A Gnotobiotic Mouse Model Demonstrates That Dietary Fiber Protects against Colorectal Tumorigenesis in a Microbiota- and Butyrate-Dependent Manner

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ABSTRACT

Whether dietary fiber protects against colorectal cancer is controversial because of conflicting results from human epidemiologic studies. However, these studies and mouse models of colorectal cancer have not controlled the composition of gut microbiota, which ferment fiber into short-chain fatty acids such as butyrate. Butyrate is noteworthy because it has energetic and epigenetic functions in colonocytes and tumor-suppressive properties in colorectal cancer cell lines. We used gnotobiotic mouse models colonized with wild-type or mutant strains of a butyrate-producing bacterium to demonstrate that fiber does have a potent tumor-suppressive effect but in a microbiota- and butyrate-dependent manner. Furthermore, due to the Warburg effect, butyrate was metabolized less in tumors where it accumulated and functioned as a histone deacetylase (HDAC) inhibitor to stimulate histone acetylation and affect apoptosis and cell proliferation. To support the relevance of this mechanism in human cancer, we demonstrate that butyrate and histone-acetylation levels are elevated in colorectal adenocarcinomas compared with normal colonic tissues.

SIGNIFICANCE: These results, which link diet and microbiota to a tumor-suppressive metabolite, provide insight into conflicting epidemiologic findings and suggest that probiotic/prebiotic strategies can modulate an endogenous HDAC inhibitor for anticancer chemoprevention without the adverse effects associated with synthetic HDAC inhibitors used in chemotherapy. Cancer Discov; 4(12); 1–10. ©2014 AACR. See related commentary by Sebastián and Mostoslavsky, p. xxxx.

INTRODUCTION

Whether dietary fiber protects against colorectal cancer is highly controversial because of conflicting results from human cohort-based epidemiologic studies (1–5). These studies have been complicated by the participants' genetic heterogeneity, differences in the composition of their gut microbiota, and the utilization of different fiber sources (6–8). How dietary fiber might protect against colorectal cancer has also not been established, but two general mechanisms have been proposed that are not mutually exclusive. First, insoluble fiber bulks luminal contents and may speed colonic...
transit to minimize the exposure of the colonic epithelium to ingested carcinogens such as nitrosamines from charred meat. Second, bacteria in the lumen of the colon ferment soluble fiber into short-chain fatty acids (SCFA) and other metabolites with potentially beneficial properties. Butyrate is an abundant (up to ≥10 mmol/L) SCFA that is transported into the colonic epithelium and localizes within two subcellular compartments (9, 10). It undergoes β-oxidation inside the mitochondria and accounts for ≥70% of the energy used by normal colonocytes (11), and it also functions as a histone deacetylase (HDAC) inhibitor inside the nucleus to epigenetically regulate gene expression (12). Butyrate is a plausible candidate for tumor suppression because it inhibits cell proliferation and induces cell differentiation or apoptosis when added to tumor-derived cell lines (9, 10). However, butyrate can also have contradictory effects (13, 14), and it is crucial that we move beyond “factor dump” experiments where relatively high doses of butyrate are added to colorectal cancer cell lines. It must be demonstrated that dietary fiber and gut microbiota can modulate butyrate levels in the colonic lumen, and that this, in turn, can inhibit colorectal tumorigenesis in vivo where the colonic crypt architecture is intact and functions in the presence of stromal cells.

