The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition

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Given the trillions of microbes that inhabit the mammalian intestines, the host immune system must constantly maintain a balance between tolerance to commensals and immunity against pathogens to avoid unnecessary immune responses against otherwise harmless bacteria. Misregulated responses can lead to inflammatory bowel diseases such as ulcerative colitis or Crohn’s disease. The mechanisms by which the immune system maintains this critical balance remain largely undefined. Here, we demonstrate that the short-chain fatty acid n-butyrate, which is secreted in high amounts by commensal bacteria, can modulate the function of intestinal macrophages, the most abundant immune cell type in the lamina propria. Treatment of macrophages with n-butyrate led to the down-regulation of lipopolysaccharide-induced proinflammatory mediators, including nitric oxide, IL-6, and IL-12, but did not affect levels of TNF-α or MCP-1. These effects were independent of toll-like receptor signaling and activation of G-protein–coupled receptors, two pathways that could be affected by short-chain fatty acids. In this study, we provide several lines of evidence that suggest that these effects are due to the inhibition of histone deacetylases by n-butyrate. These findings elucidate a pathway in which the host may maintain tolerance to intestinal microbiota by rendering lamina propria macrophages hyporesponsive to commensal bacteria through the down-regulation of proinflammatory effectors.

inflammation | microbiome

The mammalian gastrointestinal tract harbors trillions of microorganisms comprising all three domains of life. Of these, bacteria are the most abundant and can be found at concentrations as high as 10^{13} cells/mL in the small intestine and 10^{12} cells/g of luminal content in the large intestine (1). These microbiota are acquired from the environment with the initial colonization occurring at birth. Over millennia, many of these microbes have evolved to coexist within the host by assuming a symbiotic role to avoid elimination by the immune system (2). For example, some bacteria play beneficial roles for the host by supplying essential nutrients and aiding in the digestion of otherwise indigestible compounds (3). Other beneficial bacteria defend against opportunistic pathogens or play key roles in the development of the host immune system (4, 5).

The presence of such high numbers of microbes in the gut lumen renders the intestines a unique immunological site within the body. The gut epithelium forms a barrier between the host and the luminal contents of the intestines through a single layer of epithelial cells. Immune cells that reside beneath the epithelium in the lamina propria (LP) become activated if the epithelial barrier is breached by potential pathogens (6). Thus, the intestinal immune system must constantly maintain a delicate balance between tolerance to commensals and immunity to pathogenic bacteria. An imbalance in this regulation results in inflammatory conditions of the gut including inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn’s disease (7, 8).

To maintain homeostasis, the intestinal immune system must remain hyporesponsive to commensal bacteria under steady-state conditions. The cellular and molecular mechanisms that regulate this tolerance remain largely unknown. Chemical signaling through small-molecule metabolites, or quorum sensing, is a paradigm for intra- and intercellular communication between bacteria (9). Given the abundance of bacteria in the gut, the plethora of small-molecule metabolites, collectively termed the metabolome, may play additional critical roles in communicating microbial abundance and composition to the host. Indeed, emerging evidence suggests that the immune system can sense microbial products in addition to pathogen-associated molecular patterns and that recognition of these small molecules can influence the host immune response (10). As such, deciphering the chemical lexicon between intestinal bacteria and the mammalian immune system has important implications for understanding the etiology of IBD as well as for the development of prophylactics and therapeutics.

Short-chain fatty acids (SCFA) such as acetate, n-propionate, and n-butyrate are end products of bacterial anaerobic fermentation of dietary fiber and are likely candidates for regulating immune responses in the intestines. First, SCFA are secreted by bacteria from the phyla Bacteroidetes and Firmicutes and can be found at high concentrations in the large intestine (e.g., 20 mM n-butyrate in colonic lumen) (11). Second, patients with IBD have been shown to have reduced numbers of bacteria that produce SCFA (12). Furthermore, SCFA-containing enemas seem to be clinically beneficial for some patients with colitis (13). Previous studies suggest that SCFA have anti-inflammatory properties that may contribute to their ameliorative effects; however, the mechanisms by which these metabolites exert their actions remain ill-defined (14). Recent work has demonstrated that SCFA can induce regulatory T cells in the colon, thereby maintaining homeostasis in the intestines (15–18). In this study, we examined the role of SCFA in modulating immune responses by macrophages. Intestinal macrophages are the most abundant mononuclear phagocytes in the LP and play important roles in chemical signaling between intestinal bacteria and the mammalian immune system.
the induction of innate immune responses (19). In addition, through the secretion of cytokines, macrophages control the pro- or anti-inflammatory state of the intestines. Here, we find that the SCFA n-butyrate has immunomodulatory effects on intestinal macrophages.

