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Diet-derived metabolites and mucus link the gut microbiome to fever after cytotoxic cancer treatment

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Abstract

Not all patients with cancer and severe neutropenia develop fever, and the fecal microbiome may play a role. In a single-center study of patients undergoing hematopoietic cell transplant (n=119), the fecal microbiome was characterized at onset of severe neutropenia. A total of 63 patients (53%) developed a subsequent fever, and their fecal microbiome displayed increased relative abundances of *Akkermansia muciniphila*, a species of mucin-degrading bacteria (P = 0.006, corrected for multiple comparisons).

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Competing interests: R.R.J. is an inventor and M.A.J., J.K., and E.H. are co-inventors on a U.S. provisional patent application (Serial No. 63/273,05) submitted by the University of Texas MD Anderson Cancer Center that covers methods and compositions for treating cancer therapy-induced neutropenic fever or GVHD. R.R.J. is on the advisory board for MaaT Pharma, LISCure Biosciences, Seres Therapeutics, Kaleido Biosciences, and Prolacta Bioscience. R.R.J have consulted for Davolterra, Merck, Microbiome DX, and Karius. R.J.J. is an inventor on a patent (PCT/US2015/062734) that was licensed to Seres Therapeutics. **M.R.M.v.d.B** has received research support and stock options from Seres Therapeutics and stock options from Notch Therapeutics and Pluto Therapeutics; he has received royalties from Wolters Kluwer; has consulted, received honorarium from or participated in advisory boards for Seres Therapeutics, Vor Biopharma, Rheos Medicines, Frazier Healthcare Partners, Nektar Therapeutics, Notch Therapeutics, Ceramedix, Lygenesis, Pluto Therapeutics, GlaskoSmithKline, Da Volterra, Thymofox, Garuda, Novartis (Spouse), Synthekine (Spouse), Beigene (Spouse), Kite (Spouse); he has IP Licensing with Seres Therapeutics and Juno Therapeutics; and holds a fiduciary role on the Foundation Board of DKMS (a nonprofit organization). MSK Cancer Center has institutional financial interests relative to Seres Therapeutics. **R.H.V** is the founder of Bloom Sciences (San Diego, CA); the company was not involved in the funding of this work nor stands to benefit from its results. L.U.P. reports research funding, intellectual property fees, and travel reimbursement from Seres Therapeutics and holds equity in Postbiotics Plus Research, serves on an Advisory board of Postbiotics Plus Research and consulting fees from DaVolterra, CSL Behring, and MaaT Pharma. **P.C.O** reports grant or research support from Merck Sharp & Dohme Corp., Napo Pharmaceuticals, Deinove, Summit Grant, and Melinta, and paid consultancy for Napo Pharmaceuticals and Ferring Pharmaceuticals Inc.; and has filed intellectual property applications related to the microbiome (reference numbers #62/843,849, #62/977,908, and #15/756,845). **J.A.W**. is an inventor on a US patent application (PCT/US17/53.717) submitted by the University of Texas MD Anderson Cancer Center which covers methods to enhance immune checkpoint blockade responses by modulating the microbiome; reports compensation for speaker's bureau and honoraria from Imedex, Dava Oncology, Omniprex, Illumina, Gilead, PeerView, Physician Education Resource, MedImmune, Exelixis and Bristol Myers Squibb; serves as a consultant/advisory board member for Roche/Genentech, Novartis, AstraZeneca, GlaxoSmithKline, Bristol Myers Squibb, Micronoma, Merck, Biothera Pharmaceuticals; and receives research support from GlaxoSmithKline, Roche/ Genentech, Bristol Myers Squibb and Novartis; and discloses additional patents WO2020150429A1, US20200129569A1, WO2019191390A2, WO2020106983A1. **A.G.S**: is or has been a paid consultant for Diversigen, AbbVie, and Merck.

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Two therapies that induce neutropenia, irradiation and melphalan, similarly expanded *A. muciniphila* and additionally thinned the colonic mucus layer in mice. Caloric restriction of unirradiated mice also expanded *A. muciniphila* and thinned the colonic mucus layer. Antibiotic treatment to eradicate *A. muciniphila* before caloric restriction preserved colonic mucus, whereas *A. muciniphila* re-introduction restored mucus thinning.

Caloric restriction of unirradiated mice raised colonic luminal pH and reduced acetate, propionate, and butyrate. Culturing *A. muciniphila* in vitro with propionate reduced utilization of mucin as well as of fucose. Treating irradiated mice with an antibiotic targeting *A. muciniphila* or propionate preserved the mucus layer, suppressed translocation of flagellin, reduced inflammatory cytokines in the colon, and improved thermoregulation. These results suggest that diet, metabolites, and colonic mucus link the microbiome to neutropenic fever and may guide future microbiome-based preventive strategies.

Introduction

One of the most common and potentially serious complications of cancer therapy is neutropenia and subsequent infectious complications, with an estimated mortality of nearly 10% (1) as well as 100,000 hospitalizations and over $2.7 billion in hospitalization costs annually in the United States (2). At particularly high risk are patients undergoing chemotherapy for hematological malignancies including acute leukemias and high-grade lymphomas, or receiving hematopoietic cell transplantation (HCT) after myeloablative conditioning (3).

The degree and duration of neutropenia has long been identified as a critical clinical parameter predicting infection (4). More recently, the role of the intestinal microbiome in neutropenia-related infections has been increasingly appreciated, with most documented bacterial infections arising from the gastrointestinal tract (5, 6). Most patients, however, will not have an infectious etiology identified, and it is not well-understood why only some 50% of patients with profound neutropenia become febrile. Observational studies on patients with acute myeloid leukemia (AML) have described alteration to the gut microbiome, known as dysbiosis, detected at the first episode of febrile neutropenia (7). Additionally, metabolic profiling has suggested a loss of microbiota-derived protective metabolites during episodes of febrile neutropenia (8). Similar studies in the pediatric patients undergoing HCT have linked the longer duration of febrile neutropenia to a higher degree of gut dysbiosis (9)). However, pathophysiology of the potential role played by typically nonpathogenic intestinal commensal bacteria has not been extensively studied. Herein we sought to gain insight into the contribution of the gut microbiome to the pathophysiology of neutropenic fever using a combination of human samples and experimental mouse models.

Results

The composition of intestinal bacteria at onset of neutropenia is associated with subsequent development of fever

We began with an examination for a potential relationship between the composition of intestinal bacteria and fever in patients who developed neutropenia in the setting of HCT.

We examined a cohort of 119 patients at the University of Texas MD Anderson Cancer Center who all developed neutropenia after HCT conditioning. Of these, 56 (47%) remained afebrile over the next 4 days, whereas 63 (53%) developed a fever, of which 7 were found to have a bloodstream infection, including 5 with Enterobacteriaceae and 2 with oral streptococci. The initial microbiome analyses described below include these 7 patients with bloodstream infections with those who developed fever. Prior to stool collection, patients were treated with prophylactic levofloxacin given daily per our institutional standard. Additional patient characteristics are provided in Table 1. Receiving either an autologous HCT or an HCT after a preparative regimen based on both busulfan and melphalan was associated with increased fever (Table 1), likely reflecting increased conditioning intensity.

We evaluated stool samples collected at the onset of neutropenia, which preceded potential development of fever in all eligible patients. In these samples we compared the microbiome between those patients who did or did not develop fever over the next 4 days, finding a significant difference in beta-diversity ($P = 0.02$, permutational MANOVA, Figure 1A). Patients who later developed fever had increased relative abundance of bacteria from the genus *Akkermansia* ($P = 0.006$, adjusted for multiple comparisons), as well as bacteria from the genus *Bacteroides* ($P = 0.01$), whereas bacteria from the class Bacilli and from the order Erysipelotrichales were increased in patients who were afebrile (Fig. 1B-D). Applying a relative abundance threshold of 0.1% to classify patients as having high or low abundances of *Akkermansia* showed a significant association between high *Akkermansia* with subsequent fever (Fisher's test P=0.02), with 32% of afebrile patients having high *Akkermansia* in comparison to 54% of febrile patients. Similarly, an abundance threshold of 5% to classify patients as having high or low abundances of Bacteroides also showed a significant association between high *Bacteroides* with subsequent fever (Fisher's test P=0.0009), with 37% of afebrile patients having high *Bacteroides* in comparison to 68% of febrile patients. Bacterial taxa associated with bloodstream infections, including Enterobacteriales, *Streptococcus*, and *Enterococcus* (10), were not associated with fever.

