Gut microbiome–derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease

Nathan D Mathewson1,2,7, Robert Jenq3,7, Anna V Mathew4,7, Mark Koenigsknecht5,7, Alan Hanash3,7, Tomomi Toubai4, Katherine Oravec-Wilson1, Shin-Rong Wu1,2, Yaping Sun1, Corinne Rossi1, Hideaki Fujiwara1, Jaeman Byun4, Yusuke Shono2, Caroline Lindemans3, Marco Calafiore3, Thomas M Schmidt5, Kenya Honda6, Vincent B Young5,7, Subramaniam Pennathur4,7, Marcel van den Brink3,7 & Pavan Reddy1

The effect of alterations in intestinal microbiota on microbial metabolites and on disease processes such as graft-versus-host disease (GVHD) is not known. Here we carried out an unbiased analysis to identify previously unidentified alterations in gastrointestinal microbiota–derived short-chain fatty acids (SCFAs) after allogeneic bone marrow transplant (allo-BMT). Alterations in the amount of only one SCFA, butyrate, were observed only in the intestinal tissue. The reduced butyrate in CD326+ intestinal epithelial cells (IECs) after allo-BMT resulted in decreased histone acetylation, which was restored after local administration of exogenous butyrate. Butyrate restoration improved IEC junctional integrity, decreased apoptosis and mitigated GVHD. Furthermore, alteration of the indigenous microbiota with 17 rationally selected strains of high butyrate–producing Clostridia also decreased GVHD. These data demonstrate a heretofore unrecognized role of microbial metabolites and suggest that local and specific alteration of microbial metabolites has direct salutary effects on GVHD target tissues and can mitigate disease severity.

Alterations in the intestinal microbiome are associated with several disease processes1–5. However, the effect that changes in the community structure of the microbiome have on the production of microbial-derived metabolites is poorly explored. Microbial metabolites influence disease severity, but whether alterations in microbial metabolites can affect outcomes after allogeneic bone marrow transplant (allo-BMT) is not known. Allo-BMT is a critical interventional therapy for patients with aggressive hematological malignancies6–7. Although allo-BMT is a curative and widely used treatment, approximately 40–50% of patients experience severe gastrointestinal damage from graft-versus-host disease (GVHD), which leads to high transplant-related mortality2,8. Studies have shown that the intestinal microbiota is considerably altered in patients with GVHD and that the alterations correlate with GVHD severity and pathogenesis4,9. Nevertheless, the direct causality between changes in the host microbiota and GVHD severity is unclear. Moreover, it remains unknown whether changes in the microbiota result in alterations in levels of microbial metabolites and by-products that have biological effects on allo-BMT recipients.

Microbial metabolites such as short-chain fatty acids (SCFAs) are exclusively derived from the gastrointestinal (GI) microbiota and are not made by the host. Some of these fatty acids (FAs), including the histone deacetylase inhibitor (HDACi) butyrate, are a preferred energy source for intestinal epithelial cells10–13 (IECs), and administration of exogenous HDACi regulates GVHD14–16. But the effect that host indigenous microbial metabolites that function as HDACIs have on GVHD remains unknown11–13.

Here we performed unbiased profiling of the microbial metabolome with a specific focus on targeted FAs after experimental allo-BMT. We found that the amount of only one SCFA, butyrate, was significantly decreased only in the intestinal tissue of allo-BMT recipients, resulting in decreased acetylation of histone H4 in IECs. Increasing the amount of intestinal butyrate restored acetylation of histone H4, protected IECs and decreased the severity of GVHD. Furthermore, rationally altering host GI microbiota to high-butyrate producers17 mitigated GVHD.

RESULTS
Targeted microbial-metabolite profiling
We hypothesized that alterations in the composition of the microbiota in the GI lumen would result in an altered microbial metabolome after the onset of GVHD18. We determined the concentration of microbial

1Division of Hematology/Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan, USA.
2Graduate Program in Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA.
3Adult Bone Marrow Transplantation Service, Memorial Sloan Kettering Cancer Center, New York, New York, USA.
4Division of Nephrology, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, Michigan, USA.
5Division of Infectious Disease, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, Michigan, USA.
6RIKEN Center for Integrative Medical Sciences, Yokohama, Japan. 7These authors contributed equally to this work. Correspondence should be addressed to P.R. (reddypr@med.umich.edu).

Received 1 November 2015; accepted 22 January 2016; published online 21 March 2016; corrected after print 26 August 2016; doi:10.1038/ni.3400