Results

n-Butyrate Has Anti-Inflammatory Effects on Bone Marrow-Derived Macrophages. To test whether SCFA regulate macrophage function, we used bone marrow-derived macrophages (BMDM) as a model cell type. In these experiments, BMDM were stimulated with lipopolysaccharide (LPS) and either n-butyrate, acetate, or n-propionate, and then secreted amounts of the antimicrobial effector nitric oxide (NO) and the proinflammatory cytokines IL-6 and IL-12p40 were measured. Compared with acetate and n-propionate, we found that n-butyrate had the most potent effects on this cell type, as levels of NO, IL-6, and IL-12p40 were strongly decreased in the presence of n-butyrate in a dose-dependent manner (Fig. 1). However, n-butyrate had no effect on the secreted amounts of the proinflammatory cytokine TNF-α and the chemokine MCP-1, both of whose LPS-mediated expression do not require de novo protein synthesis (20). These data suggest that n-butyrate has anti-inflammatory effects on macrophages and that the regulation of proinflammatory mediators appears to be selective. We also verified that the concentrations of n-butyrate were toxic to BMDM by treating the cells with LPS and n-butyrate, followed by staining with Annexin V and propidium iodide and analysis by flow cytometry (Fig. S1).

n-Butyrate Has Anti-Inflammatory Effects on Colonic Lamina Propria Macrophages. To assess whether n-butyrate has an immunomodulatory effect on intestinal macrophages, we isolated colonic LP macrophages (CD11b<sup>+</sup>CD11c<sup>-hi</sup>SiglecF<sup>-</sup>, Gate R2) by fluorescence-activated cell sorting (FACS) (Fig. S2A) and stimulated them with LPS and n-butyrate. This population of mononuclear phagocytes appears to be the CX<sub>3</sub>CR<sub>1</sub>hiCD103<sup>-</sup> macrophages that are thought to be anti-inflammatory and hyporesponsive to the gut microbiota (Fig. S2B) (21–24). LP macrophages treated with n-butyrate showed reduced secretion of IL-6 (Fig. 2A). Treatment with n-butyrate also resulted in reduced mRNA levels of Il6, which encodes for IL-6; inducible nitric oxide synthase (Nos2), which is responsible for NO production; and genes encoding the two subunits of IL-12 (Ill2a and Ill2b) as measured by quantitative PCR (qPCR). However, n-butyrate had no effect on the primary LPS response genes Tnfa and Ccl2, which encode for TNF-α and MCP-1, respectively.

Next, we tested whether n-butyrate exerts the same effect on colon LP macrophages when it is administered orally to mice (Fig. 2B). In these experiments, mice were first given antibiotics (metronidazole and vancomycin) ad libitum in the drinking water for 10 d to eliminate major n-butyrate producers in the gut, followed by administration of n-butyrate in combination with antibiotics (25). The colon LP macrophages were isolated, and mRNA levels were determined by qPCR. Again, transcript levels for Il6, Nos2, Ill2a, and Ill2b were reduced in colon LP macrophages from mice treated with n-butyrate compared with mice given vehicle, whereas transcript levels of primary response genes were unaffected. Taken together, these results suggest that n-butyrate modulates immune responses of colon LP macrophages in vivo.

n-Butyrate Does Not Affect Signaling Pathways Downstream of Toll-Like Receptors. We next determined whether n-butyrate affects signaling pathways downstream of toll-like receptor (TLR) 4, which recognizes LPS (Fig. 3A). We found that activation of NF-κB and mitogen-activated protein kinases (MAPK) occurred to the same extent in BMDM treated with LPS in the presence or absence of n-butyrate. These results suggest that n-butyrate does not act at the level of TLR signaling.