RESULTS

Our experiments were designed to test the hypothesis that dietary fiber protects against colorectal cancer in a microbiota- and butyrate-dependent manner (Supplementary Fig. S1). To control the genetics and microbiota in our experiments, we maintained a colony of BALB/c inbred mice polyassociated with four commensal bacteria (a subset of the altered Schaedler flora or ASF; ref. 15) plus or minus the butyrate-producing bacterium *Butyrivibrio fibrisolvens* (16) in gnotobiotic isolators (Supplementary Figs. S1 and S2). These mice were provided low- or high-fiber (6% fructo-oligosaccharide/inulin) diets that were otherwise identical and calorically matched (Supplementary Figs. S1 and S3). As expected, mice colonized with the ASF plus *B. fibrisolvens* that were also provided high-fiber (“prebiotic”) diets (herein referred to as the experimental group) had significantly higher luminal butyrate levels than the other three groups of mice (herein referred to as the control groups) based on LC/MS measurements (Supplementary Fig. S4). This difference was not observed for acetate or propionate, which are the other two major SCFAs (Supplementary Table S1). We treated these mice with azoxymethane (AOM) and dextran sodium sulfate (DSS) to induce colorectal cancer and analyzed them 5 months later (17). Following one injection of AOM and two cycles of DSS, the experimental group had significantly fewer tumors than each control group. The experimental group had a mean of one tumor per mouse, whereas the control groups had three to four tumors per mouse (Fig. 1A). These results indicate that neither *B. fibrisolvens* nor high fiber had a protective effect on their own, whereas they did have a protective effect when combined.

Next, we treated mice with five injections of AOM and three cycles of DSS, which increased the tumor incidence and multiplicity as expected (Fig. 1B). More importantly, the *B. fibrisolvens* and high-fiber diet still had a protective effect in combination (3 tumors/mouse), but not in isolation (8–11 tumors/mouse; Fig. 1B). The experimental group also had significantly smaller (Fig. 1C) and less advanced (Fig. 1D and E) tumors based on their histopathologic characteristics (18). Tumors from the experimental and control groups had β-catenin gain-of-function mutations (Supplementary Fig. S5A–S5C) and were positive for nuclear β-catenin staining (Supplementary Fig. S6), which indicates that the mechanism of tumor initiation was due to aberrant Wnt signaling. These findings were expected on the basis of previous AOM studies and confirm that the mechanism of tumor initiation is similar to human colorectal cancer (17). For the 5 AOM/DSS dose regimen, we also identified squamous cell tumors derived from the surface ectoderm or glandular epithelium of the perianal region rather than the colonic mucosa (Supplementary Fig. S7A and S7B). In contrast to the colorectal tumors, the incidence, size, and histopathologic progression of the squamous tumors were not significantly different between the experimental and control groups (Supplementary Fig. S7A and S7B). Therefore, the squamous tumors, which are known to occur in the AOM/DSS model (17), served as an internal control. They demonstrate that mice from the different treatment groups received the same exposure to carcinogen, as expected, and that the tumor-suppressive effect of the fiber–microbiota–butyrate axis is specifically localized to the colonic mucosa and does not influence tumor formation outside of the lumen even at nearby sites. This dichotomy is compatible with the bioavailability of butyrate being primarily restricted to the colonic mucosa (9, 10).

To confirm that the tumor-suppressive mechanism for *B. fibrisolvens* and high fiber is due to butyrate production, we exploited a mutant *B. fibrisolvens* strain with a 0.8-kb deletion in the butyryl-CoA synthesis operon (16) that produces ≤5-fold less butyrate when cultured (Supplementary Fig. S8A–S8C; ref. 19). To validate this deficiency in butyrate production, we cultured the mutant and wild-type *B. fibrisolvens* strains in media supplemented with fructo-oligosaccharides/inulin (the same fiber source provided to the mice) and observed 7-fold lower butyrate levels in the media from mutant cultures compared with wild-type cultures (Supplementary Fig. S8A–S8C). Next, we colonized BALB/c mice with ASF plus the mutant *B. fibrisolvens* strain and evaluated AOM/DSS-induced tumorigenesis. These microbiota combined with a high-fiber diet conferred an attenuated protective effect with significantly more tumors than mice colonized with the wild-type bacterium (Fig. 1B). The mutant *B. fibrisolvens* combined with a high-fiber diet also had a diminished protective effect on tumor size and progression (Fig. 1C–E). These results demonstrate that bacterial butyrate production is commensurate with the tumor-suppressive effect. As an additional control, we colonized BALB/c mice with ASF only (in the absence of wild-type or mutant *B. fibrisolvens*) and provided them a tributyryn-fortified diet, which increases colonic butyrate levels in a microbiota-independent manner (see Supplementary Fig. S3 for the formulation of this diet and the salient attributes of tributyrin and Supplementary Fig. S4 for luminal butyrate levels in these mice; ref. 20). Following the 5 AOM/DSS dose regimen, these mice had fewer (mean of 2) colorectal tumors than any other treatment group (Fig. 1B).
These tumors were small and low grade, similar to the previous experimental group (Fig. 1C–E). Therefore, exogenous butyrate recapitulated the tumor-suppressive effect of wild-type *B. fibrisolvens* and high fiber. Taken together, these results demonstrate unequivocally that butyrate is a causative factor in the tumor-suppressive mechanism.

