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The SCFA butyrate stimulates the epithelial production of retinoic acid via inhibition of epithelial HDAC

Ronald Schilderink,[1] Caroline Verseijden,[1] Jurgen Seppen,[1] Vanesa Muncan,[1] Gijs R. van den Brink,[1,2] Tim T. Lambers,[3] Eric A. van Tol,[3] and Wouter J. de Jonge[1,2]

[1]*Tytgat Institute for Gastrointestinal and Liver Research, Amsterdam, the Netherlands;* [2]*Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, the Netherlands; and* [3]*Mead Johnson Pediatric Nutrition Institute, Nijmegen, the Netherlands*

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Schilderink R, Verseijden C, Seppen J, Muncan V, van den Brink GR, Lambers TT, van Tol EA, de Jonge WJ. The SCFA butyrate stimulates the epithelial production of retinoic acid via inhibition of epithelial HDAC. *Am J Physiol Gastrointest Liver Physiol* 310: G1138–G1146, 2016. First published May 5, 2016; doi:10.1152/ajpgi.00411.2015.—In the intestinal mucosa, retinoic acid (RA) is a critical signaling molecule. RA is derived from dietary vitamin A (retinol) through conversion by aldehyde dehydrogenases (aldh). Reduced levels of short-chain fatty acids (SCFAs) are associated with pathological microbial dysbiosis, inflammatory disease, and allergy. We hypothesized that SCFAs contribute to mucosal homeostasis by enhancing RA production in intestinal epithelia. With the use of human and mouse epithelial cell lines and primary enteroids, we studied the effect of SCFAs on the production of RA. Functional RA conversion was analyzed by Adlefluor activity assays. Butyrate (0–20 mM), in contrast to other SCFAs, dose dependently induced *aldh1a1* or *aldh1a3* transcript expression and increased RA conversion in human and mouse epithelial cells. Epithelial cell line data were replicated in intestinal organoids. In these organoids, butyrate (2–5 mM) upregulated *aldh1a3* expression (36-fold over control), whereas *aldh1a1* was not significantly affected. Butyrate enhanced maturation markers (*Mucin-2* and *villin*) but did not consistently affect stemness markers or other Wnt target genes (*lgr5*, *olfm4*, *ascl2*, *cdkn1*). In enteroids, the stimulation of RA production by SCFA was mimicked by inhibitors of histone deacetylase 3 (HDAC3) but not by HDAC1/2 inhibitors nor by agonists of butyrate receptors G-protein-coupled receptor (GPR)43 or GPR109A, indicating that butyrate stimulates RA production via HDAC3 inhibition. We conclude that the SCFA butyrate inhibits HDAC3 and thereby supports epithelial RA production.

short-chain fatty acids; butyrate; retinoic acid; epithelial cells; immune tolerance

EPITHELIAL CELLS ARE CRITICAL in maintaining mucosal homeostasis by virtue of their production of conditioning factors, such as retinoic acid (RA). RA is involved in many different physiological processes, including immune regulation. One example is the capacity of RA to imprint tolerogenic dendritic cells (DCs) (14). In the intestinal tract, specialized mucosal myeloid cells, such as CX3CR1-expressing macrophages and mucosal $CD103^+$ DCs, are sensitive to RA to maintain their tolerant phenotype (6). Furthermore, RA enhances the migratory properties of activated DCs, supports mucosal DC secretion of transforming growth factor β and IL-6, and has the capacity to induce gut-homing receptors on T cells (26).

Address for reprint requests and other correspondence: W. J. de Jonge, Tytgat Institute for Gastrointestinal and Liver Research, Meibergdreef 69, 1191BK, Amsterdam, the Netherlands (e-mail: w.j.dejonge@amc.nl).

Diet-induced changes in microbiota composition and its metabolic capacity are known to affect risks to develop cancer, or colitis, best reflected by saccharolytic fermentation and butyrogenesis (22). Major metabolic products of commensal bacteria are short-chain fatty acids (SCFAs), which are derived from fermentation of dietary fiber. Diets deficient or low in fiber exacerbate colitis development, whereas very high intake of dietary fiber or the SCFA acetate protects against colitis (18). SCFA binding to the "metabolite-sensing" receptors G-protein-coupled receptor (GPR)43 and GPR109A in nonhematopoietic cells is put forward to mediate these protective effects. However, SCFA butyrate also induces histone hyperacetylation in vivo (20) and in vitro by acting as a histone deacetylase inhibitor (HDACi) with selectivity toward class 1 HDACs (5) and as such, contributes to the generation of intestinal forkhead box P3-positive regulatory T cells (1, 26).