NATURE IMMUNOLOGY VOLUME 17 NUMBER 5 MAY 2016
FA metabolites, both SCFAs and long-chain FAs up to 18 carbons in length, from several sites in mice 7 d after bone marrow transplant (BMT) (day +7). We analyzed the serum, spleen, liver, intestines and luminal contents (stool) of the intestines using gas chromatography combined with mass spectrometry (GC-MS) (Supplementary Fig. 1a). We used a well-established, clinically relevant model of major histocompatibility complex (MHC)-mismatched BMT in which cells from C57BL/6J (H-2b) mice are transferred to lethally irradiated BALB/c (H-2d) mice and compared it to syngeneic transplant and naive animals. The animal cages were exchanged between groups on day +3 to account for any alterations in the microbial environment, and analysis after GC-MS was performed in a blinded manner. The FA concentrations in the luminal contents of the intestines were not significantly different between any of the groups (Fig. 1a and Supplementary Fig. 1b). We also found no significant differences in FA concentrations in the serum or in tissues such as the spleen and liver between allogeneic animals (Supplementary Fig. 1c and Supplementary Table 1) and syngeneic animals or naive controls. However, the greatest—and only statistically significant—difference was observed in the SCFA butyrate, which was significantly decreased in amount only in the intestinal tissue at day 7 (Fig. 1b and Supplementary Fig. 1d). We once again observed similar results on day 21 as on day 7 (Supplementary Fig. 2). Collectively these data demonstrate that butyrate levels are consistently decreased only in the intestinal tissue after allo-BMT.

**Functional impact of altered levels of SCFAs in IECs**

In light of the decreased amounts of butyrate in allogeneic animals only in the intestinal tissue, we next analyzed the potential functional impact of decreased butyrate in IECs. Because butyrate is an HDAC (H-2b) inhibitor, we examined the degree of histone acetylation by immunoblotting purified CD326+ IECs collected from mice after BMT. The degree of acetylation of histone H4 was significantly decreased on day 7 (P < 0.0025; Supplementary Fig. 3a) and day 21 (Fig. 2a) after allo-BMT, demonstrating that reductions in the amount of butyrate resulted in decreased histone acetylation. Therefore, to confirm that the decreased acetylation observed after allo-BMT (compared to syngeneic controls) was secondary to decreased HDAC inhibition resulting from decreased amounts of butyrate and not to potential alterations in HDAC and histone acetyltransferase (HAT) enzyme levels after transplant, we analyzed the expression of HDACs and HATs in IECs after BMT. We observed similar levels of several HDACs (Hdac1, -4, -7, -9 and -10) (Fig. 2b) and HATs (Ep300 and Kat5) (Fig. 2c) by qPCR in the IECs (CD326+)

**Decreased uptake of butyrate by IECs**

We next explored whether the diminished concentration of butyrate observed in the intestinal tissue was from impaired uptake of butyrate after allo-BMT. To this end, we examined the expression of the known butyrate monocarboxylate transporter SLC5A8 and of the butyrate receptor GPR43 in IECs after allo-BMT. Decreased gene (Fig. 2d) and protein (Fig. 2e) expression of SLC5A8 and GPR43 (Supplementary Fig. 3b,c) were observed in IECs from allogeneic animals compared with those from syngeneic animals after transplant on both day +21 and day +7 (Supplementary Fig. 3d), which suggested that the decreased butyrate concentration in the IECs was due to decreased uptake of microbially-derived luminal butyrate. We next cultured primary IECs with pro-inflammatory mediators (interferon-γ and/or tumor necrosis factor) and analyzed the expression of SLC5A8. Exposure of IECs to inflammatory cytokines significantly decreased the expression of SLC5A8 compared to that in vehicle-treated controls (Supplementary Fig. 3e). These data indicate that the intense inflammatory milieu after allo-BMT causes decreased expression of butyrate transporters and receptors, leading to decreased butyrate and histone acetylation in IECs.

**Rescuing the cellular effects of decreased butyrate**

We next determined whether the decreased amount of butyrate in IECs could be restored in vivo and, further, whether this would have a functional effect on histone acetylation. In addition to using transporters, butyrate can diffuse across the mucosal barrier into IECs when present in high concentrations. Therefore, we hypothesized that local administration of high amounts of butyrate would restore histone acetylation in IECs in vivo. To test this, we transferred cells from C57BL/6J mice into BALB/c mice and administered vehicle or butyrate via daily intragastric gavage. Daily butyrate administration

**Figure 1** Allo-BMT reduces amounts of intracellular butyrate in IECs. (a,b) Abundance of fatty acids (short and long chain) on day +7 in the intestinal luminal contents (stool) (a) and intestinal tissue (b) of recipients of syngeneic BMT (BALB/c→BALB/c), allo-BMT (C57BL/6J→BALB/c) or no BMT (naive (N)). Graphical results for all animals combined are shown below the heat maps for SCFAs butyrate, propionate and acetate. Representative heat maps are shown for n = 10 mice in the naive and syngeneic groups and n = 9 mice in the allogeneic group. *P < 0.05, **P < 0.01, analysis of variance (ANOVA). Bars and error bars represent mean and s.e.m., respectively.
for 21 d significantly restored acetylation of histone H4 compared with no treatment in allo-BMT recipients (Fig. 3a).

We next determined whether there was a difference in the uptake and metabolism of exogenously administered butyrate between recipients of syngeneic versus allogeneic BMT. To this end we performed metabolic flux analysis to assess the incorporation of heavy $^{13}$C-labeled butyrate into luminal and intestinal tissue butyrate pools by using GC-MS as described above. We treated recipients of syngeneic or allogeneic transplant $7 d$ after BMT with a bolus of either $^{13}$C-butyrate or $^{12}$C-butyrate, which served as the control. We collected IECs and luminal contents from the mice $6 h$ later and analyzed them for incorporation of $^{13}$C. Although we saw similar amounts of heavy $^{13}$C-labeled butyrate in the lumen of the large intestine, we saw significantly less $^{13}$C-labeled butyrate in the intestinal tissue of recipients of allo-BMT, suggesting decreased uptake (Fig. 3b).