Our previous analysis of cell-culture models suggested that the molecular mechanism of tumor suppression might involve metabolic differences between normal and cancerous colonocytes (21, 22). Unlike normal colonocytes, which use butyrate as their primary energy source (11), colorectal cancer cells primarily rely on glucose and undergo increased glycolysis with a concomitant decrease in mitochondrial oxidative metabolism because of the Warburg effect (23). Consequently, butyrate is not metabolized inside of mitochondria to the same extent and accumulates as an HDAC inhibitor in colorectal cancer cells (22). To test the validity of this model in *vivo*, we confirmed that tumors dissected from our gnotobiotic mice exhibited certain characteristics of the Warburg effect, such as increased expression of lactate dehydrogenase A (LDHA; Fig. 2A and B) and other markers (Supplementary Fig. S9A–S9C). Furthermore, compared with normal colonic tissue, the tumors produced increased levels of lactate (Fig. 2C), which is a glycolytic end product that is catalyzed by LDHA and is known to be elevated in cancer cells undergoing the Warburg effect. On the basis of LC/MS measurements, butyrate levels were significantly higher inside of tumors than normal colonic tissue (Fig. 2D and E). To investigate the basis for this difference, we performed flux experiments that measured butyrate oxidation. Consistent with the Warburg effect and our previous analysis of cell-culture models, butyrate oxidation was significantly diminished in tumors compared with normal colonic tissues (Supplementary Fig. S10A–S10E). We also analyzed several monocarboxylate transporters (MCT) responsible for butyrate influx, but they did not show consistent expression differences between tumors and normal colonic tissues (Supplementary Fig. S10A–S10E). These findings suggest that butyrate accumulates in tumors because of diminished oxidation rather than increased uptake.

The butyrate concentration inside tumors from mice in the experimental treatment groups was calculated to be >100 μmol/L, which is consistent with its acting as an HDAC inhibitor based on its IC50 (24). Therefore, we performed immunohistochemistry (IHC) to analyze panhistone 3 acetylation (H3ac) levels. H3ac levels were markedly higher in tumor cells than normal adjacent colonocytes (Fig. 2F). Because the tumor cells and normal colonocytes were present in close physical proximity on the same slide,
Butyrate can induce histone acetylation and expression of proapoptotic genes such as FAS and cell-cycle regulators such as p21 and p27 to stimulate apoptosis and inhibit proliferation in colorectal cancer cell lines (10). Therefore, we performed quantitative chromatin immunoprecipitation (ChIP) assays and qRT-PCR to evaluate promoter H3ac levels and mRNA levels of these genes. H3ac and mRNA levels were significantly higher in tumors from experimental mice than control mice, which, in turn, were significantly higher than normal colonic tissues (Fig. 3A–F). Furthermore, cleaved caspase-3 assays and Ki-67 assays demonstrated that the experimental tumors exhibited increased apoptosis and decreased cell proliferation (Fig. 3G and H and Supplementary Fig. S11). These results provide insight into gene targets and the cell biologic basis of the tumor-suppressive effect.

To provide support for the relevance of the proposed molecular mechanism in human tumor suppression, we analyzed human colorectal adenocarcinomas and adjacent normal tissues by qRT-PCR and found that these tumors also exhibited increased H3ac levels and down-regulation of mRNA levels of these genes when compared with normal colorectal tissues (Fig. 3I and Supplementary Fig. S12).
macrophoscopically normal mucosa (see Supplementary Table S2 for clinical information). We detected significantly elevated levels of butyrate (Fig. 4A and B) and H3ac (Fig. 4C and D) in the adenocarcinomas compared with the macroscopically normal mucosa samples. Although these clinical samples were obtained from two different medical centers, there was a high level of concordance between them (Fig. 4B).

