+$ T cells from OT-II mice into Treg or Th17 cell lineage. Colonic DCs and macrophages from *Niacr1*$^{-/-}$ mice were defective in inducing differentiation of naive OT-II CD4$^+$ T cells into Treg cells (Figure 2A). In line with this, CD4$^+$ T cells primed with colonic DCs and macrophages from *Niacr1*$^{-/-}$ mice produced reduced amount of IL-10 compared to those primed with WT counterparts (Figure 2B). In contrast, colonic DCs and macrophages from *Niacr1*$^{-/-}$ mice induced differentiation of CD4$^+$ T cells into cells producing higher amounts of the inflammatory cytokine IL-17 compared to those from WT mice (Figure 2B). CD103$^+$ intestinal DCs express aldehyde dehydrogenase (Aldh1a) and induce conversion of naive T cells into Treg cells (Coombes et al., 2007; Sun et al., 2007). Similar frequency of CD103$^+$ cells was present among colonic DCs from both WT and *Niacr1*$^{-/-}$ mice (data not shown), ruling out the possibility that reduced ability of Gpr109a-deficient colonic DCs to induce conversion of naive T cells into Treg cells was due to fewer number of CD103$^+$ DCs. These data indicate that colonic DCs and macrophages from *Niacr1*$^{-/-}$ mice are defective in inducing differentiation of naive T cells into Treg and IL-10-producing T cells.

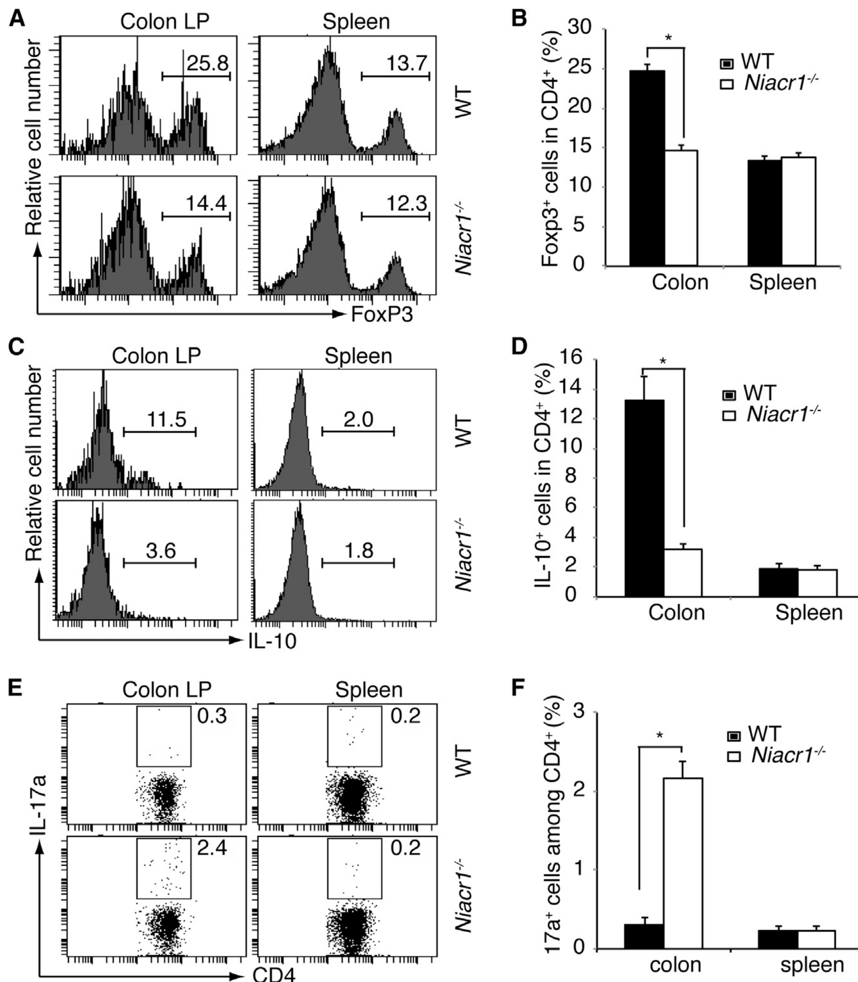

Figure 1. Proinflammatory Phenotype of Colonic CD4+ T Cells from *Niacr1*$^{-/-}$ Mice

(A) Foxp3 expression by CD4+ T cells from colonic lamina propria (LP) and spleens of WT and *Niacr1*$^{-/-}$ mice.

(B) Percent of Foxp3+ in CD4+ populations from colonic LP and spleens of WT and *Niacr1*$^{-/-}$ mice (n = 3).

(C and E) IL-10 and IL-17 expression by PMA+ ionomycin-stimulated colonic LP and splenic CD4+ T cells from WT and *Niacr1*$^{-/-}$ mice.

(D and F) Quantification of IL-10- and IL-17-producing CD4+ T cells shown in (C) and (E), respectively (n = 3).

The numbers in (A), (C), and (E) represent percentage of positive cells in indicated area. Error bars represent standard deviation (SD) of mean. *$p < 0.002$. A representative of three independent experiments is shown. See also Figure S1.

Butyrate- or Niacin-GPR109a Signaling Imparts Anti-inflammatory Phenotype on DCs and Macrophages

Intestinal DCs and macrophages express anti-inflammatory molecules such as IL-10 and Aldh1a that enable them to preferentially favor differentiation of naive T cells into Treg cells and suppress development of Th17 cells (Coombes et al., 2007; Manicassamy et al., 2010; Sun et al., 2007). *Niacr1*$^{-/-}$ colonic DCs and macrophages were defective in expression of Aldh1a1 and IL-10 (Figure 2C). In contrast, expression of the proinflammatory cytokine IL-6, which induces differentiation of naive CD4+ T cells into proinflammatory Th17 cells, was higher in colonic APCs from *Niacr1*$^{-/-}$ mice than in those from WT counterparts (Figure 2C).

We hypothesized that butyrate, which is produced in colon by gut microbiota and is an agonist for GPR109A, induces expression of IL-10 and Aldh1a1 in colonic macrophages and DCs in a Gpr109a-dependent manner. For this, we cultured splenic CD11c+ (DCs) and CD11b+ (macrophages) cells in the presence or absence of butyrate and niacin and then analyzed the expression of *Il10* and *Aldh1a1*. Both butyrate and niacin induced expression of *Il10* and *Aldh1a1* by WT splenic DCs and macrophages (Figures 2D and S2C). In contrast, butyrate and niacin failed to influence the expression levels of *Il10* and *Aldh1a1* in *Niacr1*$^{-/-}$ splenic DCs and macrophages. Consistent with this, WT splenic DCs and macrophages treated with either butyrate or niacin showed superior capability to induce differentiation of naive T cells into Treg cells than untreated counterparts (Figures 2E and S2D). In addition, CD4+ T cells primed with butyrate- or niacin-treated WT splenic DCs or macrophages produced higher amounts of IL-10 and reduced amounts of IL-17 (Figures S2E and S2F). In contrast, treatment with butyrate or niacin failed to affect the ability of *Niacr1*$^{-/-}$ splenic DCs and macrophages to influence T cell differentiation (Figures 2E and S2D–S2F).