The recently identified genus *Akkermansia* currently includes only one species present in the intestinal tract of mammals, *Akkermansia muciniphila (A. muciniphila)*, whereas *Bacteroides* is quite diverse. *A. muciniphila* and several members of *Bacteroides* are known to have mucin-degrading capabilities (11), and so we asked if intestinal bacteria from patients with febrile neutropenia had an increased ability to degrade mucin glycans. We adapted an approach where certain carbohydrates including mucin glycans can be quantified from liquid samples using periodic acid-Schiff's reagent (fig. S1A-B) (12). We found that this method could quantify the concentrations of glycans derived from commercially available porcine gastric mucin in media. After a 48-hour culture we quantified a reduction in glycan concentration in media inoculated with isolates of *A. muciniphila* (ATCC BAA-835) or *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*, ATCC 29148), but glycan concentrations were not altered by an isolate of *E. coli* that does not degrade mucin despite abundant growth (ATCC 700926, fig. S1C). We applied this assay to aliquots of patient fecal samples from Figure 1 and found that samples from patients with higher combined relative abundances of *Akkermansia* and *Bacteroides* were more effective at consuming mucin glycans (Fig. 1E).

We asked if introducing the flora of neutropenic patients who later developed fever to germ-free mice could mediate harm following cytotoxic therapy. We utilized total body radiotherapy (9 Gy RT) as a model of systemic cytotoxic therapy that induces neutropenia and non-invasively monitored ocular surface temperatures to quantify morbidity (13). In contrast to humans, mice develop hypothermia in response to exposure to inflammatory ligands such as LPS (14) as well as in models of sepsis (15), an observation attributed possibly to differences in body size. We found that, as expected, RT induced hypothermia in specific-pathogen-free (SPF) mice, with an approximately 2°C reduction seen on day 5, a time point chosen because some mice would often become moribund by days 6-7 (fig. S2A). We thus used hypothermia as a murine surrogate for studying morbidity after RT. Applying this model to patient avatar mice, we found that avatars of febrile and afebrile patients had similar degrees of hypothermia after RT (fig. S2B). In unirradiated mice, differences in the relative abundances of *Akkermansia* continued to be observed, demonstrating some compositional stability following introduction of patient fecal samples. However, following RT, afebrile avatar mice no longer showed a reduced relative abundances of *Akkermansia* compared to febrile avatar mice (fig. S2C-D). Together these results indicate that there was no impact of the introduced patient microbiome on radiation-induced hypothermia nor microbiome composition following cytotoxic therapy. Rather, RT itself led to compositional changes in mice resembling the microbiome profile of febrile patients.

We asked if, similarly to mice, cytotoxic therapy in patients could induce changes in relative abundances of bacteria of patients undergoing HCT. For most patients from our cohort (32 of 56 without fever and 44 of 63 with fever), baseline stool samples were collected at least 4 days prior to onset of neutropenia and available for comparison. We found that patients who later developed fever had significantly increased *Akkermansia* and reduced Bacilli at onset of neutropenia compared to baseline, whereas afebrile patients had no significant changes over time in any of the bacteria associated with fever ($P = 0.003$ and $P = 0.037$; Fig. 1F, fig. S2E). In summary, we identified an increase in the relative abundance of bacteria with mucolytic properties in neutropenic patients who later developed fever, and *Akkermansia* in particular was increased in patients who later developed fever.

Radiation or chemotherapy can change the composition of intestinal bacteria in mice leading to increases in *Akkermansia* and thinning of the dense colonic mucus layer

The fact that our previous analyses had been performed only in patients not receiving broad-spectrum antibiotics suggested a non-antimicrobial mechanism was mediating the increased relative abundance of mucolytic bacteria. Our patient avatar results also suggested that cytotoxic therapy could modulate the composition of the microbiome into a profile similar to that of patients who develop fever. Thus, we sought to test the hypothesis that transplant conditioning could change the composition of the intestinal microbiome, beginning with total body radiotherapy. Normal SPF C57BL/6 mice were exposed to 9 Gy RT, and their stool samples were evaluated 6 days later by 16S rRNA gene sequencing. Similar to what we observed in afebrile avatar mice, the microbiome of SPF mice on day 6 after RT was changed compared to unirradiated mice (Fig. 2A). The profile was reminiscent of that seen in patients undergoing HCT who had febrile neutropenia, showing increases in the relative abundance of *Akkermansia*, and to a lesser degree, *Bacteroides* (Fig. 2B-D). Notably, we

did not observe compensatory reductions in Bacilli or Erysipelotrichales, as was observed in patients with febrile neutropenia. Rather, we found reductions in bacteria from the family Muribaculaceae, a recently named group of bacteria that is commonly found in high relative abundance in mice but is usually a minor contributor in the intestinal tract of humans (16). We asked if this change in bacterial composition was accompanied by functional changes and found that bacteria derived from stool samples of irradiated mice more efficiently degraded mucin glycans than bacteria from unirradiated mice in vitro (Fig. 2E). We then asked if the dense colonic mucus layer, which normally separates bacteria-rich luminal contents from the colonic epithelium, was affected by irradiation in mice. To evaluate this, colonic tissue samples were cut cross-sectionally and mucus layer thickness was averaged across 8 equally-spaced circumferential sites (fig. S3). We found that the mucus layer was significantly thinner in irradiated mice compared to normal mice ($P = 0.003$; Fig. 2F).

Myeloablative RT, a foundational pillar which made HCT possible, has been progressively replaced by chemotherapy, particularly alkylating agents (17). We thus evaluated treating mice with the alkylating agent melphalan and found that this led to significant changes in the microbiome, marked by an increase in the relative abundance of *Akkermansia*, similar to that seen after RT ($P = 0.001$; Fig. 2G-J). An expansion of *Bacteroides* was not statistically significant after correction for multiple comparisons, although a loss of Muribaculaceae, similar to that following RT, was observed. Histological analysis demonstrated that the mucus layer was significantly thinner in melphalan-treated mice, similar to that after RT ($P = 0.01$; Figure 2K).

We asked why the intestinal microbiome appeared to be impacted similarly in response to different cytotoxic therapies. We first evaluated for direct effects of RT on intestinal bacterial composition by irradiating mouse fecal pellets and cultivating bacteria on agar plates. We then swabbed all bacterial colonies that grew and evaluated their taxonomical composition by 16S rRNA gene sequencing. We found that exposure to irradiation resulted in no enrichment for *Akkermansia* or *Bacteroides*, though *Akkermansia* relative abundance was low due to its relatively slow growth rate in vitro (fig. S4A). We also introduced bacteria from irradiated fecal pellets orally to mice previously treated with an oral decontaminating antibiotic cocktail. We again found that exposure to irradiation had no discernible effect on the composition of bacterial populations, including no enrichment for *Akkermansia* or *Bacteroides* (fig. S4B).

Caloric restriction in mice is sufficient to produce changes in the composition of the intestinal bacteria similar to those seen after cytotoxic therapy

Diet is known to be a major determinant of intestinal microbiome composition. We asked if RT could be impacting the microbiome composition indirectly, by causing a reduction in intake of food in mice. We individually housed RT-treated mice in metabolic cages to quantify effects on food and water intake. We found that within 2 days after RT, mice had reduced their oral intake to approximately 2 grams a day, or a 50% reduction (Fig. 3A). To evaluate whether caloric restriction (CR) could impact intestinal microbiome composition and the colonic mucus layer, we took healthy, unirradiated mice, and limited their access of chow to 2 grams per mouse per day for 7 days. We found that caloric restriction

resulted in marked changes in the microbiome characterized primarily by expansion of *Akkermansia* and, to a lesser extent, an expansion of *Bacteroides* and loss of Bacilli (Fig. 3B-E). Mucin glycan degradation was more robust after CR (Fig. 3F), and the colonic mucus layer was also significantly thinner ($P < 0.0001$; Fig. 3G). To evaluate if CR was impacting mucin production, we evaluated goblet cells, specialized epithelial cells that are the primary producers of mucin in the colon. Neither the numbers of goblet cells per crypt, nor the combined surface area of goblet cells in a cross section of colonic tissue, were impacted by CR (fig. S5A). RNA expression of the gene encoding the predominant mucin in the small and large intestine, *Muc2*, was not changed in colonic tissue homogenates from mice after CR (fig. S5B). Altogether, these results indicated that a reduction in oral nutrition was sufficient to produce a thinner colonic mucus layer, possibly through an increase in mucin degradation leading to increased consumption of mucin, whereas the production of mucin appeared to be intact.