Given the association of SCFA with disease states and the fact that intestinal epithelial cells express different SCFA receptors and transporters (24), we examined whether SCFAs influence the secretion of epithelial conditioning factors. We show that butyrate enhances expression of aldehyde dehydrogenases (aldh), a group of enzymes that catalyze the conversion of retinal to RA. Our data implicate that butyrate stimulates the epithelial capacity to produce RA via an HDAC-dependent mechanism. Together, this helps in the understanding of how SCFA can be associated with health benefits of dietary fiber.

MATERIALS AND METHODS

Cells and enteroid culture treatments. Cell lines CMT93 and Caco-2 were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM (Lonza, Cologne, Germany), supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 IU/ml) at 37°C in a humidified 5% CO_2 atmosphere. Mouse (C57BL/6) and human fetal (20 wk) small intestinal enteroid cultures were harvested and maintained, as described previously (29). This study was done in accordance with the Medical Ethical Committee guidelines of Academic Medical Center.

Reagents. Sodium butyrate, sodium propionate, sodium acetate, niacin, and Trichostatin A (TSA) were all obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). GPR43 agonist was derived from Merck-Millipore (Darmstadt, Germany). IL-1β (PeproTech, London, UK) and TNF-α (PeproTech) were used at 10 and 20 ng/ml, respectively. HDACi were derived from Italfarmaco (Milano, Italy) and used at 200 mM in 1% DMSO in DMEM medium (vehicle controls were treated with 1% DMSO in DMEM medium). Antibodies used were anti-aldh1a1 (AB23375; Abcam, Cambridge, UK), aldh1a3 (AB129815; Abcam), Ki67 (MONX10284; Monosan, Uden, the Netherlands), villin (SC-7672; Santa Cruz Biotechnology, Dallas,

TX), mucin-2 (muc2; SC-15334; Santa Cruz Biotechnology), and lysozyme (A0099; Dako, Heverlee, Belgium).

Cytokine measurements. Murine homologue keratinocyte-derived protein chemokine, CCL2, and CXCL10 and human IL-8, CCL2, and CXCL10 quantifications were performed by ELISA (R&D Systems, Abingdon, UK).

Aldefluor assay. Aldefluor kit (Stemcell Technologies, Grenoble, France) was used, according to the manufacturer's protocol, to determine ALDH activity. In short, 10^6 cells were suspended in Aldefluor assay buffer containing the fluorescent Adlefluor substrate for the ALDH enzyme, in the presence or absence of ALDH inhibitor diethylaminobenzaldehyde. After incubating for 40 min at 37°C, cells were analyzed by fluorescence-activated cell sorting (FACS).

Transcript level analysis. Cells were lysed in TRI Reagent, and total RNA was isolated using the manufacturer's instructions (Sigma-Aldrich). cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Transcript levels were measured with quantitative PCR using a LightCycler 480 (Roche Applied Science, Mannheim, Germany). Primer sequences are available upon request.

Flow cytometry. Cells stained for the appropriate markers were analyzed on LSRFortessa or FACSCalibur (all BD Biosciences, Erembodegeme, Belgium). FACS data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Lentiviral transduction. Lentiviral vectors used to introduce small hairpin (sh)RNA were essentially as described in Seppen et al. (25).

Western blotting. Cells were harvested and lysed in 50 μl ice-cold lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, and 0.1% SDS. Horseradish peroxidase-conjugated secondary antibodies and Lumilite Western blotting substrate (Roche Applied Science) were used to visualize the appropriate proteins.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v5.0a (GraphPad Software, La Jolla, CA). For multi-experimental group analysis, data were subjected to one-way ANOVA, followed by post hoc analyses for group differences.