Anti-Inflammatory Effects of n-Butyrate Are Independent of G-Protein-Coupled Receptors. One pathway by which n-butyrate may exert its effects is through the activation of G-protein-coupled receptors (GPCRs). GPCRs recognize vastly diverse ligands yet activate a small number of highly conserved signaling pathways through the recruitment of heterotrimeric G protein such as Goq, Gaq, and Gsa (26). Several GPCRs, including Gpr109a (HCA2), Gpr41 (FFA3), and Gpr43 (FFA2), have been shown to be activated by SCFA such as n-butyrate (27, 28). To determine whether these GPCRs could be involved in mediating the anti-inflammatory effects of n-butyrate, we first examined their expression in BMDM by reverse transcription–PCR (RT-PCR) and detected transcripts of both Gpr109a and Gpr43 (Fig. S3A).

Both Gpr109a and Gpr43 activate Goq proteins, and Gpr43 also signals via Gaq (28). To assess whether these receptors are activated in the presence of n-butyrate, BMDM were pre-treated with the Goq inhibitor pertussis toxin (PT), followed by stimulation with LPS and n-butyrate, and cytokine secretion was measured (Fig. S3B). The amounts of IL-6 and IL-12p40 did not differ in BMDM treated in the presence or absence of PT. These data suggest that the effects of n-butyrate do not depend on signaling through Gpr109a, and this finding was confirmed by comparing NO and cytokine secretion between

Fig. 1. n-Butyrate inhibits secretion of proinflammatory mediators. BMDM were stimulated with LPS (100 ng/mL) for 24 h and then treated with (A) n-butyrate (But, 100 μM–2 mM), (B) acetate (Ace, 100 μM–1 mM), or (C) n-propionate (Pro, 100 μM–1 mM). Cell supernatants were collected and analyzed by the Griess assay or ELISA. Data are representative of at least two independent experiments. Error bars represent mean ± SD.
BMDM from Gpr109a-deficient mice and their wild-type littermates (Fig. S3C). To address whether Gpr43 plays a role in n-butyrate’s effect on LPS-induced genes, we overexpressed Gpr43 or an empty vector control in BMDM by retroviral transduction; measured levels of secreted NO, IL-6, and IL-12p40; and found that the amount of these proinflammatory mediators did not differ between the two samples (Fig. S3D). These results suggest that Gpr43 does not mediate the effects of n-butyrate.

**n-Butyrate Exerts Its Anti-Inflammatory Effects via the Inhibition of Histone Deacetylases.** Another possibility is that n-butyrate affects gene expression by inhibiting the activity of histone deacetylases (HDACs). Previous studies in other cell types have demonstrated that n-butyrate inhibits the activity of class I and II HDACs (29). To determine whether n-butyrate behaves as an HDAC inhibitor in BMDM, we treated BMDM with the well-characterized HDAC inhibitor trichostatin A (TSA) in combination with LPS (Fig. 3B). Levels of NO and cytokine secretion decreased in a dose-dependent manner in the presence of TSA. These results suggest that n-butyrate phenocopies an HDAC inhibitor. In addition, the effects of both n-butyrate and TSA on secondary response genes appear to be controlled at the transcriptional level, as assessed by qPCR (Fig. 4A). To further demonstrate that n-butyrate acts as an HDAC inhibitor, we treated BMDM with varying amounts of n-butyrate and quantified histone H3 acetylation levels by Western blot (Fig. 3C and D). Similar to TSA, n-butyrate increased histone acetylation in a dose-dependent manner, suggesting that n-butyrate behaves as an HDAC inhibitor in BMDM. Furthermore, chromatim immunoprecipitation (ChIP) experiments revealed that treatment of BMDM with n-butyrate or TSA led to an increase of histone 3 lysine 9 acetylation (H3K9Ac) levels at the promoter regions of Nos2, Il6, and Il12b, but not Tnfa (Fig. S4). H3K9Ac is typically considered to be a mark of transcriptional activation; however, it is also thought to facilitate interactions between the PHD2 finger of the repressor complex containing Mi-2/NuRD, thereby targeting it to chromatin (30). Previous studies suggest that HDAC inhibitors such as TSA induce the expression of Mi-2 and enhance the DNA-binding activity of the Mi-2/NuRD complex at secondary response genes such as Il6, but not at primary response genes (31). Finally, we examined the presence of RNA polymerase II (pol II) and the serine 5-phosphorylated form of pol II (SSP), the presence of which indicates transcriptional initiation, at primary and secondary response genes in the presence of n-butyrate or TSA (Fig. 4B). Treatment with n-butyrate or TSA led to a decrease in the levels of pol II and pol II SSP recruitment to the promoters of Nos2, Il6, and Il12b, but not Tnfa.