Manipulating the intestinal bacteria of mice with different antibiotics and bacterial reintroduction indicates that *Akkermansia* is required for thinning of the colonic mucus layer with CR

Akkermansia is nearly universally found in fecal samples of commercially available mice (18). Germ-free mice are available but have been reported to have many derangements in mucosal immunity including an underdeveloped colonic mucus layer (19). We confirmed this to be the case, with germ-free mice having a poorly developed colonic mucus thickness. We additionally found that mono-associating mice with a murine isolate of *A. muciniphila* (MDA-JAX AM001) derived from the feces of C57BL/6 mice for two weeks was not sufficient to restore a normal-appearing colonic mucus layer (fig. S5C). Thus, to allow evaluation of the contribution of *Akkermansia* to mucus thinning during CR, we turned to narrow-spectrum antibiotics (Fig. 3H). Specifically, we evaluated streptomycin which we found depleted certain Gram-positive bacteria but spared both *Akkermansia* and *Bacteroides*; vancomycin, which depleted both Gram-positive bacteria and *Bacteroides* but spared *Akkermansia*; and azithromycin, which depleted *Akkermansia* as well as some Gram-positive populations but spared *Bacteroides* (Fig. 3I). Each of these antibiotics was administered continously in the drinking water of mice during CR. We found that neither streptomycin nor vancomycin had a significant effect on mucus thickness, whereas azithromycin treatment prevented thinning of the colonic mucus layer ($P = 0.01$; Fig. 3J) indicating that *Akkermansia* could be required for increased mucolysis during caloric restriction.

To further assess the contribution of *Akkermansia* to colonic mucus loss during CR, we evaluated antibiotic strategies that could eradicate *Akkermansia* when administered as a course of therapy that is discontinued prior to starting CR. Although even high-dose administration of azithromycin was not effective at eradicating *Akkermansia* in mice (fig. S5D-E), we found that pre-treatment with a 3-week course of tetracycline was sufficient to eradicate *Akkermansia*, as had been reported previously (18), with relative abundances remaining undetectable after completing treatment even after CR (Fig. 4A-B). Tetracycline pre-treatment resulted in no appreciable thinning of colonic mucus following CR (Fig. 4C). Further, reintroducing *Akkermansia* to tetracycline pre-treated mice was sufficient to restore

mucus thinning after CR (Fig. 4B-C). Together these data indicate that the presence of *Akkermansia* is required and sufficient for increased mucolysis during CR.

Production of short-chain fatty acids is affected by CR

To identify potential mechanistic links between diet and microbiome composition, we hypothesized that CR was perturbing normal commensal bacterial metabolism in the intestinal lumen, and began by asking if metabolic substrates entering the colon were impacted by CR. To evalute this we performed bomb calorimetry on cecal contents of mice and found that restricted mice had fewer calories entering the colon (Fig. 5A). Among the most abundant products of intestinal bacterial metabolism are organic acids, and thus we quantified pH in the colonic lumen and found that caloric restriction resulted in a raised pH of approximately 7.2 compared to 6.5, indicating overall reduced metabolism (Fig. 5B). To better characterize this rise in pH, we quantified specific bacterial metabolites using ion-chromatography mass spectometry, and found that caloric restriction led to reduced concentrations of acetate, propionate and butyrate, and increased succinate (normalized results in Fig. 5C, raw results in fig. S6A). Succinate is a metabolic precursor of propionate (20), suggesting that caloric restriction could be inhibiting enzymatic conversion of succinate to propionate.

Acidity and propionate ceoncentrations inhibit mucin utilization by *Akkermansia* in vitro

We asked if CR-induced metabolic changes in the colonic lumen affected *A. muciniphila* behavior. To study this, we turned to our in vitro mucin glycan consumption assay. We introduced murine *A. muciniphila* to liquid media supplemented with porcine gastric mucin, and evaluated the effects of varying pH either alone or with physiological concentrations of acetate, propionate, and butyrate.We found that progressively lowering the pH conditions of bacterial media led to increased inhibition of *A. muciniphila* in terms of both growth and mucin glycan degradation (Fig. 5D). We also found that higher concentration of propionate had inhibitory effects on mucin glycan utilization by *A. muciniphila* (Fig. 5E) and led to growth delays (fig. S6B) whereas acetate and butyrate each had negligible effects on *A. muciniphila* behavior. We also evaluated the effects of acetate, propionate, and butyrate at concentrations beyond physiological concentration on *A. muciniphila* in pH 6.8 conditions. We found that propionate inhibited mucin consumption at a concentration of 10 mM, and further increasing the concentration led to increased inhibition of *A. muciniphila* growth, with complete growth inhibition occuring at 40 mM (Fig. 5F-G). Butyrate was less potent, with 40 mM required to suppress mucin consumption and concentration of 100 mM required to completely growth. Acetate, even at high concentrations, had only slightly suppressive effects on *A. muciniphila* mucin degradation and growth. Taken together, these results demonstrated that propionate, and to a lesser degree butyrate, as well as acidity, have suppressive effects on the utilization of mucin by *A. muciniphila*.

We asked if this finding that propionate can inhibit mucin glycan degradation by *A. muciniphila* was potentially true for other species of bacteria. To evaluate this we examined mucin utilization in vitro by strains of *Bacteroides* (21, 22), Parabacteroides (23), Alistipes (24), Bifidobacterium (25), and *Ruminococcus* (26-28). These data demonstrated that

propionate can suppress mucin utilization by *Parabacteroides distasonis*, but not that of other mucin degraders tested (fig. S7A).

Acidified propionate administration during CR prevents thinning of colonic mucus

To see if a combination of acidity and propionate could also suppress *A. muciniphila* in vivo, we supplemented mice during CR with acidified sodium propionate in the drinking water, and found that this reduced fecal pH (fig. S7B), mitigated expansion of *Akkermansia* (Fig. 5H) and prevented thinning of the mucus layer (Fig. 5I). Similar treatment with acidified sodium acetate, despite lowering the pH in the colonic lumen, had no such preventative effect (Fig. 4H-I), whereas drinking water with sodium propionate at neutral pH was sufficient to prevent mucus thinning (fig. S7C-D). Altogether, these results indicate that a reduced concentrations of propionate following CR and a higher pH together support increased mucolytic activity.

CR or cultivation in ambient propionate produces transcriptomic changes in *Akkermansia*

To explore how CR can modulate mucin utilization by *A. muciniphila*, we profiled the *A. muciniphila* transcriptome in stool samples collected from mice that were or were not undergoing caloric restriction. We determined the circularized genomic sequence of our murine *A. muciniphila* isolate (MDA-JAX AM001) and identified 1757 putative proteins (fig. S8A), including 1373 that were not annotated as “hypothetical proteins”. We then performed RNA sequencing on stool samples from mice and considered reads that aligned to the *A. muciniphila* genome to quantify the relative abundance of individual gene transcripts expressed by *A. muciniphila*. Focusing on the 100 most variable genes not annotated as “hypothetical”, we found that 38 genes were significantly modulated by CR in vivo (DESeq2 test with Benjamini-Hochberg adjustment, $P < 0.05$, Fig. 6A).

We note that two adjacent genes (136 and 137) encoding fucose utilization proteins were both significantly upregulated in calorie-restricted mice ($P = 0.0004$ and $P = 0.008$). One is predicted to be an L-fucose transporter, whereas the other encodes an L-fucose isomerase, which converts fucose to fucolose in the first step of bacterial fucose metabolism (29). Mucins are proteins decorated by extensive glycan chains that are predominantly capped by fucose and sialic acid residues at their branching terminals. The ATCC type strain of *A. muciniphila* has previously been reported to be capable of utilizing fucose as a carbohydrate source (30). Because we had found that propionate is reduced with caloric restriction and that propionate can directly suppress mucin glycan utilization by *A. muciniphila*, we asked if propionate was sufficient to impact on expression of these fucose-utilization genes in vitro. We cultivated murine *A. muciniphila* with varying concentrations of propionate and performed RNA sequencing. This approach demonstrated that both genes 136 and 137 negatively correlated with ambient propionate concentrations (Fig. 6B), consistent with our in vivo results. We also globally evaluated the correlation of effect sizes in the in vivo and in vitro settings and found that propionate-related effects observed in vitro only explained changes seen in CR at a proportion of 0.05, though the slope was significantly non-zero ($P < 0.0001$, fig. S8B). Representations of gene expression with respect to the genome are depicted in Figure S8C and relative gene abundances of all in vivo and in vitro samples are provided in data file S1.