To determine whether there were any differences in butyrate metabolism, we further examined the presence of $\text{Slc5a8}$ (monocarboxylate transporter of butyrate) in IECs from recipients of syngeneic or allogeneic BMT (Fig. 3c). There was a significant difference between syngeneic and allogeneic recipients compared with syngeneic BMT and allo-BMT recipients in allo-BMT recipients of allo-BMT, suggesting decreased uptake (Fig. 3d).

There was significantly less $^{13}$C-labeled butyrate in the intestinal tissue of recipients of allo-BMT, suggesting decreased uptake (Fig. 3e). To determine whether there were any differences in butyrate metabolism, we further examined the presence of $\text{Slc5a8}$ (monocarboxylate transporter of butyrate) in IECs from recipients of syngeneic or allogeneic BMT (Fig. 3f). There was a significant difference between syngeneic and allogeneic recipients compared with syngeneic BMT and allo-BMT recipients in allo-BMT recipients (Fig. 3g).

Furthermore, daily intragastric gavage of butyrate resulted in increased amounts of the butyrate transporter $\text{Slc5a8}$ compared with those in untreated animals (Fig. 3h), suggesting that butyrate has a positive feedback mechanism that results in an increase in the amount of its transporter. To determine whether butyrate was directly responsible for induction of its own transporter, we analyzed the degree of histone acetylation at the promoter of $\text{Slc5a8}$ with chromatin immunoprecipitation (ChIP). We found an increased association of acetylated histone H4 in the promoter region of $\text{Slc5a8}$ in IECs (CD326$^+$) treated with butyrate compared with vehicle-treated cells (Fig. 3i). These data demonstrate that the decreased amount of butyrate after allo-BMT has functional effects on IECs.

**Increased amounts of intestinal butyrate mitigated GVHD**

Systemic administration of HDACi decreases the severity of acute GVHD$^{14,15,23}$. We therefore determined whether increasing local amounts of the endogenous HDACi butyrate would affect GVHD severity. Again using the C57BL/6J-into-BALB/c model, we administered vehicle control or butyrate via intragastric gavage daily for 1 week and then every other day for the remainder of the experiment.

Administration of butyrate resulted in decreased weight loss (Fig. 3j), decreased GVHD clinical scores (Fig. 3k) and increased survival (Fig. 3l) compared with vehicle treatment. We observed similarly improved survival using a second clinical model of BMT, an MHC-matched, minor antigen–mismatched model. We transferred C3HSLW (H-2$^b$) cells into C57BL/6J (H-2$^b$) mice, thus demonstrating strain-independent results (Supplementary Fig. 4a). Furthermore, histopathological analysis 21 d after BMT showed decreased histological scores in the intestines of the major MHC–mismatch model (Fig. 3i) and decreased histological scores in both intestines and liver in the model of minor antigen mismatch (Supplementary Fig. 4b).

To determine whether irradiation-related inflammation was critical for butyrate-induced protection, we next examined the butyrate-induced protective effects in a non-irradiated model of BMT from parent (C57BL/6J, $H-2^b$) into F1 (B6D2F1, $H-2^{b1}$)$^9$. We once again observed significantly less weight loss ($P < 0.011$; Supplementary Fig. 4c) and improved survival (Supplementary Fig. 4d) in allogeneic recipients that were treated with intragastric butyrate compared with vehicle-treated controls. Next, to further evaluate the magnitude of the butyrate-induced protective effect, we determined GVHD mortality, once again using the MHC-disparate B6-into-BALB/c model. Intragastric gavage of butyrate induced significant GVHD survival benefit in allogeneic animals that received higher doses of T cells ($P < 0.0001$; Supplementary Fig. 4e). These data collectively show that butyrate induced GVHD protective effects regardless of strain combinations, conditioning or higher alloreactive T cell doses.
Increased amounts of intracellular butyrate protected GI epithelium

We next determined whether the decreased severity of GI GVHD resulted in decreased translocation of luminal contents and improved epithelial integrity\(^{24–26}\). We used transmission electron microscopy to examine the ability of butyrate to preserve cellular junctions after allo-BMT. We found intense leakage of the electron-dense stain ruthenium red in the intestinal epithelial cells of BALB/c allo-BMT recipients (Fig. 3f). However, the integrity of the IEC junction was preserved at both day 7 and day 21 after allo-BMT (Fig. 3a). We further assessed the intestinal permeability after allo-BMT to determine whether butyrate had an effect on local \( T_{reg} \) cells. The total numbers of CD45.1\(^+\) cells recovered from the intestinal lamina propria were not different between vehicle- and butyrate-treated allo-BMT recipients (Supplementary Fig. 4g). In contrast, intestinal infiltration of donor CD4\(^+\) and CD8\(^+\) T cells and activated T cells (CD69\(^+\) or CD44\(^hi\)) was decreased in animals that received local intra-gastric butyrate administration (Fig. 4a). However, the ratio of donor \( T_{reg} \) cells to effector T cells was not different in the intestines of those animals (Fig. 4b). Microbiota-derived butyrate has been shown to increase the number of immune-regulatory macrophages in the GI tract, which increases the number of \( T_{reg} \) cells\(^{29}\). However, the total number of donor macrophages in the intestine of allogeneic recipients did not differ between vehicle- and butyrate-treated mice in our experiment (Fig. 4c).

