Butyrate Reprograms Expression of Specific Interferon-Stimulated Genes

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ABSTRACT Butyrate is an abundant metabolite produced by gut microbiota. While butyrate is a known histone deacetylase inhibitor that activates expression of many genes involved in immune system pathways, its effects on virus infections and on the antiviral type I interferon (IFN) response have not been adequately investigated. We found that butyrate increases cellular infection with viruses relevant to human and animal health, including influenza virus, reovirus, HIV-1, human metapneumovirus, and vesicular stomatitis virus. Mechanistically, butyrate suppresses levels of specific antiviral IFN-stimulated gene (ISG) products, such as RIG-I and IFITM3, in human and mouse cells without inhibiting IFN-induced phosphorylation or nuclear translocation of the STAT1 and STAT2 transcription factors. Accordingly, we discovered that although butyrate globally increases baseline expression of more than 800 cellular genes, it strongly represses IFN-induced expression of 60% of ISGs and upregulates 3% of ISGs. Our findings reveal that there are differences in the IFN responsiveness of major subsets of ISGs depending on the presence of butyrate in the cell environment, and overall, they identify a new mechanism by which butyrate influences virus infection of cells.

IMPORTANCE Butyrate is a lipid produced by intestinal bacteria. Here, we newly show that butyrate reprograms the innate antiviral immune response mediated by type I interferons (IFNs). Many of the antiviral genes induced by type I IFNs are repressed in the presence of butyrate, resulting in increased virus infection and replication. Our research demonstrates that metabolites produced by the gut microbiome, such as butyrate, can have complex effects on cellular physiology, including dampening of an inflammatory innate immune pathway resulting in a proviral cellular environment. Our work further suggests that butyrate could be broadly used as a tool to increase growth of virus stocks for research and for the generation of vaccines.

KEYWORDS ISG, interferon

Butyrate, a 4-carbon short-chain fatty acid produced from fiber metabolism by gut microbes, can reach concentrations as high as 140 mM in the colon and is also present in venous blood and peripheral tissues (1, 2). Butyrate has been known for several decades to regulate gene expression through inhibition of histone deacetylases (HDACs) (3, 4), and butyrate is largely thought to be beneficial to human health because of its ability to dampen inflammation (2, 5–16). However, it has also been suggested to promote replication of herpesviruses (17, 18) and several RNA viruses (19–21). *In vivo*, butyrate and dietary fiber were shown to be protective against influenza virus pathology in mice, despite an increase in virus titer (22). In contrast, butyrate and fiber were

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shown to be detrimental in the inflammatory disease caused by chikungunya virus, a distinct RNA virus (23). Whether butyrate broadly affects virus replication and how it affects viruses that are not directly associated with histones remain unclear and warrant investigation given the ubiquity and abundance of this metabolite.

One of the most potent innate immune mechanisms against virus infections is initiated by the induction of type I interferons (IFNs), which are secreted and signal to upregulate the expression of hundreds of IFN-stimulated genes (ISGs), many of which have antiviral functions (24, 25). Human mutations in factors needed for production of IFNs or in the signal transducer and activator of transcription (STAT) factors required for upregulation of ISGs are thus associated with severe virus infections (26–29). Despite the critical role of type I IFNs in antiviral defense, their levels and activities are held in check by dozens of cellular proteins in order to limit tissue-damaging effects that can also be caused by IFNs (30–35). Given the importance of fine-tuning the IFN response, it is likely that many regulatory mechanisms for the IFN induction and signaling pathways remain to be discovered. In addition to intrinsic regulatory components, recent evidence points to environmental and nonhereditary factors such as ambient temperature and humidity (36–38), as well as diet and microbiome composition (22, 39–43) in shaping antiviral immunity. Thus, hereditary and nonhereditary factors can both influence outcomes of virus infections, and a better understanding of how these factors independently influence antiviral immune responses is needed. Here, we report a previously unknown role for butyrate in transcriptionally reprogramming the type I IFN response.