Antibiotic treatment reduces Treg cell numbers in colon (Atarashi et al., 2011; Smith et al., 2013). We tested whether niacin induces Treg cells in antibiotic-treated mice in vivo. Compared to control mice, antibiotic-treated WT mice had fewer Treg cells in colon (Figures 2F and S2G). Niacin administration restored the Treg cell number in colons of antibiotic-treated WT mice, but niacin was ineffective in influencing the Treg cell number in *Niacr1*$^{-/-}$ mice. To test the role of niacin and Gpr109a in antigen-specific Treg cell induction in vivo, OT-II T cells were transferred into WT or *Niacr1*$^{-/-}$ mice that were treated with antibiotics in the presence or absence of niacin, and the mice were fed the cognate antigen ovalbumin in drinking water. Antibiotic treatment reduced FoxP3+ T cells among OT-II CD4+ T cell population in WT mice. Niacin increased Treg cells in adoptively transferred OT-II CD4+ T cells in colons of antibiotic-treated WT mice, whereas the effect of niacin to increase Treg cells in adoptively transferred OT-II CD4+ T cells was blunted in colons of *Niacr1*$^{-/-}$ mice (Figure S2H). Colonic macrophages and DCs promote Treg cell differentiation via retinoic acid (RA); hence, RA receptor (RAR) antagonists inhibit intestinal macrophage- or DC-induced conversion of naive T cells into Treg cells. RAR antagonist LE135 completely abrogated the butyrate- or niacin-mediated enhancement of Treg cell conversion by WT DCs (Figure S2I), indicating that butyrate or niacin instructs splenic DCs to acquire anti-inflammatory properties.

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Butyrate or Niacin Induces IL-18 Expression in Colonic Epithelium in a Gpr109a-Dependent Manner

Colons of germ-free (GF) mice expressed lower amount of *Il18* mRNA than those of conventionally housed mice (Figure S3A). Toll-like receptor (TLR) ligands do not influence *Il18* expression in the colon (Larsson et al., 2012), but butyrate does (Kalina et al., 2002). To test the role of Gpr109a in butyrate-mediated induction of *Il18* mRNA, we cultured neonatal colons from WT mice in the presence of butyrate and niacin for 1 day. Both butyrate and niacin upregulated *Il18* mRNA in neonatal colon organ cultures (Figure S3B) but lipopolysaccharide did not (Figure S3B). Butyrate- and niacin-mediated induction of *Il18* mRNA in neonatal colon was dependent on Gpr109a (Figure 3A). Expression of IL-18 in colonic epithelium protects colon against inflammation and carcinogenesis in animal models (Dupaul-Chicoine et al., 2010; Elinav et al., 2011; Salcedo et al., 2010). Therefore, we analyzed the expression of IL-18 in colonic epithelium of WT and *Niacr1*$^{-/-}$ mice. Colonic epithelium from *Niacr1*$^{-/-}$ mice contained significantly reduced amount of IL-18 mRNA and protein compared to their WT counterparts (Figure 3B). Next we tested the role of Gpr109a in butyrate- and niacin-mediated induction of IL-18 mRNA in vivo. One day after administration of butyrate or niacin, colonic epithelium from WT and *Niacr1*$^{-/-}$ mice were tested for expression of IL-18. Both butyrate and niacin induced expression of IL-18 in colonic epithelium of WT mice but failed to do so in those of *Niacr1*$^{-/-}$ mice (Figure 3C).

Gpr109a Deficiency Enhances Susceptibility to Lethal Colitis and Colonic Inflammation

Because Gpr109a regulated the expression of IL-18, IL-10, Aldh1a1, and the presence of Treg cells in colon, we examined the role of Gpr109a in colonic inflammation. WT and *Niacr1*$^{-/-}$ mice were subjected to 3% DSS in drinking water for 6 days and their survival was monitored. *Niacr1*$^{-/-}$ mice were highly susceptible to this treatment and started dying on the 5th day of DSS administration and all of them succumbed to death by day 10 (Figure S4A). In contrast, all the WT mice were alive all through the completion of the study.

To monitor both colonic inflammation and carcinogenesis in a single model, we used a well-characterized mouse model of inflammation-associated colon cancer in which DSS-mediated injury induces inflammation that contributes to colon carcinogenesis caused by azoxymethane (AOM). Mice were subjected to intraperitoneal injection of AOM, followed by cyclic DSS (2%) treatment (Figure 4A). We first evaluated the development of colonic inflammation after the first cycle of DSS treatment. Compared to WT mice, *Niacr1*$^{-/-}$ mice showed severe weight loss, diarrhea, and rectal bleeding and by day 15 they had lost ~10% of body weight (Figures 4B–4D). After AOM+DSS treatment, colons of *Niacr1*$^{-/-}$ mice shrank and showed increased weight per unit length compared to colons of WT mice (Figure S4B). Myeloperoxidase activity, a hallmark of colonic inflammation, was upregulated in colons of *Niacr1*$^{-/-}$ mice after AOM+DSS treatment (Figure S4C). Colons from untreated WT and *Niacr1*$^{-/-}$ mice showed no morphological sign of damage or inflammation. Colonic sections from AOM+DSS treatment group revealed extensive damage to mucosa with epithelial erosion and frequent ulcerations, loss of crypt structures, and infiltration by immune cells in colons of *Niacr1*$^{-/-}$ mice compared to colons of similarly treated WT mice; this resulted in a higher histological score (inflammation + epithelial damage) in the former group (Figures 4E and S4D). Colons of *Niacr1*$^{-/-}$ mice showed profoundly decreased staining for the tight junction protein claudin-3 after treatment with AOM+DSS (Figure 4F), indicating epithelial barrier breakdown. This was confirmed by increased translocation of bacteria into liver and spleen, increase in FITC-dextran in serum after oral gavage, and enhanced systemic inflammation as shown by elevated levels of serum amyloid A, IL-6, IL-17, CCL2, IL-1β, and CXCL1 in the serum (Figures S4E and S4F and data not shown).

Colons of unmanipulated *Niacr1*$^{-/-}$ mice expressed reduced amount of IL-10 compared to those of WT mice. After AOM+DSS treatment, colons of WT mice showed a modest reduction in IL-10 amounts, whereas colons of *Niacr1*$^{-/-}$ mice exhibited a severe impairment of IL-10 production (Figure 4G). Expression of several other genes that inhibit colitis and colon carcinogenesis such as *Tgfb1*, *Tgfb2*, *Tgfbr1*, and *Tgfbr2* was also drastically reduced in colons of *Niacr1*$^{-/-}$ mice compared to WT mice after AOM+DSS treatment (Figure S4G). In line with defective IL-18 production by colonic epithelium, colons of untreated *Niacr1*$^{-/-}$ animals expressed significantly decreased amount of IL-18 compared to those of WT counterparts. Compared to untreated animals, IL-18 expression was higher in colons of WT mice after AOM+DSS treatment. In contrast, IL-18 amounts in colons of *Niacr1*$^{-/-}$ mice were drastically decreased compared to colons of WT mice after AOM+DSS treatment (Figure 4G). Reduction in IL-18 production was not due to defective inflammasome activation because colons and sera of AOM+DSS-treated *Niacr1*$^{-/-}$ mice contained higher amounts of IL-1β (Figures S4F and S4H). Increased amounts of IL-17 are associated with colonic inflammation. Consistent with hyperproduction of IL-17 by colonic CD4^{+} T cells, colons of untreated and AOM+DSS-treated *Niacr1*$^{-/-}$ mice expressed higher amounts IL-17 compared to their WT counterparts (Figure 4G). Amounts of other cytokines that promote colonic inflammation and carcinogenesis such as *Il1a,* IL-6, CXCL1, CCL2, and IL-1β were also markedly increased in colons of *Niacr1*$^{-/-}$ mice after AOM+DSS treatment compared to their WT counterparts (Figures S4G and S4H). Taken together, these data demonstrate that an imbalance in the production of anti-inflammatory molecules versus proinflammatory molecules in favor of the latter increases the susceptibility of *Niacr1*$^{-/-}$ mice to colonic inflammation.