RESULTS

Butyrate affects cytokine responses in epithelial cells. SCFA butyrate affects the cytokine and chemokine secretion of intestinal epithelial cells (11). Besides the potential of SCFA to modify cytokine-induced epithelial responses, we aimed to study the effects on secretion of conditioning factors produced by epithelia under such conditions, such as RA. For the epithelial conversion of retinol to RA, the expression of aldh1a1-3 is critical. To determine whether butyrate could affect RA production, we analyzed *aldh* transcript levels after incubation with varying SCFA concentrations. In mouse CMT93 cells, butyrate significantly induced *aldh1a1* expression, whereas *aldh1a3* was not affected (Fig. 1*A*). The expression of *aldh1a1* coincided with enhanced level of *aldh1a1* protein (Fig. 1*B*). Elevated expression of *aldh1a1* was only seen upon butyrate incubation, and equimolar concentrations of other SCFAs failed to replicate this response (Fig. 1*C*). Conversely, in human Caco-2 cells, expression of aldh1a3, but not aldh1a1, was significantly upregulated upon butyrate stimulation (Fig. 1*D*).

Butyrate enhances RA production in epithelial cells. We next tested whether a similar effect was notable in primary epithelial cells grown as enteroid cultures. At 5 mM butyrate, *aldh1a3*, but not *aldh1a1*, was significantly elevated (Fig. 2*A*) in murine enteroids, with most pronounced elevation after 24 h of treatment (Fig. 2*B*). The presence of cytokines even augmented the butyrate potential to elevate *aldh1a3* expression (Fig. 2*C*). Likewise, intense *aldh1a3* protein-immune labeling was observed in 24-h butyrate-treated mouse enteroid cultures (Fig. 2*D*). In primary human fetal enteroid cultures, butyrate treatment dose dependently upregulated *aldh1a1*, whereas *aldh1a3* expression remained unaffected (Fig. 2*E*).

Notably, when analyzing expression of RA receptor (*rar*)α, *rar*β, or *rar*γ (Fig. 3*A*) in murine enteroids, expression of *rar*β was significantly enhanced by butyrate, indicating enhanced RA signaling (Fig. 3*A*). In addition, expression of SCFA transporter sodium-coupled monocarboxylate transporter 1 (*slc5a8*) was significantly enhanced by butyrate incubation (Fig. 3*B*), suggesting enhanced intracellular uptake of butyrate, confirming earlier data (20).

Subsequently, to demonstrate functional aldh protein activity, we assessed aldh activity using Adlefluor activity measurements in butyrate-treated CMT93 epithelial cells. Butyrate exposure to CMT93 epithelial cells resulted in a two-fold-increased Adlefluor activity compared with vehicle-treated epithelia (Fig. 3, *C–E*), with the maximum effect seen at 20 mM butyrate (Fig. 3*E*).

Inhibition of HDAC3 recapitulates the effect of butyrate in enteroids. Next, we examined the mechanism via which butyrate stimulated RA conversion. To this end, we tested whether specific GPR43 agonists mimicked the induction of *aldh1a3*, as seen with butyrate. However, no enhanced expression of *aldh1a1* or *aldh1a3* was observed in mouse enteroids treated with the GPR43 agonist (Fig. 4*A*). Similarly, niacin, an agonist of GPR109A, of which the physiological ligand is beta-hydroxy butyrate, failed to induce *aldh1a1* or *aldh1a3* expression at the doses tested (Fig. 4*B*). Thus we conclude that butyrate receptors GPR43 and GPR109A are not critically involved in the induction of retinaldehyde dehydrogenase (*raldh*) expression. The observation that butyrate enhanced the expression of butyrate transporter *slc5a8* (Fig. 3*B*), with the failure of GPR43 and GPR109A agonists to induce *aldh1a1* or *aldh1a3* expression, led us to hypothesize that butyrate stimulates *aldh* transcription via inhibition of HDAC (24). RARs repress transcription of target genes by recruiting the HDAC complex through silencing mediators for retinoid or thyroid-hormone receptors or nuclear receptor corepressor (8). Inversely, HDACs act on RAR function and may stimulate *aldh* expression via this mechanism (10).