RESULTS

Butyrate increases virus infection and replication. Since butyrate has been reported to promote replication of several viruses, we sought to examine whether this observation held true for additional viruses relevant to human and animal health. Given that butyrate and fiber were recently suggested to modulate inflammation during influenza virus infection (22), we first pretreated A549 lung epithelial cells with butyrate prior to H1N1 influenza A virus infection. We observed that butyrate significantly increased susceptibility of cells to influenza virus infection as measured by percent infection via flow cytometry (Fig. 1a). We also measured infectious virus levels released in cell supernatants and found that virus titers were increased by an order of magnitude in butyrate-treated cells compared to mock control cells (Fig. 1b). Since the concentration of butyrate reaches its highest level in gut tissue (1, 2), we tested whether butyrate affected susceptibility of colon cells to enteric virus infection. We observed that reovirus infection of HT-29 colon cells and resulting virus titers were both significantly increased in the presence of butyrate (Fig. 1c and d). Likewise, since human immunodeficiency virus 1 (HIV-1) can infect and subsequently deplete gut-resident CD4⁺ T cells (44–46), we also examined whether butyrate altered HIV-1 infection of cells. Like influenza virus and reovirus, we observed a significant increase in HIV-1 infection and replicative capacity in butyrate-treated THP1 monocytes compared to control monocytes (Fig. 1e and f).

As an additional test of the breadth of the effect of butyrate on RNA viruses, we also examined whether butyrate affected A549 lung epithelial cell infection with three additional viruses, human metapneumovirus (hMPV), vesicular stomatitis virus (VSV), and Sendai virus (SeV). We observed that butyrate significantly increased susceptibility of cells to hMPV (Fig. 2a) and VSV (Fig. 2b) but did not affect infection by SeV (Fig. 2c). We further confirmed the lack of effect of butyrate on SeV by repeating experiments with a lower virus dose (Fig. 2c). Interestingly, among the viruses tested, SeV is uniquely reported to be largely resistant to effects of IFN due to its dismantling of signaling downstream of the type I IFN receptor (47). Indeed, we previously reported that SeV replicates to similar titers when comparing viral loads in the lungs of wild-type (WT) mice versus IFN- α receptor knockout (KO) mice (48). Together, these data suggest that butyrate may be modulating the type I IFN pathway to affect virus infections. We further observed that butyrate lost the ability to enhance influenza virus infection in

FIG 1 Butyrate promotes virus infection and replication. Cells were pretreated with 5 mM butyrate (But) or were mock treated for 1 h prior to infection with the indicated viruses. (a) (Left) Representative flow cytometry plots from A549 cells infected overnight with influenza A virus (IAV) at an MOI of 1 in the presence or absence of butyrate. Numbers indicate the percentage of cells positive for IAV nucleoprotein (NP), indicating percent infection. (Right) Average percent infection from 4 independent infection experiments. (b) Average virus titers in supernatants from A549 cells infected at an MOI of 1 for 24 h with IAV in the presence or absence of butyrate. (c) (Left) Representative flow cytometry plots from HT29 cells infected overnight with reovirus at an MOI of 1 in the presence or absence of butyrate. Numbers indicate the percentage of cells positive for reovirus σ 3 capsid protein, indicating percent infection. (Right) Average percent infection from 3 independent infection experiments. (d) Average virus titers in supernatants from HT-29 cells infected at an MOI of 1 for 24 h with reovirus in the presence or absence of butyrate. (e) (Left) Representative flow cytometry plots from THP1 cells infected for 48 h with GFP-expressing HIV-1 at an MOI of 0.5 in the presence or absence of butyrate. Numbers indicate the percentage of cells positive for GFP, indicating percent infection. (Right) Average percent infection from 4 independent infection experiments. (f) Relative luminescent units (RLU) indicative of viral titers from TZM-bl cells infected with a 1:4 dilution of cell supernatants harvested from THP1 cells that were infected with HIV-1 for 48 h in the presence or absence of butyrate.

STAT1 KO cells lacking functional IFN signaling (Fig. 2d), further suggesting that alterations in the IFN response may underlie the effects of butyrate on viruses. As an additional control, we also measured IFN- β secretion upon SeV infection and did not observe an effect of butyrate on IFN- β production (Fig. 3).

Butyrate treatment decreases specific ISG mRNA and protein products. We next measured whether butyrate regulated levels of IFN-stimulated gene (ISG) products after a 24-h IFN treatment and found that butyrate suppressed levels of critical ISGs, such as RIG-I, IFIT2, and IFITM3, in human colon and lung cells, as well as in mouse macrophages (Fig. 4a to c). This is consistent with past reports of inhibition of candidate ISGs by HDAC inhibitors (31, 49–53). However, we also newly observed that upregulation of some ISGs, including STAT1 and STAT2, was not strongly affected by butyrate (Fig. 4b and c). While a 24-h treatment with IFN allowed detection of ISG protein products by Western blotting, ISG mRNAs are generally induced within hours of IFN- β treatment, with most ISGs being induced by 8 h posttreatment and many showing their maximal induction on a similar timescale (54, 55). Thus, we examined ISG expression at 8 h post-IFN treatment, and we similarly found that mRNA levels for RIG-I, IFIT2, and IFITM3 were suppressed by butyrate, while STAT2 mRNA induction was not significantly reduced (Fig. 4d). The reduced levels of ISG products were not a result of butyrate toxicity, as cell viability remained similar to that of control cells at the highest concentration of butyrate (5 mM) used in our experiments (Fig. 4e). Consistent with the unaffected induction of some ISGs in the presence of butyrate, we determined that phosphorylation and nuclear translocation of the STAT1 and STAT2 transcription factors upon type I IFN signaling were not perturbed by butyrate (Fig. 5a and b). Overall, our results indicate that expression of a specific subset of ISGs is inhibited by butyrate and that this occurs independently of STAT1/2 activation.