To further determine whether the salutary effects of local treatment with butyrate on GI GVHD were dependent on donor \( T_{reg} \) cells, we next performed a BMT using the same MHC-mismatched BMT model in which cells from C57BL/6 (H-2\(^b\)) mice were transferred to BALB/c (H-2\(^d\)) mice. We transferred T cell–depleted (TCD) bone marrow and purified CD4\(^+\)CD25\(^+\) T cells from donors and administered vehicle or

**Figure 3** Intra-gastric gavage with butyrate enhances uptake of the SCFA and histone acetylation in IECs. (a) Representative immunoblots of CD326\(^+\) IECs from syngeneic (BALB/c→BALB/c) and allogeneic (C57BL/6→BALB/c) BMT recipients that received vehicle or butyrate (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows amounts of acetylated histone H4 (acetyl-H4) 21 d after BMT; data are representative of three experiments combined. (b) Incorporation of \(^{13}\)C-labeled butyrate in luminal contents and intestinal tissue of the large intestine in mice gavaged with \(^{13}\)C-butyrate or (non-labeled) \(^{12}\)C-butyrate; n = 6 mice per group. ND, not detected. (c) \(^{13}\)C label incorporation in tricarboxylic acid metabolite pools from the large intestines of mice fed a bolus (2 g kg\(^{-1}\)) of \(^{13}\)C-butyrate or \(^{12}\)C-butyrate; n = 6 mice per group. In (c), symbols represent individual data points, and horizontal bars represent means. (d) Transmission electron microscopy images of intestines isolated from BMT recipients with or without intragastric gavage of butyrate. (e) Representative histopathology 21 d after BMT for recipients with or without intragastric gavage of butyrate. The intestinal mucosa and lamina propria were stained with ruthenium red (0.1%; arrows indicate cell-cell interfaces). (f) FITC-dextran translocation across the GI barrier into blood serum in butyrate-treated allo-BMT recipients compared to vehicle-treated all-BMT recipients 21 d after BMT. B6, C57BL/6J. \(* P < 0.05; ** P < 0.01; *** P < 0.0001, ANOVA (a–d,i,k). Student’s t-test (e–g) or Mantel-Cox log-rank test (h). Bars and error bars represent the mean and s.e.m., respectively.

**Figure 4** Butyrate in the gastrointestinal (GI) tract increases the number of regulatory T cells (\( T_{reg} \)) in the GI tract. (a) Flow cytometric analysis of purified CD4\(^+\) T cells 21 d after BMT from allogeneic (C57BL/6J→BALB/c) BMT recipients that received vehicle or butyrate (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (b) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (c) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (d) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (e) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (f) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (g) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (h) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (i) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (j) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population. (k) Flow cytometric analysis of purified CD4\(^+\) T cells in the intestines of mice gavaged with butyrate or vehicle (10 mg kg\(^{-1}\); “Butyrate tx allo”). The graph shows the percentage of \( T_{reg} \) cells in the total CD4\(^+\) T cell population.
Butyrate protects IECs from alloreactive T cell–mediated damage

We next sought to explore the potential mechanisms that contribute to butyrate-induced protection from severe GVHD. Because (a) butyrate was present in decreased amounts in IECs and (b) administration of butyrate mitigated GI GVHD independently of Treg cells but (c) improved junction integrity, we explored whether butyrate had direct effects in the protection of IECs from alloreactive T cell–mediated damage and conditioning. We treated IECs ex vivo with vehicle or butyrate for 24 h and subjected the cells to either 6-Gy irradiation or no irradiation, followed by 24 h of additional incubation with butyrate. We observed that butyrate was not toxic to IECs and conferred protection from irradiation-induced apoptosis (Fig. 5a).

Next we determined the ability of butyrate-treated IECs to withstand damage mediated by alloreactive T cells. We isolated and cultured primary IECs with butyrate or vehicle control overnight. The pretreated IECs were next cocultured with primed alloregenic CD8+ T cells in the absence of butyrate. Fewer butyrate-pretreated IECs succumbed to CD8+ T cell killing within 6 h and 16 h after coculture compared with controls (Fig. 5b).

Because butyrate is a primary energy source for IECs11–13, we next determined whether butyrate enhances the survival and growth of IECs in vitro. To this end, we cultured intestinal organoids in the presence or absence of butyrate. We observed that culture in the presence of butyrate significantly increased organoid size compared with untreated controls (Fig. 5c). Next we confirmed the effect of butyrate on IEC junctional function in the organoid cultures by determining the mRNA expression of Claudins. We observed that culture of organoids with butyrate significantly increased the mRNA expression of Claudins (Clun1, Clun5, Clun6, Clun10, Clun11, Clun13, Clun14, Clun17 and Clun18) (P < 0.0113; Supplementary Fig. 5a).