Cultivation in ambient propionate reduces expression of L-fucose isomerase by *Akkermansia*

These results led us to ask if propionate could affect fucose utilization by *A. muciniphilia*. We first evaluated the growth of *A. muciniphilia* in carbohydrate-poor media supplemented with fucose and found that our murine isolate exhibited better growth in the presence of fucose (Fig. 6C). We also found that the addition of 10 or 20 mM propionate resulted in dose-dependent delays in growth on fucose, as well as reduced consumption of fucose by *A. muciniphilia*, whereas the same concentrations of acetate or butyrate had no appreciable effects (Fig. 6D-E).

Next, we focused on quantifying the effects of propionate on expression of L-fucose isomerase. We prepared lysates of *A. muciniphilia* that had been cultivated with mucin in varying concentrations of propionate. These lysates were then incubated with fresh L-fucose, and resulting concentrations of both L-fucose and L-fuculose were quantified. Lysates prepared from *A. muciniphilia* grown in the absence of propionate were able to convert L-fucose to generate L-fuculose. Lysates from *A. muciniphilia* cultivated in the presence of propionate, however, did not efficiently catalyze this reaction, resulting in significantly less L-fuculose and higher concentrations of preserved L-fucose ($P < 0.0001$; Fig. 6F). Together, these results indicate that propionate can suppress the utilization and enzymatic metabolism of L-fucose by *A. muciniphilia*, potentially accounting for its reduced ability to consume mucin.

Administration of azithromycin or acidified propionate prevents colonic mucus thinning, colonic inflammation, and hypothermia in mice after radiation

Last, we asked if either of the two strategies that we had identified as effective in preventing loss of colonic mucin during caloric restriction, azithromycin or propionate, could also mediate a clinical benefit following cytotoxic therapy. To evaluate this, we returned to our RT model and tested the addition of azithromycin or propionate. We found that whereas azithromycin was effective at preventing outgrowth of *Akkermansia* in mice following RT, propionate was not (Fig. 7A). Both approaches, however, led to improved preservation of colonic mucus layer thickness (Fig. 7B). Because a thinned mucus layer quantified from fixed tissue does not necessarily imply a compromised barrier, we also characterized the effects of our interventions on intestinal barrier function after RT. We quantified serum concentration of the bacterial product flagellin, which can serve as a measure of compromised gut barrier integrity (31, 32). We found that RT resulted in a significant increase of serum flagellin that could be blocked by the addition of azithromycin or propionate treatment ($P < 0.0001$; Fig. 7C).

These results suggested that strategies to inhibit mucolytic activity of *A. muciniphila* might be effective at reducing inflammation in irradiated mice. To explore this, we characterized inflammation severity in colonic tissues by quantifying the concentration of a panel of cytokines (Fig. 7D). We found that IL-1β, CCL2, CCL7, IL-22, CXCL1, and CXCL10 were all elevated in colonic tissues of mice after RT but were reduced with the addition of azithromycin treatment. Propionate treatment also prevented elevation of each of these

cytokines, except for CXCL1 which remained elevated. We did not observe elevations of TNF after RT, nor effects of azithromycin or propionate on TNF concentrations.

We asked if strategies to inhibit mucolytic activity of *A. muciniphila*, which we had originally identified to be associated with development of fever in neutropenic patients, could potentially help to prevent fever. We thus evaluated for an impact on RT-induced hypothermia by monitoring surface ocular temperatures. We found that after RT, mice developed hypothermia as expected. The temperatures of irradiated mice supplemented with azithromycin or propionate, however, were substantially less depressed at day 6 (Fig. 7E). Ocular temperatures of mice across all experimental groups showed an inverse correlation with serum flagellin concentrations, ($r = -0.56$, P =0.0009; Fig. 7F).

Altogether, results from interventional experiments in mice after RT indicated that azithromycin therapy was effective at eliminating intestinal *Akkermansia*, preserving colonic mucus and epithelial integrity, and preventing colonic inflammation and hypothermia, whereas propionate therapy was less effective but nevertheless prevented colonic mucus thinning, improved epithelial integrity, largely abrogated much of the colonic inflammation that occurred after RT, and substantially prevented hypothermia.

Discussion

We found that bacteria with mucin-degrading capabilities, especially *Akkermansia*, were enriched in neutropenic patients who later developed fever, with 32% of afebrile patients having an *Akkermansia* relative abundance greater than 0.1% in comparison to 54% of febrile patients. Mucus degraders, however, have been previously implicated in other disease settings, including inflammatory bowel disease (28, 33), graft-versus-host disease (34) and colonic epithelial carcinogenesis (35). *A. muciniphila*, identified in 2004 as a specialized intestinal mucin-degrading commensal (36), has in particular been associated with increased colitis (37) and colonic graft-versus-host disease (34) in mouse models. Other mucin degraders include members of the genus *Bacteroides*, which have been associated with murine colitis (38) and are particularly well-studied due to the availability of methods to manipulate genetically tractable *Bacteroides* isolates (39). *Ruminococcus gnavus* is another mucin-degrading species that has been well-studied (40) and has been clinically associated with inflammatory bowel disease (41).

In other clinical settings, *A. muciniphila* is associated with potentially beneficial health effects. Loss of *A. muciniphila* has been observed in individuals with metabolic conditions, including obesity and Type 2 diabetes, and supplementation with *A. muciniphila* may mediate a clinical improvement (42). Increased *A. muciniphila* has also been associated with enhanced responses to PD1 blockade immunotherapy in patients with lung and urothelial cancers, and superior tumor responses in mice (43).

We observed that the relative abundance of *A. muciniphila* increased following cytotoxic chemotherapy in some patients undergoing HCT, reaching as high as 76.6% in some patients who later developed fever. We speculate that this may have been due to poor diet as well as possibly receiving antibiotics prior to transplant hospitalization, as any patients administered

antibiotics during transplant hospitalization were excluded from the study. One limitation of this study is that the abundance of *A. muciniphila* was highly heterogeneous in both afebrile and febrile patients and does not by itself perfectly predict who will or will not develop neutropenic fever. An important question that remains to be answered is whether there are functional strain differences within the microbiome of individual patients, as has been observed in other human cohorts (44), and whether particular subclades of *A. muciniphila* are more or less strongly associated with developing neutropenic fever.

Radiotherapy or melphalan therapy also produced increases in the relative abundance of *A. muciniphila* in mice, and we found that this was likely driven by reductions in oral dietary intake. A link between restrictions in diet and *A. muciniphila* has been observed before, including in subjects after Islamic fasting (45) or after as little as three days of deliberate underfeeding in the context of a clinical trial (46), and has been recently systematically reviewed (47). In mice, intermittent 16 hour fasting for one month resulted in increased abundance of *A. muciniphila* (48), as did 4 days after switching from oral to parenteral nutrition delivered following internal jugular vein catheterization (49). Mice consuming a fiber-depleted diet were previously found to have expansions of *A. muciniphila* and mucin-degrading *Bacteroides* as well as compromise of colonic mucus, and this led to increased susceptibility to colitis secondary to *Citrobacter rodentium* (30), findings that likely have shared mechanisms with our findings in calorie-restricted mice.

Why diet and *A. muciniphila* are closely linked is not as well understood. We found that propionate concentrations in the intestinal lumen were reduced with caloric restriction, and that propionate mediated suppressive effects on utilization of mucin glycans by *A. muciniphila*. In addition to *A. muciniphila, Staphylococcus aureus* has also been shown to be inhibited by propionate, whereas acetate and butyrate were not effective (50). *A. muciniphila* has been reported to produce propionate following metabolism of mucin-derived carbohydrates (51, 52), and thus the presence of propionate, as a metabolic end product, may serve as a feedback mechanism to suppress excessive utilization of mucin glycans. Inhibition of *A. muciniphila* by propionate was more pronounced at lower pH settings, which could be due to better penetration of propionate through bacterial cell membranes in its protonated state, as has been recently observed for acetate uptake by Enterobacteriaceae (53). The mechanism by which propionate suppresses mucin utilization by *A. muciniphila*, however, remains unclear and is a limitation of our study. Additionally, whether other colonic lumen metabolites besides SCFAs could additionally mediate suppression remains an unanswered question.

In our study we focused on intestinal bacteria, especially *Akkermansia*, and effects mediated by propionate on *Akkermansia*. It is important to acknowledge, however, that propionate likely has effects on other intestinal commensal bacteria and is also known to have an impact on a variety of host cell types, including epithelial and immune cells. Propionate has been demonstrated to mediate these effects through several signaling pathways and epigenetic mechanisms. For example, in ileal organoids, propionate has been found to modulate the expression of proteins involved in cell cycle control, adipocyte function, and lipid metabolism (54). Propionate has also been shown to inhibit histone deacetylases and

activate G protein-coupled receptor 43 and STAT3 to modulate intestinal epithelial cell migration both in vitro and in vivo (55).