**DISCUSSION**

The findings presented here strongly support the hypothesis that dietary fiber protects against colorectal cancer. Our data support a general mechanism that includes microbial fermentation of fiber rather than fiber exclusively speeding colonic transit to minimize the exposure of colonocytes to ingested carcinogens. Our data also support a molecular mechanism where microbial fermentation of fiber yields butyrate, which serves as the preferred energy source of normal colonocytes and supports homeostasis (11, 25), but accumulates in cancerous colonocytes due to the Warburg effect and functions as an HDAC inhibitor to inhibit cell proliferation and stimulate apoptosis (21, 22). These general and molecular mechanisms are depicted as a final working model in Fig. 4E.
Butyrate may have additional effects independent of HDAC inhibition in tumor suppression. For example, both fiber and butyrate can be metabolized by certain clades of bacteria, and this can influence the microbiome and host metabolism (26). Butyrate is also a ligand for certain G-protein–coupled receptors (GPR) and has anti-inflammatory effects (9, 10), which include the ability to induce the differentiation and expansion of immunosuppressive regulatory T cells (Treg; refs. 27–30). However, we did not observe a significant difference in the number of Tregs (Supplementary Fig. S12A–S12E) or the abundance of associated cytokines such as IL10 (Supplementary Fig. S13) based on flow-cytometry and cytokine-profiling experiments, respectively. In fact, there was minimal inflammation in our gnotobiotic models following DSS or AOM/AOM/ DSS treatments (Supplementary Figs. S12, S14A, S14C), which was a consequence of our experimental design. To rigorously control butyrate levels, our gnotobiotic mice were colonized with four to five commensal bacteria that have not been implicated in inflammation (including two Lactobacillus species), and they were unable to induce a robust inflammatory response. We know that this is the case based on control experiments where we transferred a subset of our AOM- treated gnotobiotic mice to a specific pathogen-free (SPF) facility where they became “conventionalized” with diverse microbiota. When these conventionalized mice were subsequently treated with DSS in the SPF facility, the inflammatory response was markedly more severe and the tumor burden was >10-fold higher than the same AOM-treated mice that received an identical DSS treatment while still maintained in gnotobiotic isolators (Supplementary Fig. S15). Butyrate is an agonist for at least one GPR (GPR109A) expressed on the apical surface of colonocytes that can mediate butyrate tumor-suppressive effects (30, 31). Although our data support a model where butyrate enters tumor cells via MCTs to inhibit HDACs (Fig. 4E), butyrate could also be activating GPR109A signaling. We did not detect significant differences in the expression of Il18 (Supplementary Fig. S16), which is a GPR109A target gene in the colonic epithelium that is relevant to tumor suppression (30), although this does not exclude a role for GPR109A or other GPRs.

Our gnotobiotic mouse model was polyassociated with several species of bacteria and provided a homogeneous diet that...
Fiber–Microbiota–Butyrate Axis in Tumor Suppression

Included a single type of fiber. This reductionist approach was necessary to demonstrate that fiber protects against colorectal cancer and interrogate butyrate function. However, it does not reflect the complex microbiota that exist within the human gastrointestinal tract or our more varied diets that include multiple sources of fiber. Other bacterial metabolites undoubtedly participate in tumor suppression but were beyond the scope of our study. Nevertheless, it is tempting to speculate that many of the conclusions drawn from our mouse model are relevant to human health, as discussed below.

As human populations have shifted away from traditional, high-fiber diets toward processed foods containing less complex carbohydrates and more refined sugars, colorectal cancer incidence has increased markedly. Yet, the link between fiber and colorectal cancer prevention has been tenuous because of conflicting results from cohort-based epidemiologic studies. By rigorously controlling genetics, the composition of gut microbiota, and other dietary factors such as fat that may mask a beneficial fiber effect, we can conclude from this study that fiber does, in fact, protect against colorectal tumorigenesis. An important aspect of this work is the central role of gut microbiota and the fermentation product butyrate. Consequently, we propose that cohort-based epidemiologic studies should be revisited and integrated with microbiome studies. We predict that if microbiome differences of participants were taken into account, it would be possible to discriminate between individuals who respond to the anticancer chemoprotective effect of fiber and those who do not respond. This would resolve some of the conflicting results from previous human studies and possibly confirm butyrate as an important molecule in human chemoprotection. This idea is supported by several microbiome studies that have reported fewer butyrate-producing bacteria in human colorectal cancer cases than in controls, even without diet being taken into consideration (32–36).