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**Fig. 2.** n-Butyrate inhibits proinflammatory mediators in colon LP macrophages in vitro and in vivo. (A) Colon LP macrophages (CD11b<sup>+</sup>CD11c<sup>−/low</sup>SiglecF<sup>+</sup>) were sorted from C57BL/6 mice and cultured with LPS (100 ng/mL) ± n-butyrate (But, 500 μM). Cell supernatants were collected and analyzed by ELISA after 24 h. In a separate experiment, RNA was isolated after 4 h, and cDNA was synthesized and analyzed by qPCR (data normalized to Rpl13a). (B) C57BL/6 mice were treated with metronidazole (1 g/L) and vancomycin (0.5 g/L) (Abx) for 10 d ad libitum, followed in combination with n-butyrate (But, 300 mM) in the drinking water for 7 d. Colon LP macrophages were sorted and analyzed by qPCR (data normalized to Rpl13a). Data are representative of two or three independent experiments. Error bars represent mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant (unpaired Student’s t test).

**Fig. 3.** n-Butyrate acts as a histone deacetylase inhibitor in BMDM. (A) Whole-cell lysates from BMDM treated with LPS (100 ng/mL) ± n-butyrate (But, 1 mM) for the indicated times were probed by Western blot with the indicated antibodies. (B) BMDM were stimulated with LPS (100 ng/mL) ± TSA (10-50 nM) for 24 h. Cell supernatants were collected and analyzed by the Griess assay or ELISA. (C) BMDM were treated with LPS (100 ng/mL) ± But (1 μM–2 mM) or TSA (10–50 nM) for 8 h. Cell lysates were probed for acetylated histone H3 (Ac-H3) by immunoblotting. Total protein loading was assessed by immunoblots for histone H3 (H3). (D) Quantification of H3 acetylation was determined by densitometry and is normalized to β-actin. Bars represent BMDM treated with LPS (-), LPS + n-butyrate (But, 2 mM), and LPS + TSA (TSA, 50 nM). Data are representative of three independent experiments. Error bars represent mean ± SD.
These data suggest that n-butyrate has an intracellular target, so it should be taken up into cells by a combination of active and passive mechanisms. To determine whether monocarboxylate transporters (MCTs) play a role in the uptake of n-butyrate, we pretreated BMDM with the pan-MCT inhibitor α-cyano-4-hydroxycinnamic acid (CHC), followed by LPS and n-butyrate at various concentrations (Fig. S5). The amount of IL-6 and IL-12p40 did not differ between cells treated with and without CHC, suggesting that n-butyrate is not actively transported by MCTs and may be taken up by a different transporter.

n-Butyrate Does Not Ameliorate a Murine Model of Colitis. Finally, we examined whether n-butyrate ameliorates a murine model of IBD. We used the dextran sulfate sodium (DSS) model of acute colitis, where inflammation is mainly due to cells of the innate immune system, including macrophages. Mice were administered antibiotics ad libitum in the drinking water throughout the duration of the experiment to deplete n-butyrate–producing bacteria. On day 13, we began gavaging n-butyrate intragastrically (i.g.). On day 14, DSS was added to the drinking water for 7 d. Severity of disease was monitored by weighing the mice daily (Fig. 5A). Surprisingly, mice treated with DSS and n-butyrate exhibited similar weight loss to that of mice given DSS only. On day 21, the colons were excised and stained with hematoxylin and eosin (H&E). Histological examination detected similar pathological changes in all DSS cohorts (Fig. 5B and C). These results may be caused by the deficient production of cytoprotective factors, including IL-6, whose role as a proinflammatory cytokine or reparative factor is context-dependent (32). We conclude that in the steady state, the anti-inflammatory effect of n-butyrate serves to promote immunological tolerance to commensals. However, during tissue injury caused by DSS, the effect of n-butyrate is overridden by the need for tissue reparative factors such as IL-6.