In this study, we presented data indicating that two antibiotics that can suppress relative abundances of Akkermansia, azithromycin and tetracycline, may mediate protection against toxicities arising from systemic cytotoxic cancer therapy including total body radiotherapy. Fluoroquinolones have been best studied as prophylaxis and are recommended by guidelines (5) but are not active against most isolates of A. muciniphila (56). Recommending azithromycin as prophylaxis, however, can be problematic. In 2018, the U.S. Food and Drug Administration (FDA) released a warning regarding a potential increased risk of cancer relapse and death with azithromycin use after allogeneic HCT based on the ALLOZITHRO randomized clinical trial, a trial which was stopped early due to an imbalance in relapsed disease (57). Although subsequent retrospective studies have not reproduced this association (58), identifying antibiotics besides azithromycin that have activity against Akkermansia and other mucin-degrading bacteria could be a translational strategy to minimize risk for fever in neutropenic patients.

In summary, we found that the intestinal microbiome of patients who developed fever in the setting of neutropenia was enriched in mucin-degrading bacteria. Further experimentation in mice identified interrelated aspects of diet, metabolites, and intestinal mucus. These results suggest that development of novel approaches, including dietary, metabolite, pH and antibacterial strategies, can potentially better prevent fevers in the setting of neutropenia following cancer therapy.

Materials and Methods

Study design

This study aimed to investigate the role of the gut microbiome and diet-derived metabolites in developing neutropenic fever among cancer patients in the setting of radiation and chemotherapy. We first examined a link between the gut microbiome and fever in a cohort of 119 patients who had undergone HCT at MD Anderson Cancer Center. To further understand the mechanism of the pathophysiology of neutropenic fever and to develop potential therapy, we used preclinical mouse models of irradiation, chemotherapy, and calorie restriction. Towards this, we conducted in vivo (SPF mice and Germ-free mice) and in vitro experiments using different approaches. Informed consent was obtained with MD Anderson Cancer Center Institutional Review Board approval (PA17-0035). The institutional Animal Care and Use Committees (IACUCs) at MD Anderson Cancer Center approved all the animal studies (IACUC 1705). More details can be found in the supplementary materials.

Human samples

Stool biospecimen collection from patients was approved by the University of Texas MD Anderson's Institutional Review Board and signed informed consent was provided by all study participants. Samples were collected from patients undergoing stem cell transplantation and stored at 4°C for up to 48 hours before they were aliquoted for long-term

storage at −80°C. Neutropenia was defined as a total white blood cell count less than 500 per microliter of blood. Neutropenic onset stool samples collected between day −2 to day +2 relative to the first day of neutropenia were eligible for inclusion in the study. Patients who received antibiotics other than standard bacterial prophylaxis with levofloxacin during the hospitalization prior to collection of neutropenic onset stool samples were excluded. Fever was defined as an oral temperature greater than 38.0°C within 4 days of neutropenic onset stool sample collection. For paired microbiome analyses, baseline stool samples were collected between 16 and 4 days (median 5.5 days) prior to neutropenic onset samples.

Mice

Studies in animal models conformed to the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. Six- to eight-week-old female C57BL/6 mice were obtained from the Jackson Laboratory. Six- to eight-week-old female C57BL/6 germ-free mice for murine studies were provided by the gnotobiotic facility of Baylor College of Medicine (Houston, TX).

Total body radiotherapy

Mice were exposed to a single myeloablative dose of total body radiotherapy (9 Gy RT) using a Shepherd Mark I, Model 30, 137Cs irradiator.

Murine temperature monitoring

Ocular temperatures were measured using an infrared FLIR E60 camera (FLIR) as previously described (9). Briefly, a 20mm lens was attached to the front of the camera using a 3D printed lens holder without any modifications to the camera for close-up imaging. Ocular temperatures were acquired with a 56 mm focal distance perpendicular to the eye being assessed. Data were analyzed by FLIR Tools+ software.

Patient avatar mice

Patient stool samples were suspended at a concentration of 50 mg of stool per mL anaerobic PBS, strained through a 100 μm cell strainer and introduced to germ-free mice by gavage (200 μL) 14 days prior to RT.

A. muciniphila mono-associated mice

All animal procedures were conducted in compliance with Baylor College of Medicine IACUC approved protocols. Germ-free C57BL/6J mice, 9-12 weeks of age, were housed in sterile flexible film isolators. Germ-free status was confirmed by fecal collection prior to starting the study. Some mice were transferred to a new isolator and treated with oral gavage of 10^8 CFU of *Akkermansia muciniphila* (MDA-JAX AM001) in 0.2 mL PBS. After 14 days, both groups of mice were euthanized via inhaled carbon dioxide.

Statistical analysis

Data were checked for normality and similar variances between groups and Student’s t-tests and Pearson’s correlation were used when appropriate. Mann-Whitney U tests and Spearman

correlation were used to compare data between two groups when the data did not follow a normal distribution. Analyses were performed using R software version 3.6.0 and Prism version 7.0 (GraphPad Software P values < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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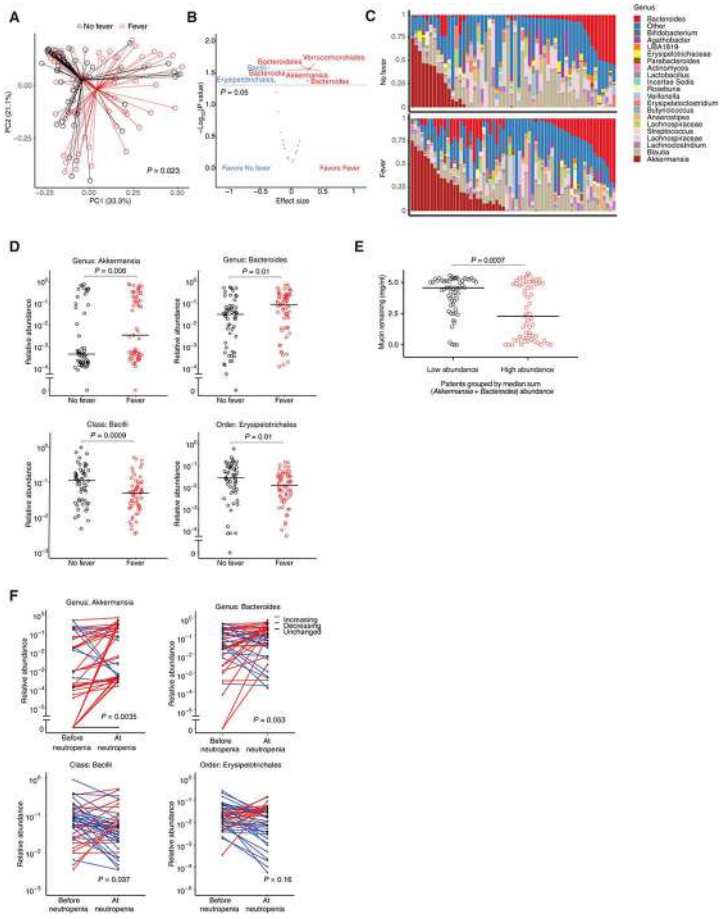

Figure 1. Mucin-degrading intestinal bacteria are associated with development of fever after onset of post-HCT neutropenia.

Intestinal microbiome parameters at neutropenia onset and subsequent fever were evaluated in a cohort of patients undergoing HCT. Stool samples were collected at onset of neutropenia (+/− 2 days), and fever outcome was determined by inpatient monitoring every 4 hours in the subsequent 4 days after collection. A) Principal Coordinates of Analysis (PCoA) was performed on weighted UniFrac distances based on 16S rRNA gene sequencing. Statistical significance was determined by permutational MANOVA testing. B) Volcano plot of bacterial taxa that were differentially abundant in A). Taxa above the green line have a p value less than 0.05; p values were adjusted for multiple comparisons using the Benjamini-Hochberg method. C) Relative abundances of bacteria at the genus level in samples from A) are indicated in stacked bar graphs. D) Relative abundances of bacteria of the indicated taxa are depicted for samples from A); p values were adjusted for multiple comparisons. E) Mucin glycan consumption by frozen aliquots of stool samples in A) was assayed. Fecal bacteria were cultivated in liquid media supplemented with porcine gastric mucin as the predominant source of carbon, followed by quantification of remaining mucin glycans after 48 hours. Samples were stratified by median sum relative abundance of *Akkermansia* and *Bacteroides*. Statistical significance was determined by the Mann-Whitney U test. F) In the subset of patients who later developed neutropenic fever, relative abundances of bacteria from the indicated taxa in stool samples collected at onset

of neutropenia were compared to results of a baseline stool sample collected earlier in the hospitalization, using the Wilcoxon signed-rank test.