GPR109a Deficiency Promotes Inflammation-Induced as well as *Apc*$^{Min/+}$-Driven Colon Carcinogenesis

We asked whether *Niacr1*$^{-/-}$ mice are more susceptible to development of colon cancer. In colons of untreated WT and *Niacr1*$^{-/-}$ mice, 80% and 20% of the crypts contained proliferating cells in lower 1/3 and 2/3 compartments, respectively. After AOM+DSS treatment, in colon of WT mice, 53% and 47% of crypts showed proliferating cells in their lower 1/3 and 2/3 compartments, respectively, and no crypt showed proliferation along its full length. In contrast, 33% of crypts in colons of AOM+DSS-treated *Niacr1*$^{-/-}$ mice showed proliferating cells all along its entire length (Figures S5A and S5B). Expression of cyclin-D1, cyclin-B1, and cyclin-dependent kinase 1, which promote development of colon cancer, was highly increased in colons of

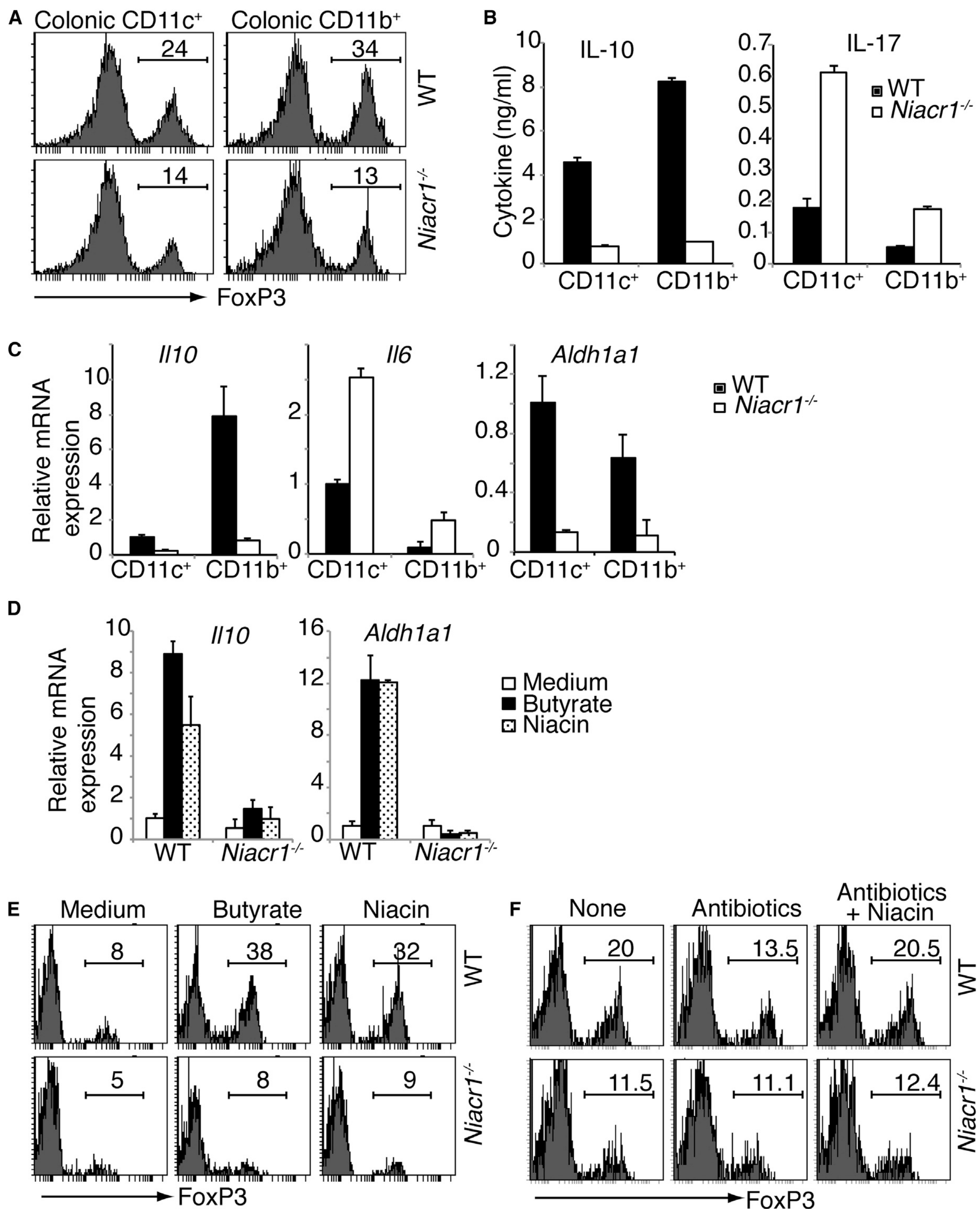

Figure 2. Butyrate and Niacin Induce Anti-inflammatory Properties in DCs and Macrophages in a Gpr109a-Dependent Manner

(A) Foxp3 expression by OT-II $CD4^+CD25^-$ T cell differentiated in the presence of TGF-β1, IL-2, and cognate peptide with colonic LP $CD11c^+$ ($CD45^+$I-A^{b+} $CD11c^+$) and $CD11b^+$ ($CD45^+$I-$A^{b+}CD11b^+$) cells from WT or *Niacr1*$^{-/-}$ mice.

(B) OT-II $CD4^+CD25^-$ T cells differentiated as described in (A) were restimulated with anti-CD3 and anti-CD28 antibodies. One day later, IL-10 and IL-17 in culture supernatants were quantified by ELISA.

(C) Expression of *Il10*, *Il6*, and *Aldh1a1* by colonic LP DCs and macrophages isolated from WT and *Niacr1*$^{-/-}$ mice was quantified by qPCR.

(D) Sorted splenic DCs were cultured with butyrate or niacin. Two days later, cells were harvested, and expression of *Il10* and *Aldh1a1* was measured by qPCR.

(E) Naive OT-II $CD4^+CD25^-$ T cells were differentiated with butyrate- or niacin-treated splenic DCs from indicated mice as described in (A). Shown is the FoxP3 expression by differentiated $CD4^+$ T cells.