FIG 2 Enhancement of virus infection by butyrate occurs only in the presence of functional interferon signaling. (a) Average percent infection data from A549 cells infected for 24 h with hMPV at an MOI of 1 in the presence or absence of butyrate. (b) Average percent infection data from A549 cells infected for 24 h with VSV at an MOI of 0.1 in the presence or absence of butyrate. (c) Average percent infection data from A549 cells infected for 24 h with SeV at an MOI of 0.1 or 1.0 in the presence or absence of butyrate. (d) (Left) Representative flow cytometry plots from wild-type (WT) or STAT1 KO MEFs infected overnight with influenza A virus (IAV) in the presence or absence of butyrate. Numbers indicate the percentage of cells positive for IAV nucleoprotein (NP), indicating the percent infection. (Right) Average percent infection from 3 independent infection experiments. Open circles indicate data points from independent experiments. Bars represent average values of individual data points, and error bars represent standard deviation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant by Student's t test for the indicated comparisons.

Suppression of ISG induction by butyrate can be mimicked by other HDAC inhibitors and countered by histone acetyltransferase inhibition. We next reasoned that if butyrate affects ISG expression via HDAC inhibition, then other HDAC inhibitors should also suppress the induction of these ISGs. Indeed, we found that the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the class I HDAC inhibitor RGFP966 both decreased levels of ISGs similarly to butyrate in lung and colon cells,

FIG 3 Butyrate treatment does not decrease production of type I IFN. RAW264.7 cells were mock treated or pretreated with 1 mM butyrate (But) followed by infection with Sendai virus (SeV) at an MOI of 10 for 24 h. Supernatants were analyzed for IFN- β levels by enzyme-linked immunosorbent assay (ELISA). Open circles represent individual replicate samples from a representative experiment. Bars are average values, and error bars represent standard deviation. Horizontal lines indicate statistical comparison of samples of interest. ns, not significant by analysis of variance (ANOVA) followed by Tukey's multiple-comparison test.

FIG 4 Butyrate decreases levels of a subset of ISG protein and mRNA products. (a to c) HT-29 cells (a), A549 cells (b), and RAW264.7 cells (c) were mock treated or were pretreated for 1 h with the indicated mM concentrations of butyrate (But) followed by IFN- β or mock treatment for 24 h in the continued presence or absence of the indicated butyrate concentrations. Western blotting for various ISGs was performed with GAPDH and tubulin serving as loading controls. All blots are representative of at least 4 similar experiments. (d) A549 cells were mock treated or pretreated for 1 h with 2.5 mM butyrate followed by mock or IFN- β treatment in the continued presence or absence of butyrate for 8 h. qRT-PCR was performed for the indicated ISGs. The fold change is expressed relative to mock-treated cells (not treated with butyrate or IFN- β). Levels are normalized to *GAPDH* for each condition. Open circles represent triplicate measurements from a representative experiment, bars represent average values, and error bars show standard deviation. The qRT-PCR data shown are representative of at least two similar experiments. Horizontal lines indicate statistical comparisons of interest. ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant by ANOVA followed by Tukey's multiple-comparison test. (e) Cell viability measurements based on trypan blue exclusion for A549 cells treated with indicated doses of butyrate. Ten percent DMSO served as a positive control for inducing cell death. Error bars represent the standard deviation of triplicate measurements.

while the class IIa HDAC-specific inhibitor TMP195 and the HDAC8-specific inhibitor 1-naphthohydroxamic acid (1-NA) did not affect induction of these ISGs (Fig. 6a and b). The decreases in ISG levels caused by butyrate, SAHA, and RGFP966 were accompanied by global increases in histone acetylation (Fig. 6c).