First, we confirmed that butyrate acts as an HDACi in the millimolar concentrations that we used in our assays, by measuring the potential of butyrate to enhance histone acetylation (Fig. 4*C*). Next, we assessed whether the treatment of HDACi mimics the elevation of *aldh1a3* expression in enteroids, as we observed with butyrate. Treatment with TSA, a general HDACi, led to significant enhancement of *aldh1a3* expression in mouse enteroid cultures (Fig. 4*D*). With the use of a more specific HDACi, we aimed to demonstrate which specific HDAC was involved in regulating *aldh1a3* gene expression. As HDAC3 is associated with RAR DNA binding and aldh activity (10), we studied the effect of HDAC3-selective inhibitors (generated by Italfarmaco) on *aldh1a3* expression. Only generally acting HDACi (Givinostat), or HDACi with activity toward HDAC3 (and to a lesser extent, HDAC6), was effective in stimulating *aldh1a3* expression in mouse enteroid cultures. In addition, selective HDACi aimed at HDAC1 and HDAC2 failed to enhance *aldh1a3* expression (Fig. 4*E*). In case butyrate's potential to induce *aldh* expression

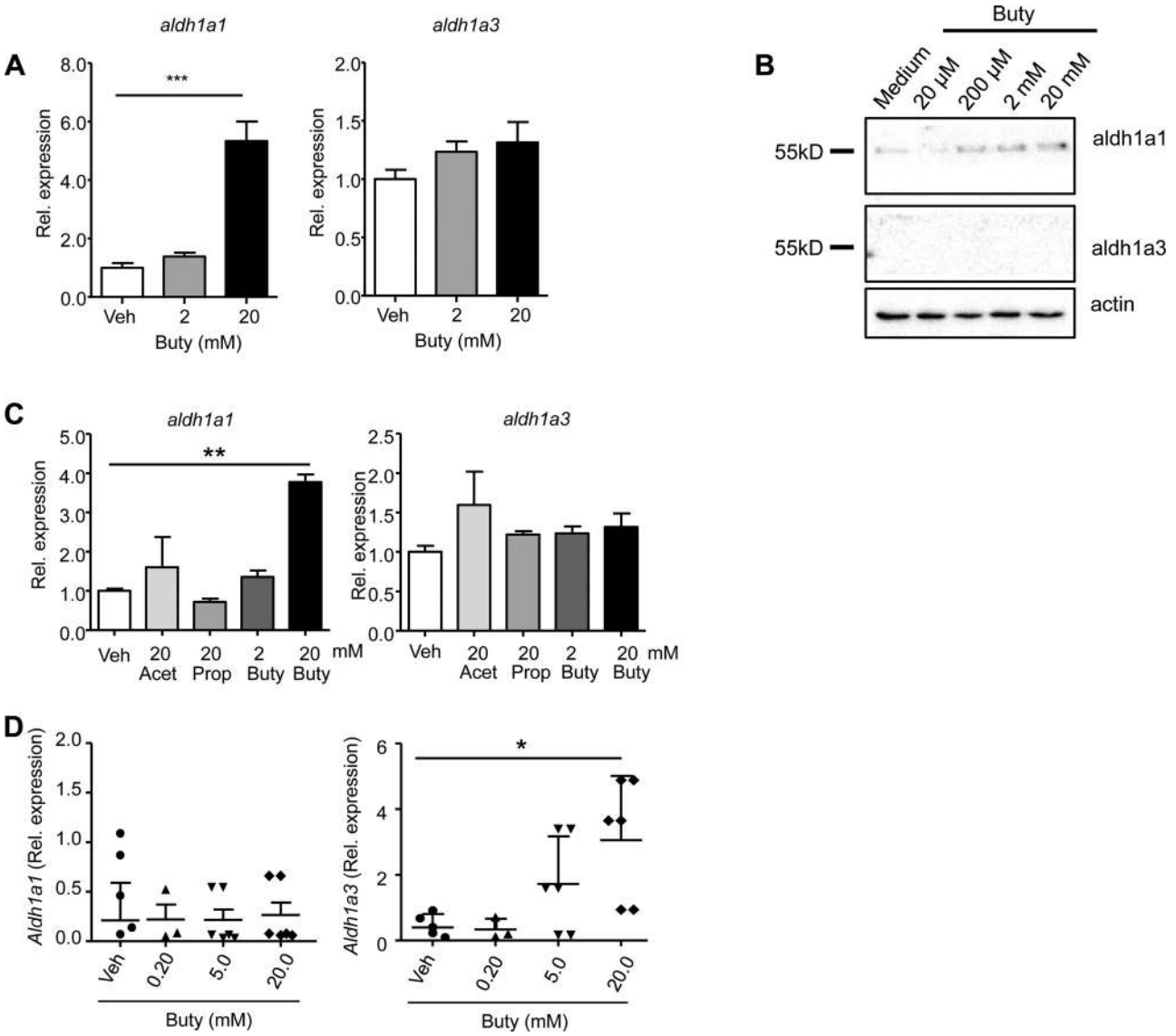


Fig. 1. Butyrate enhances expression of *aldh1a1* and *aldh1a3* in epithelial cells. *A*: expression levels of *aldh1a1* and *aldh1a3* in butyrate (Buty)-treated mouse CMT93 cells. *B*: protein levels of *aldh1a1* and *aldh1a3* in CMT93 protein lysates. *C*: expression levels of *aldh1a1* and *aldh1a3* in CMT93 cells treated with SCFA acetate (Acet), proprionate (Prop), and butyrate at indicated concentrations. *D*: expression levels of *aldh1a1* and *aldh1a3* in butyrate-treated human Caco-2 cells. Data shown are means ± SE, $n = 3$–5 for each concentration. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$. Rel., relative; Veh, vehicle; Raldh1/3, retinaldehyde dehydrogenase 1/3.