Molecular mechanisms of IEC protection

We hypothesized that butyrate would increase the expression of antiapoptotic genes via modulation of histone acetylation. We analyzed pro- and anti-apoptotic mRNA expression levels12 and found that expression of Bak1 and Bax was significantly decreased (P < 0.0012; Supplementary Fig. 5b), whereas amounts of transcripts of the antiapoptotic protein BCL-B (Bcl2l10) were significantly increased (Fig. 5d), in butyrate-treated IECs versus untreated controls. We next examined...
mRNA expression of junctional proteins such as occludin (Ocln) (P < 0.04; Supplementary Fig. 5c) and JAM (F11r) (Fig. 5e) in IECs after butyrate treatment, which significantly increased their expression. We also used ChIP to determine whether the restored acetylation of histone H4 observed in butyrate-treated allo-BMT recipients was responsible for increased BCL-B (Bcl2l10) and F11r expression. We found that acetylation of histone H4 was indeed associated with the promoter regions of Bcl2l10 (Fig. 5f) and F11r expression.

Data thus collectively suggest that butyrate has several salutary effects on IECs that may or may not be mutually exclusive, such as regulating the expression of genes involved in reducing IEC apoptosis and increasing expression of junctional proteins in IECs. To determine whether these factors are involved in an in vivo protection from GVHD, we determined the expression of pro- and anti-apoptotic proteins as well as junctional proteins in IECs isolated 21 d after allo-BMT. Amounts of pro-apoptotic transcripts of Bak1 and Bax were significantly decreased in allo-BMT recipients that received intragastric butyrate treatment (Fig. 5h), whereas expression of the anti-apoptotic BCL-B (Bcl2l10) was increased (Fig. 5i). Further, expression of junctional proteins was similarly increased in butyrate-treated animals (Fig. 5j). To determine whether these results have biological consequences for protein expression, we also examined the amounts of occludin, JAM and claudin 5. Indeed, immunoblot analysis showed increased amounts of junctional proteins in recipients of allo-BMT treated with intragastric butyrate compared with their untreated counterparts (Fig. 5k).

High butyrate–producing microbiota mitigate GVHD

The endogenous HDACi butyrate is a by-product of microbial fermentation. Therefore, we next tested the hypothesis that altering the composition of indigenous GI microbiota in hosts that can produce high levels of butyrate will mitigate GVHD. We used 17 rationally selected strains of Clostridia that have been shown to increase amounts of butyrate both in vitro and in vivo. We administered these strains via intragastric gavage every other day to naïve mice starting 14 d before allo-BMT and continuing for 21 d after BMT. We characterized the microbiota in feces collected from animals that received vehicle and 17-strain administration by 16S rRNA-encoding gene sequencing. For animals that received the 17 Clostridial strains, 16S analysis showed an important biologically significant shift in the microbiota, indicating that the introduced organisms could be detected (Fig. 6a and Supplementary Fig. 6a). Furthermore, GC-MS analysis 21 d after allo-BMT showed a significant increase in the amount of butyrate in the luminal contents (Fig. 6b) and in the intestinal tissues (Fig. 6c) of animals that received intragastric...
altering the indigenous microbiota with 17 rationally selected strains

cocktail of 17 Clostridial strains (\cite{17}), significantly increased survival in the animals treated with the

These data demonstrate that antibiotic treatment eliminated benefi
ciations of mice, we also determined whether mice housed at another
stitution (Memorial Sloan Kettering Cancer Center) and treated

Additionally, because clinical BMT patients are often treated with
antibiotics, and as an alternative approach to colonizing the indig

obligate anaerobes. The mice were then colonized 4 and 6 d later

by intragastric gavage. Once again, we

with either human
Clostridia
compared with recipients of
E. faecium
by intragastric gavage. Once again, we

recolonized with bacteria died significantly faster (\cite{17})

for survival. Animals that were treated with antibiotics but were not
recolonized with bacteria died significantly faster (P < 0.0001) than
mice not treated with the antibiotic mixture (\textit{Supplementary Fig. 6b}).
These data demonstrate that antibiotic treatment eliminated benefi
microbiota, similar to a previous report \cite{5}. We once again observed
significantly increased survival in the animals treated with the
cocktail of 17 Clostridial strains (\textit{Fig. 6g}). These results suggest that
altering the indigenous microbiota with 17 rationally selected strains

of Clostridia known to produce high amounts of butyrate \cite{17, 34} can
decrease the severity of GVHD and improve survival across multiple
institutions with strain-independent results.

\section*{DISCUSSION}

The community structure of the microbiota is altered after allo-
BMT \cite{4, 5}. Our results now provide a novel perspective on microbial
metabolites and their effect on GVHD. Our study showed that
butyrate was the only SCFA to decrease significantly in amount after
allo-BMT, and that the decrease occurred in the intestinal tissue.
Decreased amounts of butyrate in the IECs of allo-BMT recipients led
to decreased acetylation of histones, whereas increasing the amount
of butyrate via intragastric gavage restored histone H4 acetylation
and GVHD. An important observation was the lack of changes in
amounts of luminal (stool) butyrate, despite a documented shift in the
microbiome species that produce low amounts of butyrate after
allo-BMT \cite{3}. We posit that this may be because of decreased butyrate
uptake into IECs due to decreased amounts of butyrate transporter.
In such a case the overall amount of butyrate would not be signifi-
cantly decreased in the lumen despite decreased production, because
less would be taken into the IECs owing to the shift in the microbiota
after allo-BMT.