We next posited that if butyrate affects ISGs by inhibiting HDACs, then a histone acetyltransferase (HAT) inhibitor may counteract this effect. We pretreated cells with C646, an inhibitor of the HAT p300 (56), prior to butyrate treatment and IFN stimulation, and found that C646 partially reversed the effect of butyrate on ISG protein levels (Fig. 6d) while also countering its increase of global histone acetylation (Fig. 6e). Comparing

FIG 5 Butyrate does not prevent STAT phosphorylation or nuclear translocation upon IFN- β stimulation. (a) A549 cells were mock treated or treated with 2.5 mM butyrate for 1 h prior to mock stimulation or IFN- β stimulation for 15 min. Western blotting was performed for the indicated STAT proteins and phosphorylated (P) STAT proteins, with GAPDH blotting serving as a loading control. (b) Confocal microscopy images of A549 cells treated as in panel a with staining for STAT1 and STAT2. DAPI was used to visualize the nuclei. Scale bar, 10 μ m.

FIG 6 Effects of butyrate on ISGs can be mimicked by other HDAC inhibitors and can be countered by HAT inhibition. (a) A549 cells were mock treated or pretreated with the indicated HDAC inhibitors, butyrate (2.5 mM), SAHA (3 μ M), TMP195 (10 μ M), RGFP966 (20 μ M), or 1-naphthohydroxamic acid (1-NA, 10 μ M) for 1 h before 16 h of IFN- β stimulation with continued presence of chemical inhibitors. Western blotting was performed for specific ISGs, and blotting for GAPDH served as a control for loading. (b) HT-29 cells were treated as in panel a. (c) A549 cells were mock treated or pretreated with the indicated HDAC inhibitors, butyrate (2.5 mM), SAHA (3 μ M), TMP195 (10 μ M), RGFP966 (20 μ M), or 1-naphthohydroxamic acid (1-NA, 10 μ M) for 1 h before 16 h of IFN- β stimulation with continued presence of chemical inhibitors. Western blotting was performed for acetylated histones, and GAPDH served as a control for loading. (d) A549 cells were mock treated or pretreated for 2 h with HAT inhibitor C646 (10 μ M), followed by addition of butyrate (2.5 mM) or vehicle control for 1 h, followed by 16 h of IFN- β stimulation with continued presence of C646 and/or butyrate. Western blotting was performed for RIG-I and IFITM3 as representative ISGs, and blotting for GAPDH served as a control for loading. (e) A549 cells were mock treated or pretreated for 2 h with HAT inhibitor C646 (10 μ M), followed by addition of butyrate (2.5 mM) or vehicle control for 1 h, followed by 16 h of IFN- β stimulation with continued presence of C646 and/or butyrate. Western blotting was performed for histones and acetylated histones, and blotting for GAPDH served as a control for loading. (f) A549 cells were individually mock treated or pretreated for 1 h with 2.5 mM saturated fatty acids ranging in carbon chain length from 3 to 7 carbons (labeled C3 to C7), followed by IFN- β or mock treatment for 24 h in the continued presence or absence of the indicated fatty acids. Western blotting for various ISGs was performed with GAPDH serving as a loading control. Blots are representative of at least two similar experiments in all cases.

the effects of fatty acids of 3 to 7 carbons in chain length revealed that 4-carbon butyrate was uniquely able to repress ISGs (Fig. 6f) in accord with its more potent ability to inhibit HDACs compared to other short-chain fatty acids (57). Knockdowns with small interfering RNAs (siRNAs) targeting HDACs 1, 2, and 3 individually or in combinations resulted in compensatory mechanisms in which nontargeted HDACs were highly upregulated, making effects on ISGs difficult to interpret in genetic targeting experiments (Fig. 7a and b). Nonetheless, our results overall suggest that a complex interplay between HDACs and HATs likely contributes to the regulation of ISG induction and the effects of butyrate.

Butyrate decreases and increases specific ISG expression. Since past studies on the regulation of ISGs by HDACs largely focused on specific candidate ISGs, we sought to determine the extent to which butyrate globally affects ISGs. RNA-seq analyses revealed that butyrate alone regulated basal transcription of 882 genes, of which 821 genes were upregulated (4-fold or greater increase and false-discovery rate [FDR] of <0.05) and 61 were downregulated (4-fold or greater decrease and FDR of <0.05)

FIG 7 Effects of HDAC knockdown on ISG levels. (a and b) siRNAs targeting HDACs 1, 2, and 3 (siHDAC) or control (C) siRNA were transfected (a) individually or (b) in combinations into A549 cells for 24 h prior to treatment with butyrate (But) (2.5 mM) or vehicle control for 1 h, prior to addition of IFN- β as indicated. Western blotting was performed for RIG-I and IFITM3 as representative ISGs, HDACs to examine knockdown efficiencies and expression compensation of other HDACs, and GAPDH as a control for loading.