Discussion

Maintaining homeostasis with commensal bacteria in the intestines is important for preventing unnecessary immune responses to otherwise harmless microbes. The cellular and molecular mechanisms by which this regulation is achieved are relatively unknown. Here, we demonstrated that n-butyrate, a secreted bacterial metabolite abundant in the colon, plays a role in modulating immune responses of intestinal macrophages via the inhibition of histone deacetylases (Fig. 5D). By down-regulating proinflammatory mediators such as NO, IL-6, and IL-12, n-butyrate...
may contribute to the maintenance of tolerance to commensals by rendering colon LP macrophages hyporesponsive to the microbiota. SCFA, including n-butyrate, are the main by-products of bacterial fermentation of dietary fiber and are generally thought to be clinically beneficial for patients suffering from IBD (33). Despite this knowledge, there is a lack of understanding of how SCFA exert their anti-inflammatory effects. In this study, we hypothesized that SCFA have immunomodulatory effects on colonic macrophages, which are important cell types because they regulate inflammation in the intestines. Indeed, when we treated either isolated colon LP macrophages or mice with n-butyrate, there was a reduction in the gene expression of proinflammatory mediators including Nos2, Il6, Il12a, and Il12b. It appears that the bioactivity of n-butyrate is selective because it did not affect primary LPS response genes such as Tnfa and C12.

We next investigated the mechanism by which n-butyrate exerts its effects on intestinal macrophages. We first eliminated the possibility that n-butyrate may affect signaling downstream of TLRs by examining the activation of MAPK. Other studies suggest that n-butyrate inhibits nuclear translocation of NF-κB, an inhibitor of NF-κB that prevents its nuclear translocation. Multiple GPCRs, including Gpr109a, Gpr43, and Gpr41, are known to be activated by n-butyrate. Although Gpr109a and Gpr43 are expressed by macrophages, we found that these receptors do not mediate the anti-inflammatory effects of n-butyrate. However, it is possible that n-butyrate may affect other downstream targets through these receptors.

We also examined the possibility that n-butyrate acts as an HDAC inhibitor in macrophages. It is widely known that n-butyrate inhibits HDAC activity in many cell types. In this study, we showed that n-butyrate phenocopies a well-characterized HDAC inhibitor, TSA, in macrophages. Like n-butyrate, TSA also inhibited the amounts of NO, IL-6, and IL-12p40 secreted by macrophages but did not affect secretion of TNF-α or MCP-1. Both n-butyrate and TSA increased the overall levels of histone H3 acetylation, suggesting that n-butyrate acts as an HDAC inhibitor.

In addition, we performed ChIP experiments and demonstrated that treatment of macrophages with either n-butyrate or TSA led to an increase in H3K9Ac levels at the promoter regions of genes involved in transcription. H3K9Ac is typically regarded as a chromatin modification that leads to transcriptional activation. However, H3K9Ac is also thought to recruit the repressor complex Mi-2/NuRD through its PHD2 domain (30). It has been shown that HDAC inhibitors such as TSA induce the expression of Mi-2 and enhance the DNA-binding activity of the Mi-2/NuRD complex at secondary response genes such as Il6 but not at primary response genes such as Tnfa (31). Together, these data suggest that n-butyrate could also cause repression of secondary response genes through the recruitment of the Mi-2/NuRD repressor complex because Mi-2 is known to promote chromatin remodeling that is inhibitory to secondary response genes, whereas primary response gene induction is independent of chromatin remodeling (20).