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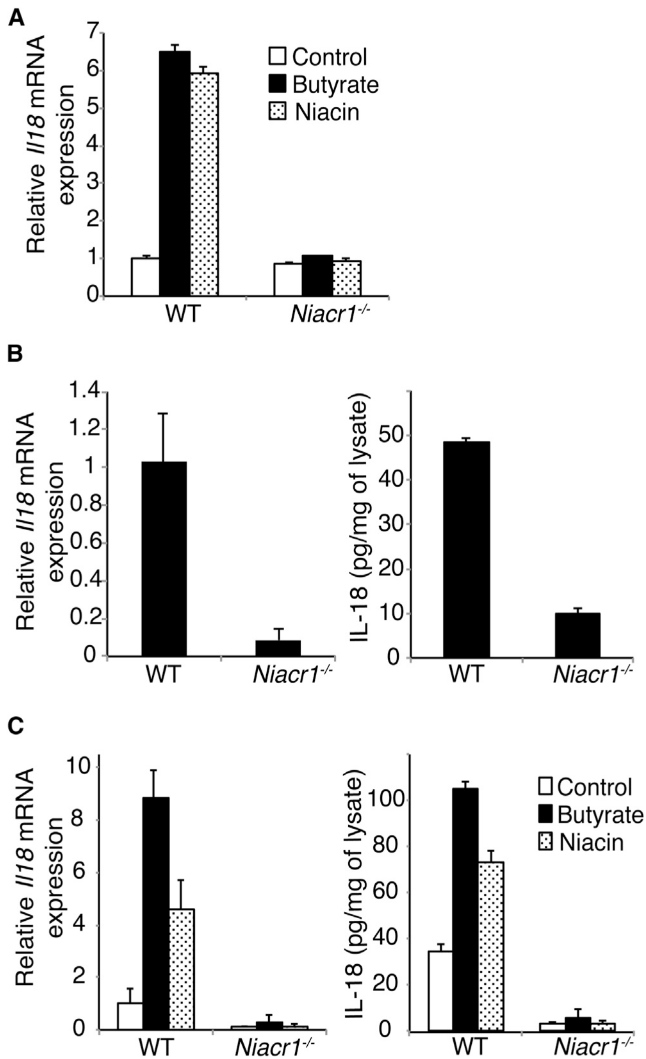

Figure 3. Gpr109a Is Required for Butyrate- and Niacin-Mediated IL-18 Induction

(A) Induction of IL-18 in neonatal colon. Colons of 6-day-old WT or *Niacr1*$^{-/-}$ mice were cultured in medium with or without butyrate or niacin for 24 hr, and *Il18* mRNA was quantified by qPCR (n = 3).

(B) Expression of IL-18 mRNA and protein in colonic epithelial cells isolated from WT or *Niacr1*$^{-/-}$ mice were quantified by qPCR and ELISA, respectively (n = 3).

(C) One day after oral administration of butyrate or niacin in vivo, expression of IL-18 in colonic epithelium of WT or *Niacr1*$^{-/-}$ mice was quantified (n = 3).

Error bars represent SD of mean. A representative of three independent experiments is shown. See also Figure S3.

Niacr1$^{-/-}$ mice after AOM+DSS regime. In contrast, expression of tumor suppressors *Slc5a8*, *Msh2*, and *Msh3* were decreased in colons of AOM+DSS-treated *Niacr1*$^{-/-}$ mice compared to WT mice (Figure S5C). At the end of the AOM+DSS treatment regime, colons of *Niacr1*$^{-/-}$ mice showed increased number of large polyps (22.8 ± 4.0 polyps/mouse) compared to those of WT mice (2.3 ± 1.8 polyps/mouse) (Figures 5A–5C). Furthermore, at completion of experiment, *Niacr1*$^{-/-}$ animals also exhibited anemia (Figure S5D). Mutations in adenomatous polyposis coli (*APC*) gene cause an inherited form of colon cancer in humans. Because IL-10 and Treg cells suppress whereas IL-17 enhances colon carcinogenesis in *Apc*$^{Min/+}$ mice (mice expressing a point mutation in one copy of *Apc* gene) (Erdman et al., 2005; Grivennikov et al., 2012), we also analyzed *Apc*$^{Min/+}$-driven intestinal and colon carcinogenesis in *Niacr1*$^{-/-}$ mice. At 3 months of age, *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice had strikingly more polyps in the colon and small intestine than did *Apc*$^{Min/+}$ mice (Figures 5D and 5E). These data from two different animal models demonstrating accelerated progression of colon carcinogenesis upon deletion of *Niacr1* provide strong evidence for the tumor-suppressive role of this receptor in colon.

Gpr109a Expressed in Immune Cells as well as in Colonic Tissue Is Necessary for Protection against Colitis and Colon Carcinogenesis

To address the role of hematopoietic versus nonhematopoietic cells expressing Gpr109a, reciprocal bone marrow (BM) chimeras between WT and *Niacr1*$^{-/-}$ mice were generated and subjected to AOM+DSS treatment. Gpr109a expressed in hematopoietic cells played a critical role in AOM+DSS-induced colonic inflammation and carcinogenesis because a WT or *Niacr1*$^{-/-}$ host that received *Niacr1*$^{-/-}$ BM exhibited significantly more AOM/DSS-induced weight loss, diarrhea, and colonic polyps than corresponding recipients of WT BM cells (Figures 6A–6C). Similarly, Gpr109a expressed in nonhematopoietic cells was also important in AOM+DSS-induced colonic inflammation and carcinogenesis because *Niacr1*$^{-/-}$ mice receiving WT or *Niacr1*$^{-/-}$ BM cells developed more severe weight loss, diarrhea, and colon carcinogenesis than did a corresponding WT host receiving the same BM cells (Figures 6A–6C). The contribution by nonhematopoietic Gpr109a was quantitatively greater than that by immune cell Gpr109a because the WT→*Niacr1*$^{-/-}$ group developed more colonic polyps and showed more weight loss and diarrhea than did the *Niacr1*$^{-/-}$ →WT group ($p < 0.004$). These data demonstrate that Gpr109a expressed by both hematopoietic and nonhematopoietic cells is necessary for effective protection against colonic inflammation and colon cancers.

Activation of Gpr109a Suppresses Colonic Inflammation and Carcinogenesis in the Absence of Gut Microbiota or Dietary Fiber

We then examined the relevance of niacin, a pharmacologic agonist for GPR109A, to colonic inflammation. For this, we first depleted gut microbiota with antibiotics, which reduces the production of butyrate, the endogenous agonist for GPR109A. Antibiotic treatment resulted in >300-fold reduction in aerobic and anaerobic bacterial counts in the stool (data not shown). Antibiotic treatment increased DSS-induced weight loss,

(F) WT and *Niacr1*$^{-/-}$ mice were treated with antibiotics in the presence or absence of niacin in drinking water. Four weeks later, colons were harvested and FoxP3 expression by colonic CD4^{+} T cell was analyzed.

The numbers in (A), (E), and (F) represent percentage of positive cells in indicated area. Error bars represent SD of mean (n = 2–3). A representative of at least two independent experiments is shown. See also Figure S2.