(Fig. 8a; see Table S1A in the supplemental material). Gene ontology term analysis revealed diverse biological associations among butyrate-upregulated genes, including inflammatory and immune responses (Fig. 8b; Table S1B), while downregulated gene associations included type I IFN signaling and responses to virus (Table S1C). RNA-seq analysis of IFN- β -treated cells identified 263 genes that were upregulated 4-fold or more ($FDR < 0.05$) compared to mock-treated cells, which we classified as ISGs (Table S1D). Baseline (without IFN stimulation) expression of 34 of these ISGs was upregulated 4-fold or greater with statistical significance ($P < 0.05$) by butyrate alone, and these included antiviral restriction factors OASL and BST2 (Fig. 8c; Table S1E). Conversely, baseline expression of 4 ISGs, including IFIT2 and OAS2, was downregulated by 4-fold or greater with statistical significance ($P < 0.05$) by butyrate alone. Thus, butyrate reprograms the baseline expression of several important ISGs.

Of the 263 ISGs, IFN- β -induced upregulation of 160 (60%) was classified as being inhibited by butyrate, whereas the upregulation of 96 ISGs (37%) was not strongly affected by butyrate (Fig. 8 and e; Table S1D). Seven ISGs (3%), all of which were upregulated by butyrate treatment alone, were classified as having their IFN-mediated induction increased by butyrate (Fig. 8d and e; Table S1D). Of the 160 ISGs that showed diminished induction in the presence of butyrate, we confirmed regulation of IFITM3 and RIG-I and newly identified butyrate regulation of other antiviral ISGs, such as CXCL10 and TRIM22 (Fig. 8f). In similar agreement with our previous results, butyrate did not repress STAT2 induction (Fig. 8f). We additionally validated RNA-seq results by performing reverse transcription-quantitative PCR (qRT-PCR) for representative ISGs whose induction was newly found to be inhibited, unaltered, or upregulated by butyrate (Fig. 8g). Overall, we found that butyrate reprograms the magnitude of induction for at least 63% of ISGs, identifying a new mechanism by which butyrate may affect virus infections.

DISCUSSION

Recent advances in our understanding of the microbiome-immunity axis have spurred interest in how the gut microbiota and their metabolic products affect human health (10, 58–64). We report here that the short-chain fatty acid butyrate reprograms the response to type I IFN by tuning the magnitude of induction of specific ISGs both

FIG 8 Butyrate differentially regulates baseline and IFN-induced expression of ISGs. A549 cells were mock treated or pretreated with butyrate (2.5 mM) for 1 h prior to stimulation with IFN- β or vehicle control for 8 h in the continued presence or absence of butyrate. (a to f) RNA was extracted and subjected to RNA-seq analysis. Three biological replicates for each condition were analyzed by RNA-seq. (a) Volcano plot depicting basal gene expression changes induced by butyrate treatment. Data are plotted as butyrate-treated cells normalized to mock-treated cells, with dots representing expression changes of individual genes. Dashed lines indicate cutoff values used to determine the significance of gene expression differences (adjusted P value < 0.05 and Log₂ fold change greater than 2 [positive or negative]). Gray dots, adjusted P value > 0.05 with log₂ fold change < 2 ; green dots, adjusted P value > 0.05 with log₂ fold change > 2 ; blue dots, adjusted P value < 0.05 with log₂ fold change < 2 ; red dots, adjusted P value < 0.05 with log₂ fold change > 2 . Select genes of interest (highly up- or downregulated by butyrate) have been annotated on the volcano plot. (b) Gene ontology term analysis of the top 5 biological processes associated with genes upregulated by butyrate. (c) Venn diagram comparing baseline butyrate-regulated genes (4-fold or greater increase or decrease in response to butyrate treatment) to genes classified as ISGs (genes showing a 4-fold or greater increase in expression stimulated by IFN- β). (d) Pie chart representing the number of ISGs categorized based on the effect of butyrate on their mRNA levels. Downregulated genes were classified as a 4-fold or greater decrease in expression stimulated by IFN- β in the presence of butyrate. Upregulated genes were classified as a 4-fold or greater increase in expression stimulated by IFN- β in the presence of butyrate. "Not strongly affected" indicates all other ISGs. (e) Heat map of all ISGs under the indicated conditions compared to mock-treated cells (without butyrate or IFN- β treatment). Data represented are the mean of 3 biological replicates per condition. (f) Magnification of specific regions in the heat map shown in panel e to visualize representative example ISGs belonging to each of the 3 ISG categories based on the effect of butyrate on their induction. (g) Independent experimental samples were prepared as in panels a to f for validation of RNA-seq results via qRT-PCR. Fold change is expressed relative to mock-treated cells (without butyrate or IFN- β treatment). Gene expression was normalized to GAPDH for each sample. Open circles represent triplicate samples in a single experiment. Bars represent average values, and error bars represent standard deviation. Horizontal lines indicate statistical comparisons of interest. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant by ANOVA followed by Tukey's multiple-comparison test.