would solely involve HDAC3, the silencing of HDAC3 expression would enhance baseline *aldh* expression and render epithelial cells insensitive to butyrate-induced *aldh* upregulation. shRNA-mediated silencing class I HDAC1, -2, or -3 affects pathways that are critical in enteroid stem cell survival and growth of the enteroids, likely by affecting Bcl-xl family member expression (33). Hence, we could not use enteroid cultures to test this. Instead, we used CMT93 cells to transduce shRNA targeting HDAC3. There is a difference between these model systems. In CMT93 cells, butyrate stimulates aldh1a1 but not aldh1a3, whereas in mouse organoids, butyrate stimulates aldh1a3 but not aldh1a1. When repressing HDAC3 expression using lentiviral HDAC3-targeted shRNA in CMT93 cells, no effect on baseline *aldh1a1* expression was seen, and butyrate was equally effective in stimulating *aldh1a1* expression compared with scrambled shRNA (Fig. 4*F*). This indicates that in contrast to mouse enteroids, expression of HDAC3 protein is not critical for the capacity of butyrate to enhance *aldh1a1* in mouse CMT93 cells, which could reflect the difference between these two intestinal models.

Finally, we examined whether enhanced *aldh* expression in enteroids actually reflected dedifferentiation and stemness. Aldh is highly expressed in stem cells of the hematopoietic system and the intestine, where it is assumed that aldh activity is necessary for stem cell differentiation (9). We thus tested whether incubation of enteroids with butyrate affected proliferation and differentiation. When incubating mouse enteroid cultures with 0.5 mM butyrate for 24 and 48 h, no apparent morphological changes in growth were observed. However, clear indications of enhanced differentiation and cell death at 48 h were evident at incubations of 5 mM butyrate (Fig. 5*A*). This was corroborated by elevated expression of differentiation