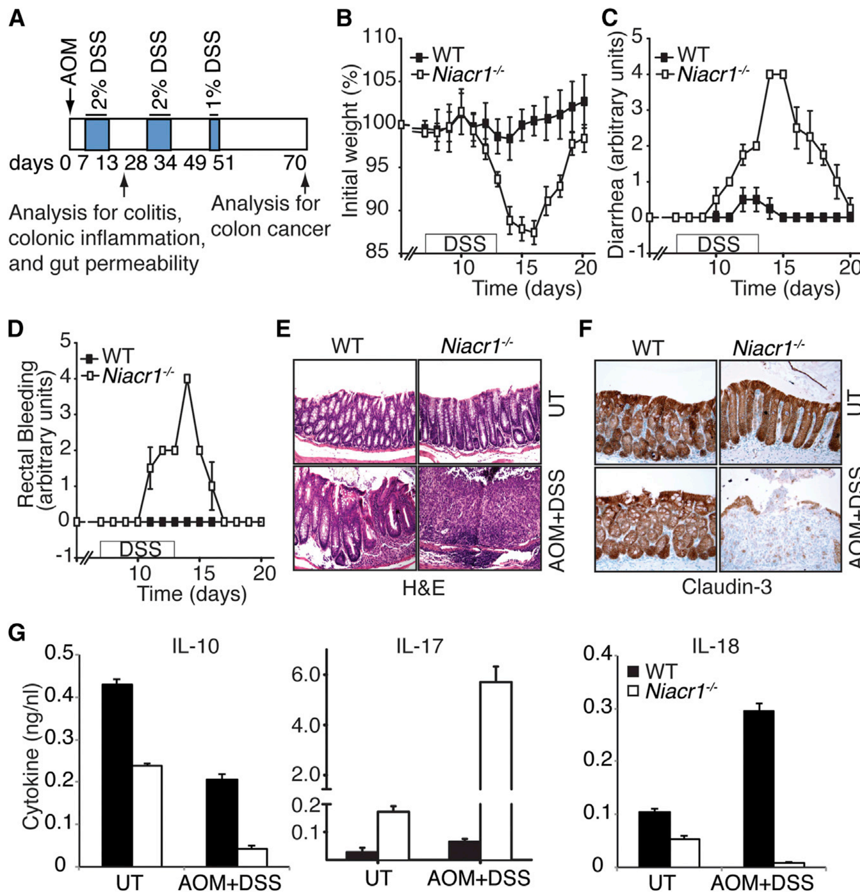

Figure 4. Increased Susceptibility of *Niacr1*$^{-/-}$ Mice to Colonic Inflammation

(A) Experimental paradigm for induction of colonic inflammation and inflammation-associated colon cancer in mice; azoxymethane (AOM) by intraperitoneal injection; DSS in drinking water. At days 20 and 70, colons of mice were analyzed for inflammation and cancer, respectively.

(B–D) Change in body weight, diarrhea, and rectal bleeding of WT and *Niacr1*$^{-/-}$ mice subjected to AOM+DSS (n $\geq$ 4).

(E) Representative images of H&E-stained colonic sections from untreated or AOM+DSS-treated (20th day) WT and *Niacr1*$^{-/-}$ mice (original magnification, 200×).

(F) Claudin-3 staining of colonic sections from untreated (UT) or AOM+DSS-treated (20th day) WT and *Niacr1*$^{-/-}$ mice (original magnification, 200×).

(G) IL-10, IL-17, and IL-18 levels in colons of WT and *Niacr1*$^{-/-}$ mice before and after AOM+DSS treatment (n = 5). Error bars represent standard deviation of mean.

Values are mean ± SD or representative of at least two independent experiments. See also Figure S4.

diarrhea, and bleeding in WT mice (Figures 7B and S6A). Consistent with increased inflammation, we found that antibiotic treatment increased the number of polyps (8.2 ± 2.2 polyps/mouse with antibiotics; 1.6 ± 1.5 polyps/mouse without antibiotics) in WT mice (Figures 7C and 7D). We then tested whether administration of niacin protects antibiotic-treated mice against colonic inflammation and carcinogenesis. Niacin was added to drinking water along with antibiotic cocktail. Niacin ameliorated AOM+DSS-induced weight loss, diarrhea, and bleeding and reduced colon cancer development in antibiotic-treated WT mice (Figures 7B–7D and S6A). Consistent with a role of niacin in IL-18 induction, the protective effect of niacin in DSS-induced weight loss and diarrhea in antibiotic-treated *Il18*$^{-/-}$ mice was significantly blunted (Figure S6B). Niacin did not alter the development of weight loss, diarrhea, rectal bleeding, and colon cancer in antibiotic-treated *Niacr1*$^{-/-}$ mice, suggesting an essential role of Gpr109a in niacin-mediated promotion of colonic health (Figures 7B–7D and S6A). Antibiotic treatment reduced colonic inflammation and number of polyps in *Niacr1*$^{-/-}$ mice. This may be due to the presence of altered colitogenic gut microbiota in *Niacr1*$^{-/-}$ animals. Increased representation of *Prevotellaceae* and TM7 groups of bacteria are associated with enhanced risk of colitis in IL-18-deficient mice (*Casp1*$^{-/-}$, *Asc*$^{-/-}$, *Nlrp6*$^{-/-}$, or *Il18*$^{-/-}$) (Elinav et al., 2011). We found that feces of *Niacr1*$^{-/-}$ mice exhibited increased representation of *Prevotellaceae* and TM7 groups of commensal bacteria compared to WT mice (Figure S6C). *Bacteroides* and *Bacillus* groups of bacteria were present in comparable numbers in feces of both WT and *Niacr1*$^{-/-}$ mice. To test whether Gpr109a deficiency promotes preferential accumulation of *Prevotellaceae* and TM7 bacterial groups in their intestines independent of parental contribution, we performed similar studies with fecal samples from littermates of *Niacr1*$^{+/-}$ and *Niacr1*$^{-/-}$ genotypes. We observed that feces from *Niacr1*$^{-/-}$ mice contained significantly more *Prevotellaceae* and TM7 bacteria than did feces from *Niacr1*$^{+/-}$ littermates (Figure S6D). This provides evidence for significant alterations in relative abundance of indicated microbial species in the colons of *Niacr1*$^{-/-}$ mice and explains the reduction of colonic inflammation and colon carcinogenesis by antibiotic treatment in *Niacr1*$^{-/-}$ animals (Figures 7B–7D and S6A). Both exogenously administered rIL-10 and rIL-18 were able to reduce AOM+DSS-induced weight loss, diarrhea, and colon carcinogenesis in *Niacr1*$^{-/-}$ mice (Figures 7E and 7F).