positively and negatively depending on the gene (Fig. 4 and 8). Unlike the previously reported mechanism by which butyrate regulates the type II IFN response (65, 66), butyrate does not inhibit type I IFN-mediated STAT activation or nuclear translocation (Fig. 5).

Although histone acetylation is a posttranslational modification often associated with transcriptional activation (67–69), chromatin acetylation has also been shown to be repressive in certain contexts (49, 70). Indeed, HDACs are known to be required for induction of several ISGs (31, 49–53). Our results newly suggest that different subsets of ISGs have different requirements for HDAC activity, identifying a currently unappreciated layer of complexity in the regulation of these genes. Our experiments do not rule out the possibility that butyrate's effect on ISGs is a result of increased acetylation of nonhistone proteins, which can also be substrates of HDACs (71–73).

Butyrate is well characterized as having anti-inflammatory effects (2, 10, 13, 61, 74), which might lessen tissue damage resulting from viral infection. A recent study demonstrated that mice fed a butyrate-rich diet had higher influenza virus titers during early stages of infection compared to control mice but experienced less tissue damage to lungs later in infection (22). Together with our findings, this suggests that butyrate might reduce tissue damage caused by type I IFN or other proinflammatory mechanisms at the cost of increasing overall virus replicative capacity via ISG repression.

Interestingly, we observed that SeV was unique among the viruses we tested in that it was not affected by butyrate treatment (Fig. 2). This finding may further implicate the effects of butyrate on IFN signaling and ISG induction in promoting virus infection because SeV is known to be less sensitive to IFN than many other viruses due to its multifaceted ability to dismantle the type I IFN signaling system in infected cells (47, 48). We also note that among the viruses that we examined, SeV is uniquely unsusceptible to inhibition by IFITM3 (75), one of the potent antiviral ISGs that is decreased by butyrate.

It is also possible that some intrinsic proviral genes are upregulated by butyrate, which may be additive or synergistic with the diminished ISG response to provide a net result of increased virus replication in the presence of butyrate. Additionally, butyrate may in some instances directly affect virus replication independent of, or in addition to, its effect on the type I IFN response, as is reported for HIV-1 (76–78). Further study exploring the multifaceted regulation of the type I IFN response by butyrate and its differential effects on virus infections is warranted.

Since butyrate can increase virus titers in most cases, it could be employed as an inexpensive tool for increasing yields of viral vaccines or research virus stocks. Our results also suggest that treatment with butyrate or butyrogenic bacteria, which is being increasingly considered for therapeutic purposes (64, 79), should be evaluated in terms of a balance between anti-inflammatory and proviral effects.

MATERIALS AND METHODS

Cell culture, interferon treatments, and drug treatments. A549, HT-29, THP1, MDCK, Vero, and RAW264.7 cells were purchased from the ATCC. TZM-bl cells were obtained from the NIH AIDS Reagent Program. WT and STAT1 KO mouse embryonic fibroblasts (MEFs) (80) were generated by Alexander Ploss and Charles Rice (Rockefeller University). THP1 cells were grown in RPMI supplemented with 10% Equafetal fetal bovine serum (Atlas Biologicals). All other cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% Equafetal fetal bovine serum. Cells were grown at 37°C with 5% CO₂ in a humidified incubator. Where indicated, cells were treated with human IFN- β (EMD Millipore) at a concentration of 40 units/ml or with mouse IFN- α 2 (eBioscience) at a 1:1,000 dilution for 24 h. Treatment with the fatty acids propionate (P1386, Sigma-Aldrich), butyrate (B103500, Sigma-Aldrich), valerate (240370, Sigma-Aldrich), hexanoic acid (21530, Sigma-Aldrich), and heptanoic acid (75190, Sigma-Aldrich) and the HDAC inhibitors suberoylanilide hydroxamic acid (SAHA, 149647-78-9, Sigma-Aldrich), TMP-195 (23242, Cayman Chemical), RGFP966 (16917, Cayman Chemical), and 1-naphthohydroxamic acid (6953-61-3, Sigma-Aldrich) was done for 24 h or 1 h prior to IFN treatment, with drugs kept in culture medium for the remainder of the experiments. The HAT inhibitors C646 (328968-36-1, Sigma-Aldrich) and NU9056 (4903, Tocris) were added to culture medium 2 h prior to butyrate treatment and 3 h prior to IFN treatment and were kept in culture medium for the remainder of the experiments.