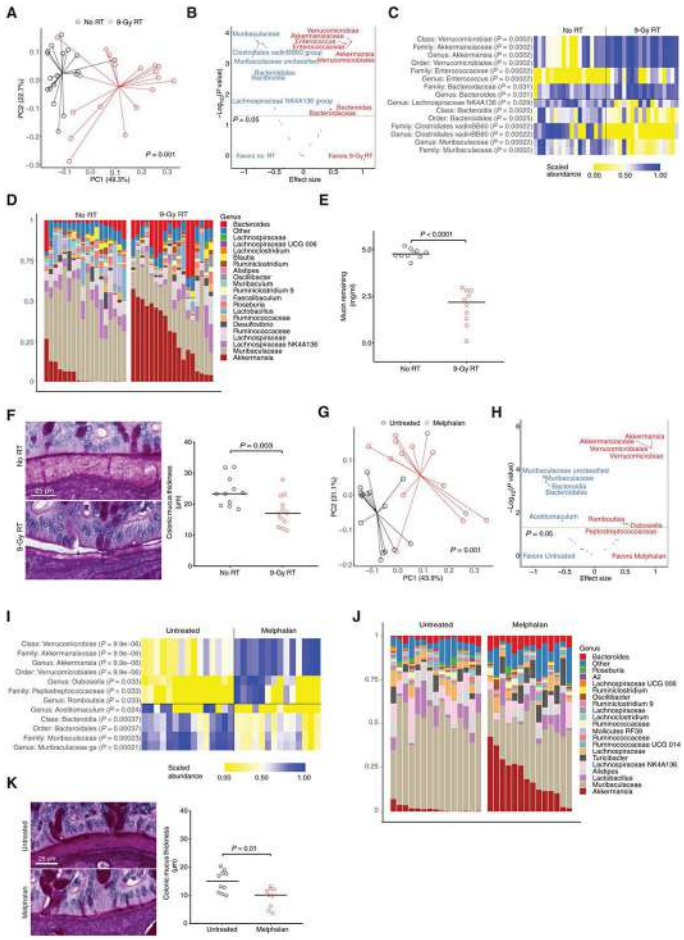

Figure 2. Systemic cytotoxic therapy increases the relative abundance of mucin-degrading intestinal bacteria in mice.

Evaluation of intestinal microbiome parameters was performed in adult C57BL/6 female mice 6 days after total body radiotherapy (9 Gy RT, panels A-E) or 6 days after melphalan therapy (20 mg/kg, panels G-K). A) After 9 Gy RT, PCoA was performed on weighted UniFrac distances; combined results of 3 experiments. Statistical significance was determined by permutational MANOVA testing. B) Volcano plot of bacterial taxa that were differentially abundant in A); p values were adjusted for multiple comparisons using the Benjamini-Hochberg method. C) Heatmap of scaled relative bacterial relative abundances of the indicated taxa are depicted for samples from A). D) Relative abundances of bacteria at the genus level in samples from A) are indicated in stacked bar graphs. E) Bacteria from frozen stool samples collected from mice in A) were evaluated for mucin glycan consumption; combined results of 2 experiments. Statistical significance was determined by the Mann-Whitney U test. F) Thickness of the dense inner colonic mucus layer was evaluated histologically in mice in A). Representative images are provided with combined results of 3 experiments. Statistical significance was determined by the Mann-Whitney U test. G) After melphalan therapy, PCoA was performed on weighted UniFrac distances; combined results of 3 experiments. Statistical significance was determined by permutational MANOVA testing. H) Volcano plot of bacterial taxa that were differentially abundant in G); p values were adjusted for multiple comparisons using the Benjamini-Hochberg method. I)

Heat map of scaled relative bacterial relative abundances of the indicated taxa are depicted for samples from G). J) Relative abundances of bacteria at the genus level in samples from G) are indicated in stacked bar graphs. K) Thickness of the dense inner colonic mucus layer was evaluated histologically in mice in G). Representative images are provided with combined results of 2 experiments. Statistical significance was determined by the Mann-Whitney U test.

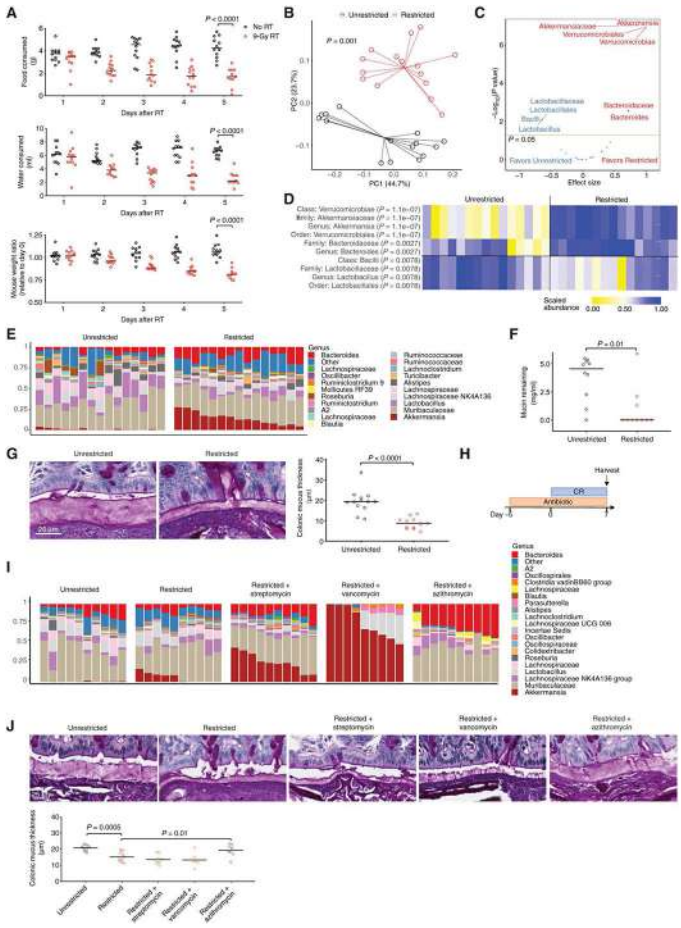

Figure 3. Caloric restriction increases the relative abundance of mucin-degrading intestinal bacteria in mice.

A) After 9 Gy RT, mice were individually housed in metabolic cages and monitored daily for food consumption, water consumption, and weight. Statistical significance was determined by the Mann-Whitney U test. B) Intestinal microbiome parameters were evaluated in normal mice after undergoing caloric restriction (2 g/mouse/day) for one week. PCoA was performed on weighted UniFrac distances; combined results of 3 experiments. Statistical significance was determined by permutational MANOVA testing. C) Volcano plot of bacterial taxa that were differentially abundant in B); p values were adjusted for multiple comparisons using the Benjamini-Hochberg method. D) Heat map of scaled relative bacterial relative abundances of the indicated taxa are depicted for samples from B). E) Relative abundances of bacteria at the genus level in samples from A) are indicated in stacked bar graphs. F) Bacteria from frozen stool samples collected from mice in B) were evaluated for mucin glycan consumption; combined results of 2 experiments. Statistical significance was determined by the Mann-Whitney U test. G) Thickness of the dense inner colonic mucus layer was evaluated histologically in mice in B). Representative images are provided with combined results of 3 experiments. Statistical significance was determined by the Mann-Whitney U test. H) Experimental schema. Mice underwent caloric restriction as in B), with the addition of narrow-spectrum antibiotics administered in the drinking water starting 5 days prior to onset of restriction. I) Relative abundances of bacteria at the genus

level in samples are indicated in stacked bar graphs; combined results of 2 experiments. J) Thickness of the dense inner colonic mucus layer was evaluated histologically in mice in I). Representative images are provided with combined results of 2 experiments. Statistical significance was determined by the Mann-Whitney U test.