Antibiotic treatment reduces the number of colonic polyps in mice with *Apc* mutation, implying a cancer-promoting role of gut microbiota in this model (Grivennikov et al., 2012; Li et al., 2012). Our data indicate that gut microbial metabolite butyrate and hence gut microbiota reduce colon carcinogenesis in *Apc*$^{Min/+}$ mice. We hypothesized that gut microbiota has both cancer-promoting and -suppressing effects in *Apc*$^{Min/+}$ mice, and microbiota-depleted animals show the balance between cancer-promoting and -suppressing properties of gut microbiota. To define the protective role of Gpr109a in promoting colonic health through butyrate and gut microbiota, we fed *Apc*$^{Min/+}$ mice and *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice with fiber-free (FF) diet, which eliminates butyrate production in colon. Figures 7G and 7H show that *Apc*$^{Min/+}$ mice fed FF-diet had significantly increased number of colonic polyps, which was

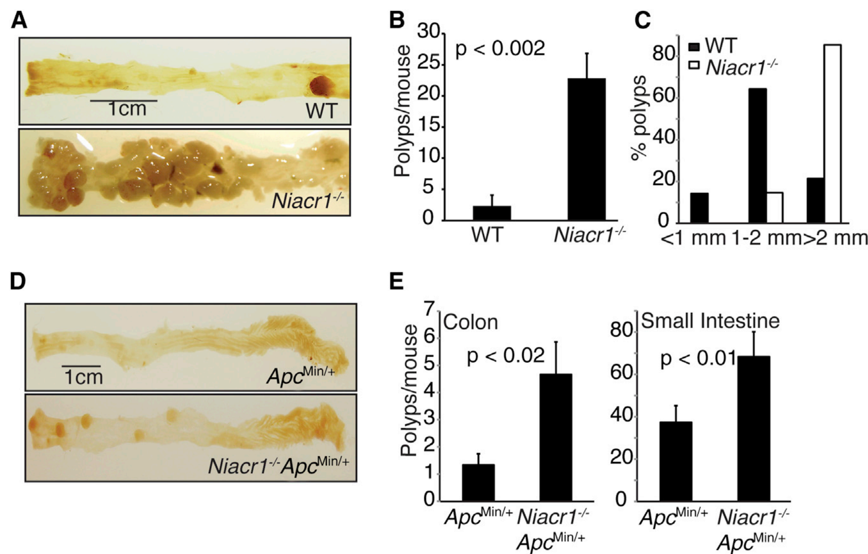

Figure 5. Increased Susceptibility of *Niacr1*$^{-/-}$ Mice to Colon Cancer

(A) Representative photographs of dissected colons from WT and *Niacr1*$^{-/-}$ mice on day 70 after animals were treated with AOM+DSS as described in Figure 4A.

(B and C) Number (B) and size distribution (C) of colonic polyps induced by AOM+DSS treatment in WT and *Niacr1*$^{-/-}$ mice (n = 6).

(D) Representative photographs of dissected colons of 3-month-old *Apc*$^{Min/+}$ and *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice.

(E) Number of polyps in colons and small intestines of *Apc*$^{Min/+}$ and *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice (n = 4).

Values are mean ± SD or representative of two independent experiments. See also Figure S5.

effectively suppressed by the Gpr109a agonist niacin. FF diet in the presence or absence of niacin did not affect development of colonic polyps in *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice. Collectively, these data clearly indicate that Gpr109a agonists suppress development of colon cancer. Butyrate, the endogenous Gpr109a agonist in colon, is produced after fermentation of dietary fiber by commensal bacteria. Therefore, the data presented here suggest a role of Gpr109a in suppression of colonic inflammation and carcinogenesis by butyrate-producing commensals and dietary fibers.

DISCUSSION

The current study defines an essential role of Gpr109a in the suppression of colonic inflammation and carcinogenesis. Commensals induce Treg cells and IL-10-producing T (Tr1) cells in colon (Atarashi et al., 2011; Geuking et al., 2011; Mazmanian et al., 2008; Round and Mazmanian, 2010). They also induce IL-10 expression in colonic DCs and macrophages, which promote differentiation of Tr1 cells (Jeon et al., 2012; Ueda et al., 2010). Our studies provide a molecular mechanism by which the commensals elicit these effects. The bacterial metabolite butyrate functions as a messenger between the commensals and the host. This SCFA induces expression of anti-inflammatory molecules in macrophages and DCs and enables them to support differentiation of Treg and IL-10-producing T cells. The present study also implicates a tumor-suppressive role of Gpr109a-butyrate signaling in colon and suggests that commensals in the gut provide protection to the host not only against colonic inflammation but also against colon cancer. Our conclusion that butyrate is responsible, at least partly, for the actions of gut microbiota on the host colon with regard to suppression of inflammation and carcinogenesis is congruent with previous findings that the frequency of butyrate-producing bacteria and rate of butyrate production are greatly diminished in the colon during ulcerative colitis and colon cancer (Frank et al., 2007; Wang et al., 2012). Butyrate enemas decrease colonic inflammation in ulcerative colitis (Hamer et al., 2008). The present studies also highlight the biological significance of dietary fiber and its relevance to butyrate production. Dietary fiber suppresses colonic inflammation and colorectal cancer (Davis and Milner, 2009; Hamer et al., 2008). The most important aspect of the present studies is the identification of Gpr109a as one of the mediators of the biological effects of butyrate.

Niacin is a vitamin, which, when taken in pharmacological doses, suppresses atherosclerosis by acting as a GPR109A agonist in immune cells. At these high doses, niacin is likely to reach the colon at concentrations high enough to exert GPR109A-dependent effects. Therefore, the present studies suggest that pharmacological doses of niacin may have anti-inflammatory and tumor-suppressive effects in the colon.

Although it has been known for decades that the commensal metabolite butyrate suppresses inflammation and carcinogenesis in colon, the exact identity of molecular target(s) of butyrate in this process remained elusive. The present studies identify Gpr109a as an important mediator of butyrate effects in colon and also as a critical molecular link between colonic bacteria and dietary fiber and the host. These findings have important implications for prevention as well as treatment of inflammatory bowel disease and colon cancer and suggest that under conditions of reduced dietary fiber intake and/or decreased butyrate production in colon, pharmacological doses of niacin might be effective to maintain GPR109A signaling and consequently protect colon against inflammation and carcinogenesis.

EXPERIMENTAL PROCEDURES

Mice

Age-matched conventional and germ-free mice (Swiss Webster strain) were obtained from Taconic Farms and used for experiments on day of arrival. *Il18*$^{-/-}$ and *Apc*$^{Min/+}$ mice on C57BL/6 background were obtained from Jackson Laboratory. Fiber-free diet (TD00278) was from Harlan Laboratories. *Niacr1*$^{+/-}$*Apc*$^{Min/+}$ mice were derived from intercross of *Niacr1*$^{-/-}$ mice and *Apc*$^{Min/+}$ mice. *Niacr1*$^{+/-}$*Apc*$^{Min/+}$ mice were bred with *Niacr1*$^{-/-}$ mice to obtain *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice. All animal procedures were approved by the Institutional Animal Care and Use Committee, Georgia Regents University.

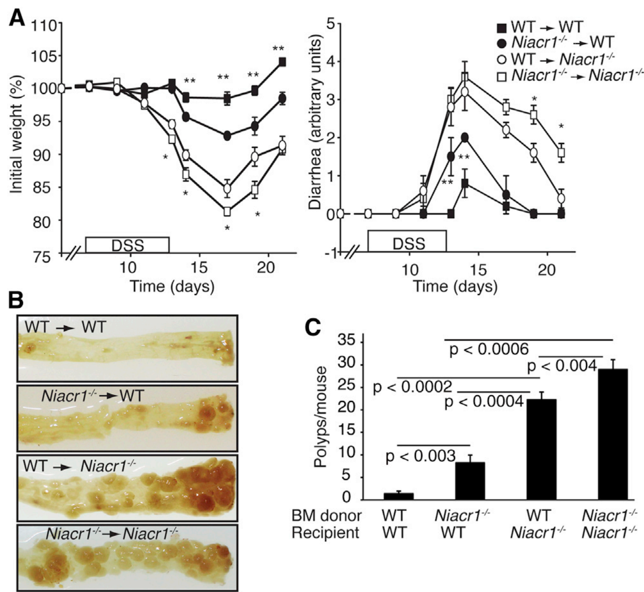

Figure 6. Role of Gpr109a Expressed in Hematopoietic and Nonhematopoietic Cells in Regulation of Colitis and Colitis-Associated Colon Cancer

Reciprocal bone marrow chimeras of WT and *Niacr1*$^{-/-}$ mice were subjected to AOM+DSS-induced model of colitis-associated colon cancer.