siRNA knockdowns. Gene knockdown was achieved using Dharmacon ON-TARGETplus SMARTpool siRNAs targeting human HDAC1 (L-003493), HDAC2 (L-003495), HDAC3 (L-003496), and CNOT7 (L-

012897) or nontargeting control (D-001810-10-20) with Lipofectamine RNAiMAX reagent (Life Technologies) according to the manufacturer's protocol.

Virus propagation, infection, and flow cytometry. Influenza virus A/Puerto Rico/8/34 (H1N1, PR8) was provided by Thomas Moran (Icahn School of Medicine at Mount Sinai), and stocks were propagated in 10-day-old embryonated chicken eggs (Charles River Laboratories) for 48 h at 37°C, and the virus titers were determined on MDCK cells as we have done previously (81–83). For influenza virus replication assays, virus inoculum was washed from cells after 1 h, and TPCK [L-(tosylamido-2-phenyl) ethyl chloromethyl ketone]-treated trypsin (Worthington Biochemical) was included in the cell medium. Sendai virus expressing green fluorescent protein (GFP) and vesicular stomatitis virus expressing GFP were provided by Dominique Garcin (University de Geneve). SeV was propagated in 10-day-old embryonated chicken eggs at 37°C for 40 h, and the virus titers were determined on Vero cells. VSV was propagated in and the virus titers were determined on HeLa cells. Human metapneumovirus expressing GFP was generated with a reverse genetics system based on the NL/1/00 (A1) strain utilizing a previously described methodology (84) and propagated in Vero cells and concentrated by ultracentrifugation through a 20% sucrose cushion, and the virus titers were determined on LLC-MK2 cells. Reovirus (Dearing strain) was purchased from the ATCC and propagated and titered using Vero cells. A549 cells were treated with 2.5 mM butyrate for 1 h prior to infection with IAV, SeV, VSV, or hMPV. HT29 cells were treated with 2.5 mM butyrate for 1 h prior to infection with reovirus. GFP-expressing HIV-1 pseudoviruses were generated in HEK293T cells as previously described (85). Briefly, replication-competent HIV-1 was generated using NL4-3 proviral plasmid (NIH AIDS Reagent Program, catalog no. 114), and single-cycle HIV-1 GFP reporter virus was generated by pseudotyping NL4-3ΔEnvEGFP (NIH AIDS Reagent Program, catalog no. 11100) with NL4-3 gp160 (86). THP1 cells were treated with 2.5 mM butyrate for 1 h prior to infection with HIV-1 pseudoviruses in duplicate wells at a multiplicity of infection (MOI) of 0.5. After 48 h, cells were washed, fixed, and analyzed for GFP expression. THP1 cells were treated with 2.5 mM butyrate for 1 h prior to infection with replication-competent HIV-1 at an MOI of 0.5. Cell supernatants were harvested at 48 h postinfection, and virus titers were determined by infecting TZM-bl cells and measuring β-galactosidase activity using a Galacto-Lite system (Applied Biosystems). TZM-bl cells are a commonly utilized indicator cell line that produces luciferase under the control of the HIV-1 promoter. These cells allow relative levels of HIV-1 present within samples to be quantified via infection of the cells and subsequent measurement of luciferase activity. For flow cytometry quantification of infection, IAV-infected cells were stained with anti-influenza NP (BEI Resources, NR-19868) at a 1:1,000 dilution, reovirus-infected cells were stained with anti-reovirus σ3 antibody (Developmental Studies Hybridoma Bank, 4F2, deposited by Terence Dermody) at a 1:1,000 dilution, and cells infected with GFP-expressing HIV, SeV, VSV, or hMPV were analyzed for GFP fluorescence directly. Flow cytometry was performed on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Western blotting and confocal microscopy. For Western blotting, cells were lysed with buffer containing 0.1 mM triethanolamine, 150 mM NaCl, and 1% SDS at pH 7.4 supplemented with EDTA-free protease inhibitor cocktail (Roche). For phosphorylated protein Western blots, PhosSTOP (Sigma Aldridge) phosphatase inhibitor was added to lysis buffer. Primary antibodies for IFITM1 (13126, Cell Signaling Technology), IFITM3 (11714, ProteinTech), RIG-I (20566, ProteinTech), GAPDH (39-8600, Invitrogen), IFIT2 (PA3-845, Thermo Scientific), STAT1 (9172, Cell Signaling Technology), STAT2 (72604, Cell Signaling Technology), pSTAT1 (7649, Cell Signaling Technology), pSTAT2 (4441, Cell Signaling Technology), tubulin (Antibody Direct), HDAC1 (34589, Cell Signaling Technology), HDAC2 (57156, Cell Signaling Technology), HDAC3 (85057, Cell Signaling Technology), H4K16ac (13534, Cell Signaling Technology), H3K9ac (9649, Cell Signaling Technology), H3K27ac (8173, Cell Signaling Technology), H3 (4499, Cell Signaling Technology), H4K8ac (2594, Cell Signaling Technology), and H4 (13919, Cell Signaling Technology) were used at 1:1,000 dilutions or according to the manufacturer's protocol in both Western blotting and confocal imaging. For confocal microscopy, cells grown on coverslips were treated for 15 min with IFN-β fixed for 20 min in 4% paraformaldehyde/phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100/PBS for 20 min, blocked with 2% FBS/PBS for 20 min, and consecutively labeled with primary and Alexa Fluor-conjugated secondary antibodies (Life Technologies) in 0.1% Triton X-100 in PBS for 20 min. Coverslips were mounted on glass slides using Prolong Gold antifade mountant with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). Imaging was performed on an Olympus Fluoview confocal microscope.