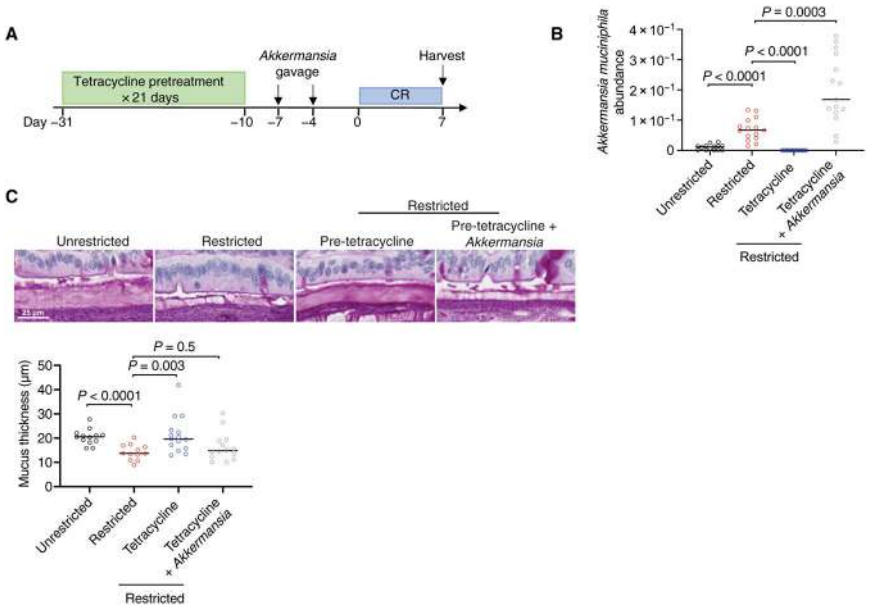

Figure 4. A) Experimental schema.

Mice were pre-treated with tetracycline administered in the drinking water for 21 days to clear the intestines of *A. muciniphilia*. A murine isolate of *A. muciniphila* (MDA-JAX AM001) was re-introduced 5 days before the onset of restriction. B) Relative abundances of *Akkermansia* on day 7 after CR was quantified by 16S rRNA gene sequencing. Combined results of 4 experiments. Statistical significance was determined by the Mann-Whitney U test. C) Thickness of the dense inner colonic mucus layer was evaluated histologically in mice in B). Statistical significance was determined by the Mann-Whitney U test.

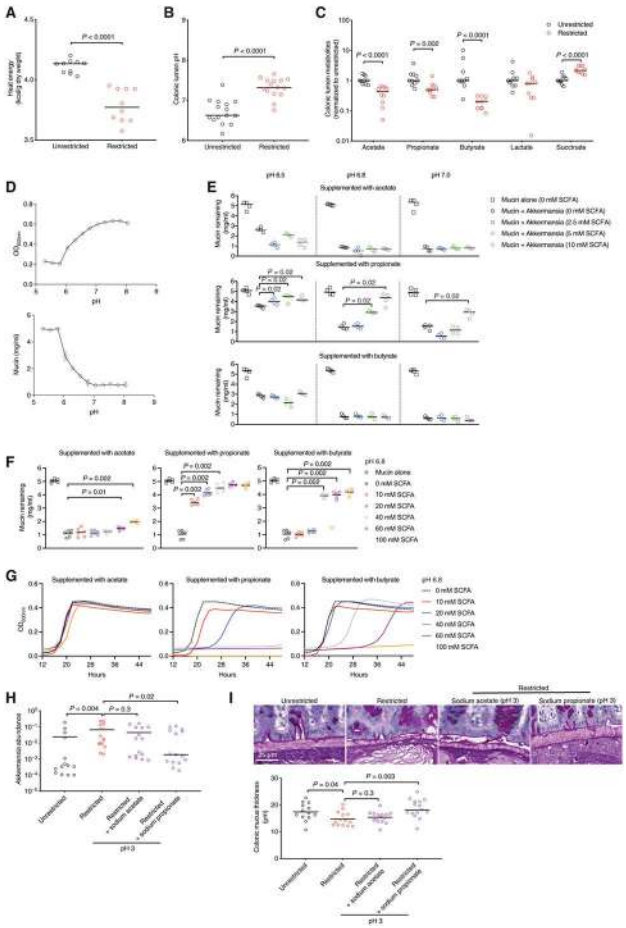

Figure 5. Bacterial metabolites link caloric restriction to mucolytic bacteria.

A) In mice that underwent one week of caloric restriction, cecal luminal contents were assessed for caloric content by bomb calorimetry; combined results of 2 experiments. B) Colonic luminal contents were assessed for pH in mice after one week of caloric restriction; combined results of 3 experiments. C) Metabolites from samples in B) were quantified using ion chromatography-mass spectrometry (IC-MS); combined results of 2 experiments. D) Murine *A. muciniphila* (MDA-JAX AM001) was cultivated under anaerobic conditions of varying pH in 4 replicates, and growth and mucin glycan consumption were quantified after 48 hours of culture; results of one of two experiments with similar results. $p < 0.0001$, growth of *A. muciniphila* at pH 5.0 vs. pH 6.75; $p = 0.03$, mucin degradation at pH 5.0 vs. pH 6.75. E) Murine *A. muciniphila* (MDA-JAX AM001) was cultivated under varying pH and varying concentrations of sodium acetate, sodium propionate, and sodium butyrate in 4 replicates, and mucin glycan consumption was quantified after 48 hours of culture; results of one of two experiments with similar results. pH 6.8: $P = 0.03$, 0 mM vs. 5 & 10 mM propionate; P = NS, 0 mM vs. 2.5 mM propionate; P = NS, 0 mM vs. 2.5, 5, and 10 mM acetate and butyrate. F) Murine *A. muciniphila* (MDA-JAX AM001) was cultivated with varying concentrations of sodium acetate, sodium propionate, and sodium butyrate. Mucin glycan consumption was quantified after 24 hours of culture. Values are shown as averages; results of 3 experiments. $P = 0.002$, 0 mM vs. 10- and 20-mM propionate; P = NS, 0 mM vs. 10 and 20 mM acetate and butyrate. G) Murine *A. muciniphila* was cultivated with SCFAs

as in F). Growth was monitored continuously up to 48 hours. Values are shown as averages; results of one of two experiments with similar results; H) Normal mice received one week of caloric restriction, as well as supplementation with sodium acetate or sodium propionate in the drinking water, acidified to pH3. Relative abundances of *Akkermansia* was quantified by 16S rRNA gene sequencing; combined results of 3 experiments. I) Thickness of the dense inner colonic mucus layer was evaluated histologically in mice in F). Representative images are provided with combined results of 3 experiments.

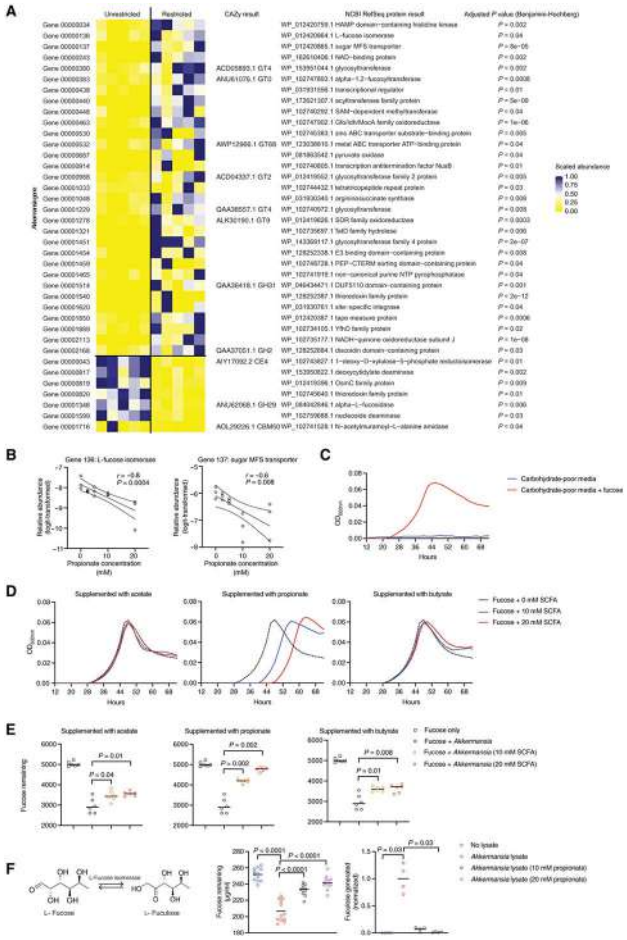

Figure 6.