(A) Weight loss and diarrhea after the first cycle of DSS ($n \geq 4$). Single asterisk (*) indicates significant difference between WT → *Niacr1*$^{-/-}$ and *Niacr1*$^{-/-}$ → *Niacr1*$^{-/-}$ group for weight loss ($p < 0.05$) and diarrhea ($p < 0.02$). Double asterisk (**) indicates significant difference between WT → WT and *Niacr1*$^{-/-}$ → WT group for weight loss and diarrhea ($p < 0.02$).

(B) Representative photographs of dissected colons from bone marrow chimeric mice.

(C) Tumor burden in bone marrow chimeras subjected to AOM+DSS-induced colon cancer ($n \geq 4$).

Error bars represent ±SD of mean. A representative of two independent experiments is shown.

Antibodies

Antibodies against mouse CD4 (clone GK1.5), CD45 (clone 104), Foxp3 (clone FLK-16 s), IL-10 (clone JES5-16E3), IL-17 (clone eBio17B7), CD11b (clone M1/70), CD11c (clone N418), I-A^b (clone 25-9-17), CD90 (clone 53-2.1), CD19 (clone SJ25C1), and Gr1 (clone RB6-8C5) and against human CD3 (clone UCHT1), CD14 (61D3), CD123 (6H6), and CD56 (CMSSB) were from eBioscience. Anti-human GPR109A (clone 245106), anti-Ki67 (clone TEC-3), and anti-claudin3 (34-1700) were from R&D Systems, Dako, and Life Technologies, respectively.

Cell Isolation and Analysis

B cells (CD19$^+$), T cells (Thy1.1$^+$), macrophages (CD11b$^+$CD11c$^-$), dendritic cells (CD11c$^+$CD11b$^-$), and CD4$^+$CD25$^-$ T cells from spleens and CD45$^+$I-A^{b+}CD11c$^+$ (DCs) or CD45$^+$I-A^{b+}CD11b$^+$ (macrophages) cells from colonic LP were sorted with MoFlo (Dakocytomation) or FACS Aria Flow cytometers (BD Biosciences). Alternatively, splenic CD11c$^+$ or CD11b$^+$ cells were sorted with CD11b or CD11c antibodies coupled to magnetic beads (Miltenyi Biotec). For isolation of colonic epithelium, washed and everted colons were incubated in Hanks balanced salt solution (HBSS) containing 2 mM EDTA at 37°C with gentle shaking. Supernatants were centrifuged and pellets were resuspended in 40% percoll and centrifuged. The cells on top layer were collected and used as colonic epithelium. For lamina propria lymphocytes, colons and small intestines were opened and shaken with HBSS containing 2 mM EDTA at 37°C to remove epithelial cells. After that, pieces of colon or small intestine were incubated with collagenase D (1 mg/ml) and DNase (0.1 mg/ml) for 20 min. The cell suspension was collected, washed, and stained with antibodies specific for mouse CD4 and Foxp3 and analyzed by FACS. In some experiments, mononuclear cells from colonic LP or spleen of WT and *Niacr1*$^{-/-}$ mice were cultured with phorbol myristate acetate plus ionomycin in the presence of GolgiStop and Golgiplug for 5 hr. Cells were fixed and stained for CD4, IL-10, and IL-17. Peripheral blood mononuclear cells were obtained with institutional approval from Institutional Review Boards, Georgia Regents University.

Cell Cultures

Sorted colonic LP CD45$^+$I-A^{b+}CD11c$^+$ or CD45$^+$I-A^{b+}CD11b$^+$ cells were cultured with naive OT-II (CD4$^+$CD25$^-$) T cells in the presence of egg albumin peptide (ISQVHAAHAEINEA) (0.5 μg/ml), TGF-β1 (1 ng/ml), and IL-2 (5 ng/ml). After 4 days, cells were harvested and stained for CD4 and FoxP3. Alternatively, cells were rested overnight and restimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml). After 24 hr, supernatants was collected and analyzed for IL-10 and IL-17a by ELISA. Splenic CD11c$^+$ (DCs) and CD11b$^+$ (macrophages) were cultured (10^5 cells/well) in the presence or absence of butyrate (0.5 mM) or niacin (0.5 mM). After 2 days, cells were harvested and analyzed for IL-10 and Aldh1a expression or tested for their ability to induce differentiation of naive OT-II CD4$^+$ T cells as above. In some experiments, retinoic acid receptor (RAR) inhibitor LE135 was used (1 μM).

Bone Marrow Chimeras

WT (CD45.1) or *Niacr1*$^{-/-}$ (CD45.2) mice were irradiated (900 rads) and injected intravenously with donor bone marrow cells (2×10^6 cells/mouse). Reconstitution was confirmed by staining for donor-specific CD45 allele (CD45.1 versus CD45.2) in blood. Two months after reconstitution, mice were used for colonic inflammation-associated colon cancer experiments.

Antibiotics and Niacin Treatment

Mice were given a cocktail of antibiotics (0.2 μg/ml of gentamicin, 0.15 μg/ml of ciprofloxacin, 2 mg/ml streptomycin, and 1 mg/ml bacitracin) in drinking water at the indicated period of time. Where indicated, antibiotic cocktail was supplemented with 25 mM of niacin. For IL-18 induction assays, mice were treated with butyrate or niacin (25 mM) as described (Kalina et al., 2002).

Induction of Colonic Inflammation and Inflammation-Associated Colon Cancer

Inflammation-associated colon cancer was induced by intraperitoneal injection of azoxymethane (10 mg/kg body weight). Seven days later, DSS (36–50 kDa) was added in a cyclic manner to drinking water at indicated doses. Some mice were also given rIL-10 or rIL-18 intraperitoneally (50 ng/mouse) at indicated points. Mice were monitored for weight changes, diarrhea, and rectal bleeding. Diarrhea was scored as (0) normal stool, (1) soft but formed pellet, (2) very soft pellet, (3) diarrhea (no pellet), or (4) dysenteric diarrhea. Rectal bleeding was recorded as (0) no bleeding, (2) presence of occult blood in stool, or (4) gross macroscopic bleeding.

Histopathology and Immunohistochemistry

Sections (5 μm) from formalin-fixed and paraffin-embedded colons or polyps were placed onto glass slides. H&E-stained sections were blindly scored for severity of colonic inflammation. The degree of inflammation was scored as follows: (0) no inflammation, (1) mild inflammation or prominent lymphoid aggregates, (2) moderate inflammation, (3) moderate inflammation associated with crypt loss, and (4) severe inflammation with crypt loss and ulceration. Crypt destruction was graded as follows: (0) no destruction, (1) 1%–33% of crypts destroyed, (2) 34%–66% of crypts destroyed, and (3) 67%–100% of crypts destroyed. The individual scores from inflammation and crypt damage were summed to derive histological score for colonic inflammation (maximum score 7). For immunohistochemistry, sections were deparaffinized with xylene and antigen retrieval was performed with target antigen retrieval solution (Dako). The staining was visualized with Vectstain ABC kit and diaminobenzidine.