Metagenomic sequencing projects have made a number of observations regarding the microbiome and the incidence of certain cancers. However, these studies are correlative so it is difficult or impossible to know whether the microbiome differences are a cause or a consequence of the disease state. In contrast, relatively few studies have investigated the function of microbiota in gnotobiotic mouse models of cancer (37, 38). Furthermore, most or all of these studies have focused on bacteria that promote oncogenesis by causing inflammation or genotoxicity (37, 38). In contrast, butyrate-producing bacteria confer a tumor-suppressive effect, which arguably has greater translational potential for chemoprevention via probiotics or prebiotics. The molecular mechanism described here involves butyrate functioning as an HDAC inhibitor, which is noteworthy because synthetic HDAC inhibitors are being used as anticancer chemotherapeutic agents, with some having already received FDA approval and others being evaluated in phase III clinical trials (4). The translational implication of our model is attractive because diet (prebiotics: fiber) and pos-biotic or prebiotics. Food, water, and all other materials, including cages and bedding, were autoclaved and imported into the isolators using aseptic technique following standard procedures. Each diet was from Test Diet. The low-fiber diet (553R; cat. no. 18136800) contained 2% cellulose, whereas the high-fiber diet (5510; cat. no. 18139010) contained 2% cellulose plus 6% fructo-oligosaccharides/inulin (Sigma-Aldrich; #F8052 and 12255). The tributyrin diet (54VC; cat. no. 18149610) contained 2% cellulose plus 6% tributyrin (Sigma-Aldrich; #W222305). The initial colonization of germ-free BALB/c mice with specific bacteria (which were the only imported materials not autoclaved except filter-sterilized AOM/DSS) was performed by oral gavage following standard procedures.

Methods

Mice

BALB/c mice were maintained in isolators (Class Biologically Clean) and bred in house at the National Gnotobiotic Rodent Resource Center (NGRRC) at the University of North Carolina at Chapel Hill (Chapel Hill, NC). All mouse experiments were approved by the Institutional Animal Care and Use Committees (IACUC) review board at the University of North Carolina at Chapel Hill and were performed in accordance with federal guidelines.

Gnotobiotic Mouse Husbandry

Food, water, and all other materials, including cages and bedding, were autoclaved and imported into the isolators using aseptic technique following standard procedures. Each diet was from Test Diet. The low-fiber diet (553R; cat. no. 18136800) contained 2% cellulose, whereas the high-fiber diet (5510; cat. no. 18139010) contained 2% cellulose plus 6% fructo-oligosaccharides/inulin (Sigma-Aldrich; #F8052 and 12255). The tributyrin diet (54VC; cat. no. 18149610) contained 2% cellulose plus 6% tributyrin (Sigma-Aldrich; #W222305). The initial colonization of germ-free BALB/c mice with specific bacteria (which were the only imported materials not autoclaved except filter-sterilized AOM/DSS) was performed by oral gavage following standard procedures.

Bacteria

Bacteria were cultured in BBL Schaedler broth containing vitamin K (cat. no. 221541) that was supplemented with 5% FBS in an anaerobic chamber (ThermaForma) filled with an anaerobic mixture (Airgas; #750333 consisting of 5% CO₂ and 10% H₂ balanced with N₂) at 37°C. For experiments measuring butyrate production in culture, the Schaedler broth was supplemented with 0.5% or 5% fructo-oligosaccharides/inulin (the same additive that was used for the high-fiber mouse diet). The following bacteria were cultured: ASF360 (Lactobacillus acidophilus), ASF 361 (Lactobacillus salivarius), ASF457 (Flexibacter phylimum), and ASF519 (Bacteroides distasonis), which were obtained from Taconic, and Butyrivibrio fibrisolvens (type I, ATCC 19171; type II, ATCC 51255), which was obtained from the ATCC.