The reasons for decreased amounts of transporter proteins after
allo-BMT are intriguing. Previous reports showed a decrease in the
amount of SLC5A8 after alterations in the microbiota \cite{38}. Thus, our
findings that amounts of SLC5A8 and GPR43 are decreased in IECs
after allo-BMT are consistent with previous reports \cite{4, 5}. Furthermore,
we demonstrate that exposure of IECs to inflammatory cytokines
leads to decreased expression of butyrate transporters. These data
suggest that the inflammatory milieu early after allo-BMT decreases
expression of the butyrate transporter SLC5A8, leading to decreased amounts of SLC5A8 in IECs and further decreasing transporter expression and butyrate intake in a feedback mechanism.

Administered butyrate was more rapidly metabolized in allo-BMT recipients than in syngeneic BMT recipients, as shown by the greater incorporation of carbon from butyrate into the TCA cycle. These data point to a novel observation on the role of energy requirements of IECs in the context of inflammation and GVHD. Our data collectively provide new insights into the role and interactions of the microbiome-derived metabolite butyrate after allo-BMT.

Previous studies have shown that 17 rationally selected strains of Clostridia that produce high amounts of butyrate increase numbers of \( T_{reg} \) cells in the intestine\(^{17} \). Although we did not observe an increase in the number of \( T_{reg} \) cells in the intestine or dependence on donor \( T_{reg} \) cells for reduction of GVHD severity after butyrate treatment, we cannot formally rule out the contribution of butyrate-mediated effects on \( T_{reg} \) cells (directly or indirectly via macrophages) to decreased GVHD severity when the cells are present in the donor inoculum. Furthermore, it is possible that the beneficial effect of the administration of the 17 Clostridial strains was secondary to factors other than butyrate. Nonetheless, when considered in light of the data demonstrating GVHD protection by direct intrastraginal administration of exogenous butyrate, the results suggest that butyrate is sufficient for GVHD protection.

Our data suggest that butyrate has direct salutary effects on IECs. Butyrate altered the ratio of the expression of anti-apoptotic to pro-apoptotic molecules and increased the expression of proteins relevant for junctional integrity. Nonetheless, the direct physiological relevance of only one of these mechanisms, apoptosis or junctional integrity, cannot be ascertained from our data. Furthermore, our data do not directly address the effects of butyrate on the various other cells that make up the GI epithelium. Clearly, butyrate and HDACis do not directly address the effects of butyrate on the various other IECs in the context of inflammation and GVHD. Our data collectively provide new insights into the role and interactions of the microbiome-derived metabolite butyrate after allo-BMT.

The relevance of only one of these mechanisms, apoptosis or junctional integrity, cannot be ascertained from our data. Furthermore, our data do not directly address the effects of butyrate on the various other cells that make up the GI epithelium. Clearly, butyrate and HDACis do not directly address the effects of butyrate on the various other IECs in the context of inflammation and GVHD. Our data collectively provide new insights into the role and interactions of the microbiome-derived metabolite butyrate after allo-BMT.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Metabolomic data, mass spectral analytical parameters and spectral raw data from the study, and metadata have been deposited in the NIH Common Fund’s Data Repository and Coordinating Center under Metabolomics Workbench Project ID PR0000270.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

This work was supported by the US National Institutes of Health (NIH) (National Heart, Lung and Blood Institute, grants HL-090775 and HL-128046 (to P.R.); National Cancer Institute, grant CA-173878 (to P.R.)); Metabolomic studies were performed through the Molecular Phenotyping Core, Michigan Nutrition and Obesity Center (grant DK089503 (to S.P.)) and Michigan Regional Metabolomics Resource Core (grant DK097153 (to S.P.)). We acknowledge use of the Microscopy & Image-analysis Laboratory (MIL) of the University of Michigan’s Biomedical Research Core Facilities for preparation of samples and images. Support for the MIL core is provided by the University of Michigan Cancer Center (NIH grant CA46592) and the University of Michigan Gut Peptide Research Center (NIH grant DK34933).