Quantitative Real-Time PCR

cDNA was synthesized from 2 μg of total RNA by Superscript III reverse transcription system (Invitrogen). qPCR was performed with SYBR green PCR mix

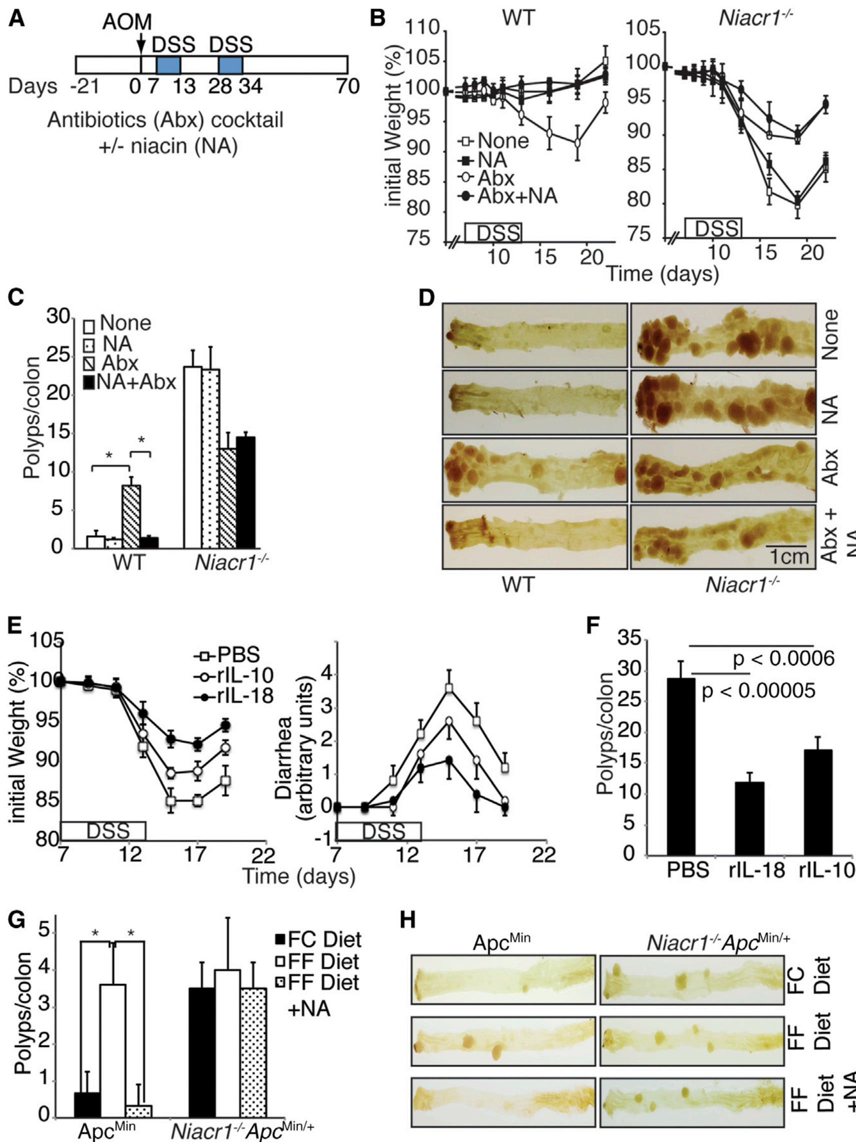

Figure 7. Niacin Suppresses Colonic Inflammation and Carcinogenesis in Absence of Gut Microbiota and Dietary Fiber via Gpr109a

(A) Experimental paradigm for antibiotic treatment (gentamicin sulfate, ciprofloxacin, streptomycin, and bacitracin in drinking water) and niacin administration to mice in the AOM+DSS-induced colon cancer model.

(B) Weight loss in WT and *Niacr1*$^{-/-}$ mice treated with antibiotics in the presence or absence of antibiotics and subjected to AOM+DSS as in (A).

(C) Tumor burden in WT and *Niacr1*$^{-/-}$ mice under various treatment conditions (n $\geq$ 4).

(D) Representative photographs of dissected colons of WT and *Niacr1*$^{-/-}$ mice subjected to various treatments as described in (A).

(E) *Niacr1*$^{-/-}$ mice were treated as in Figure 4A. Some mice also received rIL-10 or rIL-18 intraperitoneally (50 ng/mouse/injection) starting day 2 and every alternate day till day 60. Shown are the weight loss and diarrhea during first cycle of DSS.

(F) Tumor burden on day 70 in *Niacr1*$^{-/-}$ mice treated as described in (E) (n = 4).

(G) Two-month-old *Apc*$^{Min/+}$ and *Niacr1*$^{-/-}$*Apc*$^{Min/+}$ mice were fed with dietary fiber containing normal chow (FC) or fiber-free (FF) chow. Some mice in FF diet group also received niacin in drinking water ad libitum. Five weeks later, mice were sacrificed and colonic polyps were counted (n = 2–5). *p < 0.007.

(H) Representative photographs of dissected colons from mice subjected to experimental protocol described in (G).

Values represent mean ± SD or representative of at least two independent experiments. See also Figure S6.

and StepOnePlus machine (Applied Biosystems). PCR primers are listed in Table S1. *Gapdh* was used as internal control.

ELISA

Colonic tissue or colonic epithelium extracts were prepared in PBS containing 0.1% NP-40 and protease inhibitors (Thermo Fisher Scientific). ELISA was performed with antibody pairs for IL-6, IL-1β, and IL-17. ELISA kits for IL-18 and Ccl2 were from R&D systems and eBioscience, respectively. Cxcl1 was quantified with a kit from Peprotech. Serum amyloid A was detected via Kit from Immunology Consultants Laboratory.

Measurement of Intestinal Permeability

Mice were given fluorescein isothiocyanate (FITC)-dextran by oral gavage at a dose of 0.5 mg/g of body weight. Four hours later, mice were bled and FITC-dextran was quantified in the serum via a fluorescence spectrophotometer.

Myeloperoxidase Activity

Pieces of colon (100 mg weight) were homogenized in phosphate buffer (20 mM [pH 7.4]) and centrifuged. Pellet was resuspended in phosphate buffer (50 mM [pH 6.0]) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The sample was freeze-thawed, sonicated followed by warming to 60°C for 2 hr, and centrifuged. Redox reaction of 3,3′,5,5′-tetramethylbenzidine (Sigma) by supernatant was used to determine myeloperoxidase (MPO) activity. Reaction was terminated with 2N HCl and absorbance was read at 450 nm.

Organ Culture

Colons from 7-day-old pups were opened, cut into ~0.5 cm pieces, and cultured with butyrate (0.5 mM) or niacin (1 mM) for 24 hr. The tissues were then used for analysis of *Il18* mRNA by qPCR. For DSS-treated animals, colon segments (100 mg weight) were chopped into smaller pieces (1–2 mm) and cultured in medium containing penicillin and streptomycin. Twenty-four hours later, supernatants were collected and cytokines were measured by ELISA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.12.007.

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