AOM/DSS

AOM (Sigma-Aldrich; #A486) was dissolved in PBS at a concentration of 50 mg/mL and stored as aliquots at –80°C. Aliquots were subsequently thawed and diluted in sterile saline (0.9% NaCl) at a concentration of 1.25 mg/mL. This final AOM solution was then...
filter sterilized in a tissue-culture hood, imported into gnotobiotic isolators, and delivered by i.p. injection at a final concentration of 10 mg/kg body weight. AOM injections were performed on a weekly basis for a total of up to 5 injections. Five days after the final AOM treatment, the mice were provided drinking water containing 2.5% DSS (ICN; MW 36–50 kDa) for 5 days. The DSS-containing water was then replaced with normal drinking water for 16 days. This cycle was repeated one or two more times, resulting in either two or three DSS treatments. The DSS was prepared by dissolving it in autoclaved water followed by filter sterilization in a tissue-culture hood and importation into gnotobiotic isolators.

**Scoring of Tumors**

Tumor scoring was performed in a blinded manner by two veterinary pathologists. Colons were flushed and then splayed open. Colonic mucosal masses counted as tumors grossly appeared as variably sized, irregular, asymmetrical, shiny to roughened, tan to red, sessile to pedunculated, occasionally coalescing nodular masses. For coalescing tumors, the number of individual tumors was estimated. Colonic mucosal masses considered lymphoid nodules and not included in the tumor count were approximately 0.5 cm in diameter, 0.2 cm in height, and grossly appeared as symmetrical, shiny, translucent, flat, plaque-like masses.

**Histopathology and IHC**

Swiss-rolled colons and other tissues (e.g., lymph nodes and liver) were fixed in 4% paraformaldehyde or 10% formalin and processed for the production of 5-μm paraffin sections. Sections were stained with hematoxylin and eosin (H&E) or processed for IHC using standard procedures. Antibodies included pan-acetyl H3 (Millipore; #06-599), total H3 (Millipore; #05-928), LDHA (Cell Signaling Technology; #3582), α-tubulin (Sigma-Aldrich; #T6793), phospho-AKT (Ser473; Cell Signaling Technology; #4060), total AKT (Cell Signaling Technology; #2965), and phospho-PDH (Ser293 of E1α isofrom; Calbiochem; #AP1062).

**HDAC Assays**

Nuclear extracts were prepared from frozen tissues using a kit (Sigma; #NXTRACT) and dounce homogenization. HDAC colorimetric assays were performed using a kit (Epigentek; #P-4034). HDAC activities were normalized to protein levels for each sample.

**ChIP Assays**

Tissues were pulverized in liquid nitrogen using a mortar and pestle and then cross-linked in prewarmed 0.4% formaldehyde in PBS for 10 minutes at 37°C. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 mol/L and then sonicated with four 10-second pulses at 30% of maximum power. IP procedures, and antibodies that were used included pan-acetyl H3 (Millipore; #06-599), total H3 (Millipore; #05-928), or rabbit IgG (Santa Cruz Biotechnology) as a negative control. Protein A/G agarose beads (Santa Cruz Biotechnology; #AP1062) were added and incubated for at least 2 hours at 4°C. Dissociation curves and agarose gels demonstrated a single PCR product. qRTPCR

RNA was prepared using TRIzol reagent (Invitrogen) and reverse transcribed using random hexamers and SuperScript II RT (Invitrogen) according to standard procedures. Twenty-microliter reactions that included 10 μL of either 2× TaqMan or 2× Power SYBR Green Master Mix (Applied Biosystems) with 50 to 100 ng of cDNA and the appropriate primers were run on an ABI 7300 instrument. Product accumulation was monitored by PAM or SYBR Green fluorescence. Control reactions lacking reverse transcriptase yielded very low to no signal. Relative expression levels were determined using the ΔΔCt method and normalized to Gapdh.