AUTHOR CONTRIBUTIONS

N.D.M. designed experiments, performed experiments, analyzed data and wrote the paper. A.V.M. and M.K. performed experiments and analyzed data. R.J. and A.H. designed and performed experiments. T.T., K.O.-W., S.-R.W., Y.S., C.R., H.F., J.B., Y.S., C.L. and M.C. performed experiments. T.M.S., V.B.Y. and M.v.d.B. wrote the paper. K.H. provided reagents and wrote the paper. S.P. analyzed data and wrote the paper. P.R. designed experiments, analyzed data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
28. Arpaia, N. et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* **504**, 451–455 (2013).
29. Chang, P.V., Hao, L., Offermanns, S. & Medzhitov, R. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. USA* **111**, 2247–2252 (2014).
30. Lahl, K. et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* **204**, 57–63 (2007).
31. Lahl, K. & Sparwasser, T. In vivo depletion of Foxp3+ Tregs using the DEREG mouse model. *Methods Mol. Biol.* **707**, 157–172 (2011).
32. Topham, C.H. & Taylor, S.S. Mitosis and apoptosis: how is the balance set? *Curr. Opin. Cell Biol.* **25**, 780–785 (2013).
33. Wong, J.M.W., de Souza, R., Kendall, C.W.C., Emam, A. & Jenkins, D.J.A. Colonic health: fermentation and short chain fatty acids. *J. Clin. Gastroenterol.* **40**, 235–243 (2006).
34. Narushima, S. et al. Characterization of the 17 strains of regulatory T cell-inducing human-derived Clostridia. *Gut Microbes* **5**, 333–339 (2014).
35. Schloss, P.D. et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75**, 7537–7541 (2009).
36. Schloss, P.D., Gevers, D. & Westcott, S.L. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *PLoS ONE* **6**, e27310 (2011).
37. DeSantis, T.Z. et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **72**, 5069–5072 (2006).
38. Cresci, G.A., Thangaraju, M., Mellinger, J.D., Liu, K. & Ganapathy, V. Colonic gene expression in conventional and germ-free mice with a focus on the butyrate receptor GPR109A and the butyrate transporter SLC5A8. *J. Gastrointest. Surg.* **14**, 449–461 (2010).
39. Takashima, S. et al. The Wnt agonist R-spondin1 regulates systemic graft-versus-host disease by protecting intestinal stem cells. *J. Exp. Med.* **208**, 285–294 (2011).
40. Iyengar, S., Zhan, C., Lu, J., Korngold, R. & Schwartz, D.H. Treatment with a rho kinase inhibitor improves survival from graft-versus-host disease in mice after MHC-haploidentical hematopoietic cell transplantation. *Biol. Blood Marrow Transplant.* **20**, 1104–1111 (2014).
Mice were intragastrically gavaged with a bolus (2 g/kg) of either labeled $^{13}$C$_2$-butyrate or nonlabeled $^{12}$C$_2$-butyrate. The small and large intestines were collected 6 h later and prepared for analysis as described above. The incorporation of $^{13}$C$_2$-labeled butyrate (sodium butyrate-$1,2$-C$_2$; Sigma) into the butyrate pools in the lumen and the intestinal tissue was measured by liquid chromatography–mass spectrometry performed on an Agilent 6520 high-resolution Q-TOF (quadrupole–time of flight) instrument coupled with an Agilent 1200 HPLC system (Agilent Technologies, New Castle, DE), equipped with an electrospray source. The extract was subjected to hydrophilic interaction chromatography with a Phenomenex Luna NH$_2$ column (particle size, 3 µm; i.d. 150 mm) at a flow rate of 0.07 µL/min. Solvent A was 5 mM ammonium acetate, pH 9.9, and solvent B was acetonitrile. The column was equilibrated with 80% solvent B. The gradient was as follows: 20–100% solvent A over 15 min; 100% solvent A over 5 min; 20% solvent B for 0.1 min; and 20% solvent A for 15.9 min. Liquid chromatography–electrospray ionization mass spectrometry in the negative mode was done on a Q-TOF instrument with the following parameters: spray voltage, 3,000 V; drying gas flow, 10 l/min; drying gas temperature, 350 °C; and nebulizer pressure, 20 psi. The fragmentor voltage was 150 V in full scan mode. We scanned a mass range of m/z 100–1,500 to obtain full scan mass spectra. We used two reference masses at m/z 121.050873 and m/z 922.009798 to obtain accurate mass measurements within 5 ppm. We extracted and deconvoluted all chromatograms and corresponding spectra of TCA metabolites (citrate, succinate and malate) and their $^{13}$C$_2$-labeled counterparts using the MassHunter software (Agilent Technologies, New Castle, DE). We rechecked the retention time consistency manually and compared it to that of authentic compounds that were injected under similar chromatographic conditions. For tissue extracts, we normalized the metabolite concentrations to the protein content, which was determined by the Bradford-Lowry method. For the flux analyses, peak areas of the labeled compounds were normalized to the natural abundance of the label and represented as ratios to the total compound peak area.

**16S deep sequencing.** We collected fecal pellets from mice on specified days (shown in figures) and stored them at −80°C for 3 d prior to analysis. DNA was extracted and purified with phenol-chloroform after bead-based lysis. We carried out sequencing and analysis as described$^{45}$. We sequenced the V4 region of the 16S rRNA gene using Illumina MiSeq technology. We trimmed and analyzed sequencers using mothur$^{45}$. We added the 16S rRNA gene sequence from each strain in the 17-strain cocktail to the version 9 trainset sequences from the Ribosomal Database Project$^{46}$. We classified the resulting sequences by comparing them to the described trainset with the requirement that the confidence score be 100%.

We analyzed Memorial Sloan Kettering Cancer Center 16S experiments using the Illumina MiSeq platform to sequence the V4–V5 region of the 16S rRNA gene. Sequence data were compiled and processed with mothur version 1.34 (ref. 35), screened and filtered for quality$^{36}$, and then classified to the species level$^{47}$ according to the Greengenes reference database$^{47}$.