Quantitative RT-PCR. RNA was extracted from A549 cells treated with DMSO (Mock) or 2.5 mM butyrate, with and without IFN-β treatment for 6 h, using the RNeasy minikit (74104, Qiagen). cDNA was prepared from extracted RNA using the AffinityScript qPCR cDNA synthesis kit (600559, Agilent). PCRs for each sample were performed in triplicate with specific primers using iQ SYBR green Supermix (1708887, Bio-Rad). Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method (87). PCRs were performed using the CFX96 Touch real-time system (Bio-Rad). Normalization was performed using GAPDH levels. The primer sequences used can be found in Table S1F.

RNA-seq transcriptomics and data analysis. RNA was extracted from A549 cells treated with DMSO (Mock) or 2.5 mM butyrate, with and without IFN-β treatment for 8 h, using the RNeasy minikit (74104, Qiagen). Three biological replicates were sequenced per experimental condition. RNA sequencing was performed at The Ohio State University Comprehensive Cancer Center Genomics Shared Resource. The mRNA libraries were generated using a NEBNext Ultra II directional RNA library prep kit for Illumina (E7760L, New England Biolabs) and NEBNext poly(A) mRNA magnetic isolation module (E7490, New England Biolabs). Then, 200 ng of total RNA (quantified using a Qubit fluorometer) was used to construct sequencing libraries. Libraries were sequenced with an Illumina HiSeq 4000 instrument in paired-end 150-bp read mode; 17 million to 20 million passing filter (PF) clusters (equivalent to 34 million to 40

million PF paired reads) were sequenced per sample. Each sample was inspected for quality using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Alignment of reads was performed using Spliced Transcripts Alignment to a Reference (STAR) v.2.6.1 with human genome hg38 (88). The Bam files obtained from alignment with STAR were processed using HTSeq-count (89) to obtain the counts per gene in all samples. The read counts obtained from HTSeq-count were analyzed for differential gene expression using the DESeq2 function from DEBrowser (90) (<https://debrowser.umassmed.edu/>). Heatmaps were constructed using Morpheus software (<https://software.broadinstitute.org/morpheus/>). Volcano plots were generated with the EnhancedVolcano package from Bioconductor (<https://github.com/kevinblighe/EnhancedVolcano>) using R programming software version v.3.5.3.

Statistical analysis. Data are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism v.8.3.0 (GraphPad Software). Student's *t* tests were used for single comparisons between two groups. Other data were analyzed using one-way analysis of variance with Tukey's multiple-comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant. Only statistical comparisons of direct interest to effects of butyrate are labeled, and a lack of labeling does not indicate a lack of statistical significance.

Data availability. RNA sequencing FASTQ files are available at the NCBI Sequence Read Archive under accession number [PRJNA633674](https://www.ncbi.nlm.nih.gov/sra/PRJNA633674).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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