**Flux Experiments**

Approximately 1 × 10⁶ cells were incubated inside Exetainer breath storage tubes (Labco Limited) in 1 mL of PBS containing 0.5 mmol/L 13C₄-butyrate and 5 mmol/L 12C-glucose for 1 hour at 37°C.
Reactions were stopped with sodium azide treatment. Butyrate oxidation was measured by analyzing isotopic CO₂ derived from butyrate to nonisotopic CO₂ derived from glucose using a 20/20 gas isotope ratio mass spectrometer (Europa Scientific) at Metabolic Solutions. Dissolved CO₂ in solution was liberated into the tube headspace by the addition of 100 μL of saturated citric acid. The ratio of ¹³CO₂ to ¹²CO₂ (mass 45 to 44) was measured directly from the sample tube headspace. All samples were compared with an internal reference gas (5% CO₂, balance 75% N₂, and 20% O₂) that had been calibrated against the International Standard PetDeeBemhtne (PDB). The results were expressed as % ¹³C/¹²C from the ATCC and split into Seahorse 24-well cell culture plates in PBS containing 2% FBS and kept at 4 °C to maintain viability. The cell suspension was then washed to remove media for each cell was replaced with fatty acid oxidation media, which contains 110 mmol/L NaCl, 4.7 mmol/L KCl, 2 mmol/L MgSO₄, 1.2 mmol/L Na₂HPO₄, 0.5 mmol/L, and 2.5 mmol/L glucose adjusted to a pH of 7.4. FHC and HCT116 cells were incubated in this media for 1 hour in a non-CO₂ incubator at 37 °C and then run on the XF24 Analyzer (Seahorse Bioscience). After three baseline measurements of the oxygen consumption rate (OCR), butyrate was injected into each well. Six more OCR measurements were then made with final concentration of 5 μmol/L) was injected into each well. Six more OCR measurements were then made. OCR was then suppressed by injecting 10% sodium azide into each well. Contribution of butyrate oxidation to OCR was determined after injection of 2DG, which inhibited glucose oxidation, and before sodium azide treatment.

Flow Cytometry

Colonic epithelial cells were isolated by incubating splayed-open colons in PBS containing 5 mmol/L EDTA at 37 °C. After a 30-minute incubation while being rotated, the colonic tissue was transferred to a new tube for the isolation of lamina propria cells, and the epithelial cells and lamina propria cells were then combined. Tissue homogenates were then centrifuged at 14,000 rpm. Supernatant was collected. Thirty milligrams of colonic tissue was homogenized in 0.5 ml of PBS buffer containing 0.1% Tween 20 with protease inhibitors. Tissue homogenates were then centrifuged at 14,000 rpm for 10 minutes at 4 °C to pellet debris, and the supernatant was used for cytokine/chemokine detection. The levels of cytokines and chemokines were detected in the serum using a Milliplex Mouse Cytokine/Chemokine Immunoassay (Millipore) per the manufacturer’s instructions. The assay included reagents to measure the following: Eotaxin, G-CSF, GM-CSF, IFNγ, IL1α, IL1β, IL2, IL3, IL4, IL5, IL6, IL7, IL9, IL10, IL12(p40), IL12(p70), IL13, IL15, IL17, IP-10, IFN-γ, LIX, MCP-1, M-CSF, MIS, MIP-1β, MIP-1α, RANTES, TNFα, and VEGF.

The Milliplex protocol was altered for the colonic tissue, substituting the provided matrix solution with the buffer used for colonic homogenization. All samples were analyzed in triplicate with detection and analysis performed on a Bio-plex 200 detection system (Bio-Rad).

Human Clinical Samples

Following Institutional Review Board (IRB) review and approval, normal and cancerous colon tissue was obtained as frozen samples and paraffin-embedded sections from the Tissue Procurement Facility at the University of North Carolina at Chapel Hill and Poudre Valley Hospital (Fort Collins, CO) in collaboration with Colorado State University (Fort Collins, CO). Each specimen was distributed with a unique identification number (no patient identifying information was distributed) in accordance with patient privacy and confidentiality guidelines.

Statistical Analysis

Supplementary Table S3 lists the statistical test used for each figure. In figures where a hypothesis was tested by comparing two groups of samples, a two-tailed t test was used to determine significant differences. For more than two groups of samples, ANOVA followed by the Tukey post hoc test was used. In experiments where the data did not follow a normal distribution, a Kruskal-Wallis nonparametric statistical test was used. It was used instead of the Mann-Whitney test because more than two groups of samples were compared in our experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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