**17-strain mixture.** The 17-strain mix was prepared as described$^{17}$. All strains were grown in 5 ml of EG media for 24 h at 37°C under anaerobic conditions. Each strain was grown to confluence, with the exception of strains 3, 8, 13, 26 and 29. Cells were scraped from EG agar plates and added to the 5-ml culture to obtain the same approximate optical density as the other strains.
Bars and error bars represent the mean and s.e.m., respectively.

Experiments were repeated at least two times with three biological replicates. Immunoprecipitation experiments were performed using the EZ-Magna ChIP kit from EMD Millipore (Billerica, MA) according to the manufacturer’s instructions. We cross-linked the cells with 1% formaldehyde and sonicated extracted chromatin using a sonicator (Bioruptor Pico (Diagenode, Denville, NJ)) to achieve an average fragment size of 200–1,000 bp. We immunoprecipitated the sonicated lysates using ChIP-grade specific antibodies purchased from EMD Millipore for acetylated histone-H4 and RNA polymerase II or IgG control antibody. Next we examined de–cross-linked DNA by qPCR using primers targeting the promoter regions of the target genes Bcl2l10 (forward, CCAAGGGAGTTACCAGAG; reverse, ACCCTTCTGAGTCCCTGAGA), Slc5a8 (forward, CACGACACAG CCTCTCTTGT; reverse, TACAGTGAATCCAGTGTCGT) and F11r (forward, TCCGGGATTAAAGCAGTG; reverse, ACAGGGGACACGAGATTGGG). We verified the immunoprecipitation efficiency of all samples by qPCR analysis of the promoter region of Gapdh (forward, CTGCGAGTACTGTGGGGAG; reverse, CAAAAGCAGATTTACAG). Data analysis was determined as a percentage of input using the following equations: ChIP = (GΔCtCHIP − GΔCtinput − log(6.644)) and % input = 2^(-ΔΔCt(normalized CHIP)).

Flow cytometry. To analyze immunophenotype surface markers, we collected lymphocytes from the IEC fraction or spleen, stained them using the recommended dilutions indicated on the manufacturer product sheets, and gated them on anti-CD4 conjugated with PerCP/Cy5.5 (clone GK1.5) or anti-CD8 conjugated with allophycocyanin (clone 53-6.7) and configurations of the following per mouse in duplicate: anti-CD69-phycocerythrin (clone H1.2F3), anti-CD25-phycocerythrin (clone ME-14), anti-CD25-phycocerythrin (clone 3C7), anti-CD44-PerCP/Cy5.5 (clone IM7), anti-CD44-allophycocyanin (clone IM7) and anti-Foxp3-allophycocyanin (clone FJK-16s). Antibodies to CD4, CD8, CD69, CD62L, CD25, CD44 and CD326 were purchased from BioLegend. Stained cells were then analyzed with an Accuri C6 flow cytometer (BD Biosciences).

IECs were stained with CD326-allophycocyanin (clone G8.8) and DAPI and sorted to >98% purity with a FACSAria III (BD Biosciences) gating on live cells.

All antibodies used have been validated for this species and application as shown in the respective 1DegreeBio validation profile. Antibodies to CD4, CD8, CD69, CD62L, CD25, CD44 and CD326 were purchased from BioLegend, anti-Foxp3 was from ebioscience, and DAPI was from Life Technologies.

Cytotoxic T lymphocyte assay. We isolated CD8+ T cells from BALB/c (H-2d) mice using anti-CD8 microbeads and LS columns (Miltenyi Biotec) according to the manufacturer’s instructions. We primed CD8+ T cells in the presence of irradiated (30 Gy) splenocytes from C57BL/6J (H-2b) mice for 6 days before culturing them with primary IECs for 6 h and 16 h. We collected primary IECs from C57BL/6J mice and incubated them overnight in the presence or absence of 1 mM butyrate in gelatin-coated (Cell Biologics, Chicago, IL) 100-mm culture dishes (Fisher Scientific).

Reproducibility. Experiments were repeated at least two times with three sample replicates, bringing the n to at least 6; sample sizes are indicated in figure legends. For analysis of one variable in one BMT experiment, at least two groups of three recipients were required.

Statistics. Bars and error bars represent the mean and s.e.m., respectively. We performed non-survival analysis using Student’s unpaired t-test between two groups. We used ANOVA for comparisons with more than two groups. We performed survival data analysis using a Mantel-Cox log-rank test.
Corrigendum: Gut microbiome–derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease

Nathan D Mathewson, Robert Jenq, Anna V Mathew, Mark Koenigsknecht, Alan Hanash, Tomomi Toubai, Katherine Oravec-Wilson, Shin-Rong Wu, Yaping Sun, Corinne Rossi, Hideaki Fujiwara, Jaeman Byun, Yusuke Shono, Caroline Lindemans, Marco Calafiore, Thomas C Schmidt, Kenya Honda, Vincent B Young, Subramaniam Pennathur, Marcel van den Brink & Pavan Reddy

*Nat. Immunol.* 17, 505–513 (2016); published online 21 March 2016; corrected after print 26 August 2016

In the version of this article initially published, the middle initial (C) for the sixteenth author was incorrect. The correct author name should be Thomas M. Schmidt (and the corresponding initials in the Author Contributions section should be T.M.S.). The error has been corrected in the HTML and PDF versions of the article.