Propionate suppresses L-fucose utilization by *A. muciniphilia*. A) Transcriptomic profiling identifies *A. muciniphila* (MDA-JAX AM001) genes similarly regulated by diet in vivo and propionate in vitro. RNA sequencing was performed on murine *A. muciniphila* cultivated at pH 6.8 with varying concentrations of sodium propionate (as in Figure 5E) in 3 replicates (left panel), and on fecal pellets from mice after one week of dietary restriction (n=5, right panel). Sequences aligning with the genome of murine *A. muciniphila* were quantified, and the scaled abundances of the subset of genes similarly regulated by diet and propionate are depicted in the heat map, along with annotations obtained using both the CAZy and NCBI RefSeq Protein databases. B) Relative abundance of fucose isomerase gene (A) and MFS transporter gene obtained by RNA sequencing performed on murine *A. muciniphila* cultivated at pH 6.8 with varying concentrations of sodium propionate. C) Murine *A. muciniphila* was cultivated in carbohydrate-poor BYEM10 media with and without L-fucose supplementation. Growth was monitored continuously up to 72 hours. D) Murine *A. muciniphila* was cultivated with fucose and varying concentrations of sodium acetate, sodium propionate, and sodium butyrate. Growth was monitored continuously up to 72 hours. Values are shown as averages; results from 3 experiments. E) Murine *A. muciniphila* was cultivated with fucose and varying concentrations of sodium acetate, sodium propionate, and sodium butyrate. Fucose remaining was quantified after 40 hours

of culture. Values are shown as averages; results of 3 experiments. F) Schematic diagram for the conversion of L-fucose to L-fuculose mediated by L-fucose isomerase (Left panel). (Center and right panel) In vitro enzymatic activity of L-fucose isomerase from murine *A. muciniphila* grown in mucin with or without propionate. L-fucose remaining and L-fuculose generated was quantified after a 1-hour incubation. Values are shown as averages; results of 3 experiments. L-fuculose concentrations were normalized to median L-fuculose concentration of the *Akkermansia* lysate group. Statistical significance for each graphical data set was determined by the Mann-Whitney U test.

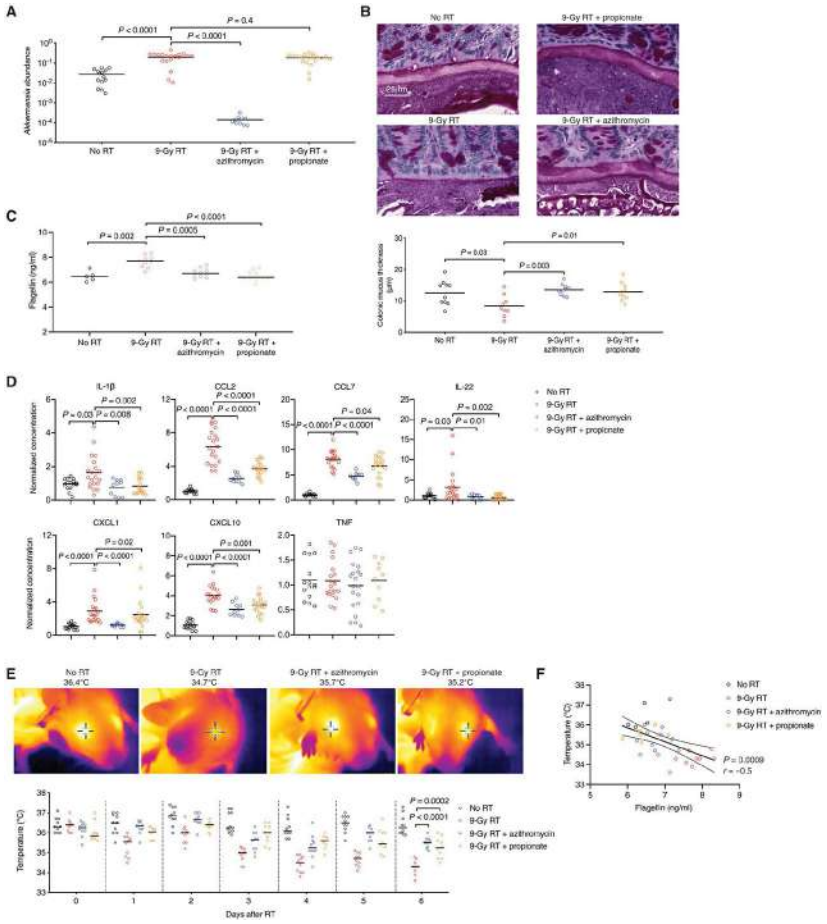

Figure 7. Strategies targeting mucolytic bacteria in mice receiving RT preserve colonic mucus, reduce hypothermia, and reduce colonic inflammation.

In the setting of 9 Gy RT, mice were treated with azithromycin or sodium propionate. A) Relative abundances of *Akkermansia* on day 6 after RT was quantified by 16S rRNA gene sequencing. Combined results of 3 experiments. Statistical significance was determined by the Mann-Whitney U test. B) Thickness of the dense inner colonic mucus layer was evaluated histologically. Representative images are provided with combined results of 2 experiments. Statistical significance was determined by the Mann-Whitney U test. C) Serum flagellin concentrations were quantified on day 6 after RT. Statistical significance was determined by the Mann-Whitney U test. D) On day 6 after RT, mice were harvested, and colonic tissues were processed to quantify concentrations of cytokines. Combined results of 3 experiments. Statistical significance was determined by the Mann-Whitney U test. E) Ocular temperatures were monitored daily. Representative images 6 days after RT are provided with combined results of 2 experiments. Statistical significance was determined by the Mann-Whitney U test. F) Quantification of the correlation between flagellin concentrations and ocular temperature on day 6 after RT by Pearson coefficient.

Table 1.

Patient characteristics

	Combined	No fever	Fever	p value
Total patients	n=119	n=56	n=63	
Median age (range)	58 years (23-80)	62 years (23-80)	56 years (23-75)	0.12
Gender				
Female	n=49 (41.2%)	n=22 (39.3%)	n=27 (42.9%)	0.69
Male	n=70 (58.8%)	n=34 (60.7%)	n=36 (57.1%)	
Graft type				
Autologous HCT	n=62 (52.1%)	n=22 (39.3%)	n=40 (63.5%)	0.008
Allogeneic HCT	n=57 (47.9%)	n=34 (60.7%)	n=23 (36.5%)	
Nentropenia depth				
WBC 100-500/∝L	n=34 (28.6%)	n=15 (26.8%)	n=19 (30.2%)	0.84
WBC <100/∝L	n=85 (71.4%)	n=41 (73.2%)	n=44 (60.8%)	
Disease				
Multiple myeloma/PCD	n=43 (36.1%)	n=16 (28.6%)	n=27 (42.9%)	0.11
Acute myeloid leukemia	n=23 (19.3%)	n=12 (21.4%)	n=11 (17.5%)	0.58
Non-Hodgkin lymphoma	n=20 (16.8%)	n=9 (16.1%)	n=11 (17.5%)	0.84
MDS/MPN/MF	n=12 (10.1%)	n=8 (14.3%)	n=4 (6.3%)	0.22
Acute lymphocytic leukemia	n=11 (9.2%)	n=7 (12.5%)	n=4 (6.3%)	0.34
Hodgkin lymphoma	n=4 (3.4%)	n=0 (0%)	n=4 (6.3%)	0.12
Olher	n=6 (5%)	n=4 (7.1%)	n=2 (3.2%)	0.42
Conditioning regimen				
Busulfan-based	n=33 (27.7%)	n=18 (32.1%)	n=15 (23.8%)	0.41
Melphalan-based	n=64 (53.8%)	n=32 (57.1%)	n=32 (50.8%)	0.58
Busulfan and melphalan-based	n=17 (14.3%)	n=2 (3.6%)	n=15 (23.8%)	0.0015
Other	n=5 (4.2%)	n=4 (7.1%)	n=1 (1.6%)	0.19
Conditioning intensity				
Myeloablative	n=101 (84.9%)	n=44 (78.6%)	n=57 (90.5%)	0.08
Nonmyeloablative	n=18 (15.1%)	n=12 (21.4%)	n=6 (9.5%)	

Abbreviations: HCT (hematopoietic cell transplantation), WBC (white blood cell), PCD (plasma cell disorder), MDS (myelodysplastic syndrome), MPN (myeloproliferative neoplasm), MF (myelofibrosis). “Other” includes blastic plasmacytoid dendritic cell neoplasm (n=2), chronic myelogenous leukemia (n=1), germ-cell tumor (n=1), systemic sclerosis (n=1), and T-cell-prolymphocytic leukemia (n=1).