Further supporting the hypothesis that n-butyrate behaves as an HDAC inhibitor, we showed that both n-butyrate and TSA inhibit these proinflammatory mediators at the transcriptional level, consistent with the well-established function of HDACs as regulators of gene transcription. Finally, we also examined the presence of pol II and the serine 5-phosphorylated form of pol II, the presence of which indicates transcriptional initiation, at primary and secondary response genes in the presence of n-butyrate or TSA. We found that treatment with n-butyrate or TSA led to a decrease in the levels of pol II and SSP recruitment to the promoters of Nos2, Il6, and Il12b, but not of Tnfa. Collectively, these data strongly support the hypothesis that n-butyrate acts as an HDAC inhibitor in macrophages.

Given that n-butyrate has an intracellular target, we next examined the uptake mechanism of n-butyrate into macrophages. The monocarboxylate transporters MCT1 and MCT4 are known to transport n-butyrate in colon epithelial cells and are also expressed by macrophages (36, 37). To test whether these transporters are involved in n-butyrate import, we treated macrophages with a pan-inhibitor of MCTs; however, our results suggest that MCTs are not involved in n-butyrate transport in this context. Several mechanisms of SCFA uptake have been proposed, including passive absorption of the undissociated form (i.e., butyric acid) by diffusion through the plasma membrane, so it is possible that other pathways may be involved in n-butyrate assimilation (38).

Finally, we tested whether n-butyrate has ameliorative affects in a murine model of colitis induced by chemical damage using DSS. Based on our results, we expected that n-butyrate would down-regulate the amount of proinflammatory mediators, thereby preventing severe inflammation in the treated mice. However, administration of n-butyrate to mice given DSS had no effect on the severity of DSS colitis. These data are consistent with rodent and human studies that have shown conflicting results in the outcome of colitis between cohorts treated with or without n-butyrate (39, 40). These results may be due to the fact that n-butyrate inhibits production of IL-6, a pleiotropic cytokine that has both pro- and anti-inflammatory effects depending on the context of its expression. Previous studies have determined that IL-6 is involved in tissue repair and cytoprotection in the gut, and prevention of its production could inhibit regeneration of the tissue after chemical insult (41). We note that Maslowski et al. have shown that the SCFA acetate ameliorates DSS colitis (14). However, this study does not contradict our current results describing the effect of n-butyrate on macrophage function because the effect of acetate was attributed to activation of Gpr43 on neutrophils, and furthermore, acetate does not inhibit HDAC activity (29).

In our study, we demonstrate that n-butyrate, a bacterial metabolite found in high abundance in the colon, regulates macrophage function through the inhibition of HDACs. n-Butyrate may behave as a microbial signal to inform the host immune system that the relative levels of n-butyrate–producing bacteria are within the desired range. In this model, HDACs act as sensors for n-butyrate, and their inhibition by high amounts of this metabolite causes intestinal macrophages to reduce production of proinflammatory mediators such as NO, IL-6, and IL-12. This effect ultimately renders the intestinal immune system hyporesponsive to the beneficial, n-butyrate–producing bacteria. In the absence of these beneficial bacteria, lamina propria macrophages remodel the intestinal microbial communities by eliminating unwanted populations of bacteria through the production of proinflammatory mediators until the optimal microbial balance is re-established and the levels of n-butyrate return to the desired concentrations. These findings provide a possible mechanism for the long-standing question of how the intestinal immune system maintains homeostasis in the gut.

Materials and Methods

For details, see SI Materials and Methods.

Administration of n-Butyrate in Vivo. Mice were preadministered metronidazole (1 g/l) and vancomycin (0.5 g/l) in the drinking water for 10 d ad libitum. n-Butyrate (300 mM) and antibiotics were administered via the drinking water on day 10 for 7 d. On day 17, colonic LP macrophages were prepared and analyzed in a blinded manner by a trained gastroenterologist.

DSS Colitis and Histological Scoring. Following treatment of mice with the appropriate combinations of antibiotics, DSS, and n-butyrate, colon sections were prepared and analyzed in a blinded manner by a trained gastroenterologist. The samples were given a score of 0–4 (where 0 = none; 1 = mild; 2 = moderate; 3 = severe; 4 = very severe) for epithelial injury, mononuclear infiltrate, and polymorphonuclear infiltrate.

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