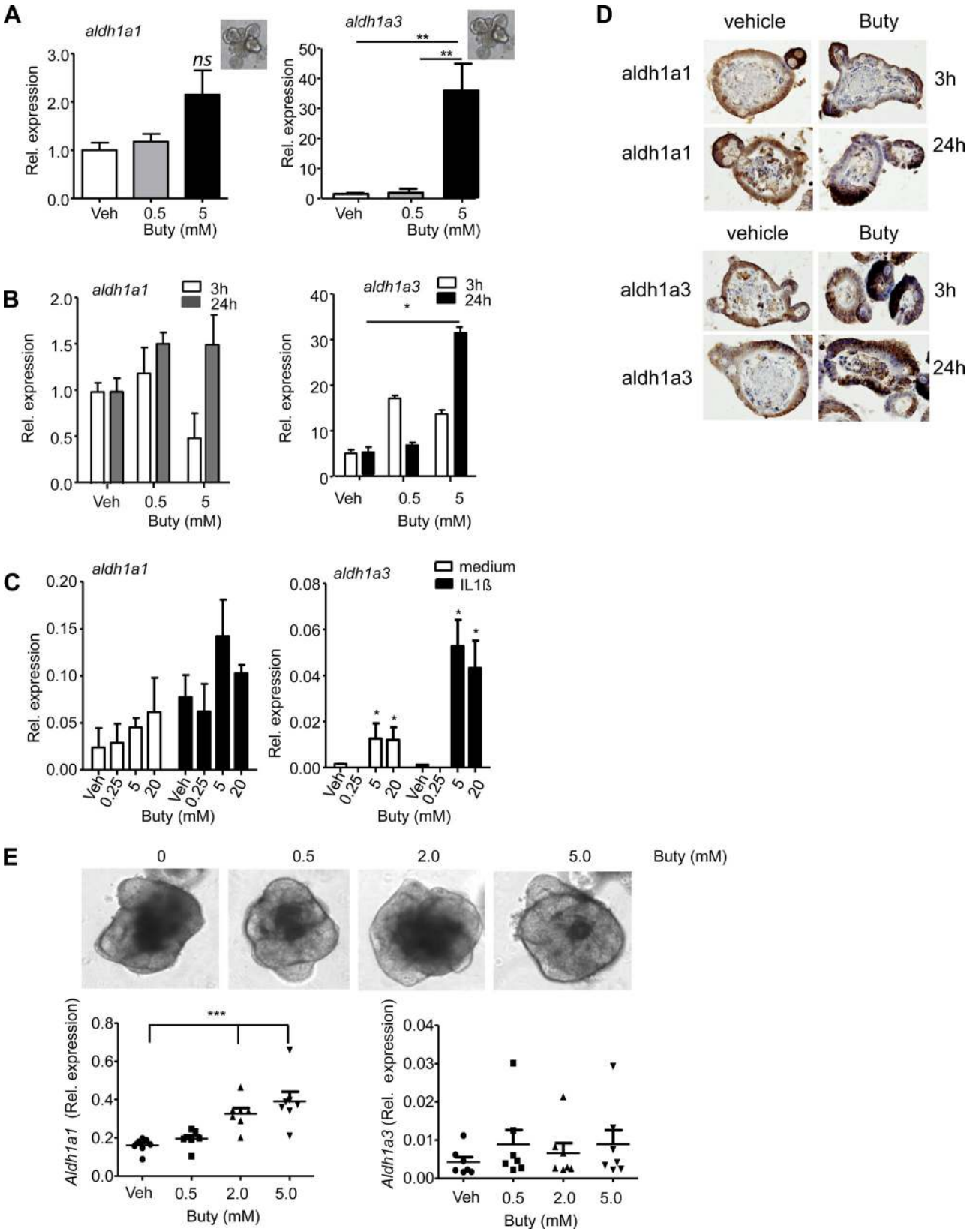


Fig. 2. Butyrate enhances RA signaling in primary epithelial cultures. *A*: *aldh1a1* and *aldh1a3* transcript expression levels in butyrate-treated murine enteroid cultures (*insets*: micrographs showing enteroid). *B*: *aldh1a1* and *aldh1a3* expression in murine enteroids treated with butyrate for 3 h (white bars) or 24 h (gray or black bars). *C*: expression of *aldh1a1* and *aldh1a3* in murine enteroids treated with butyrate in medium (white bars) and in medium containing IL-1β (10 ng/ml; black bars). *D*: murine enteroids treated with butyrate for 3 h (*top rows*) or 24 h (*bottom rows*) and immune labeled for *aldh1a1* and *aldh1a3* where indicated. *E*: *aldh1a1* and *aldh1a3* transcript expression levels in butyrate-treated human enteroid cultures (*top*; butyrate-treated enteroids). Representative enteroid cultures are shown. Data shown in graphs are means ± SE, $n = 3$–5 for each concentration tested. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P \leq 0.001$.

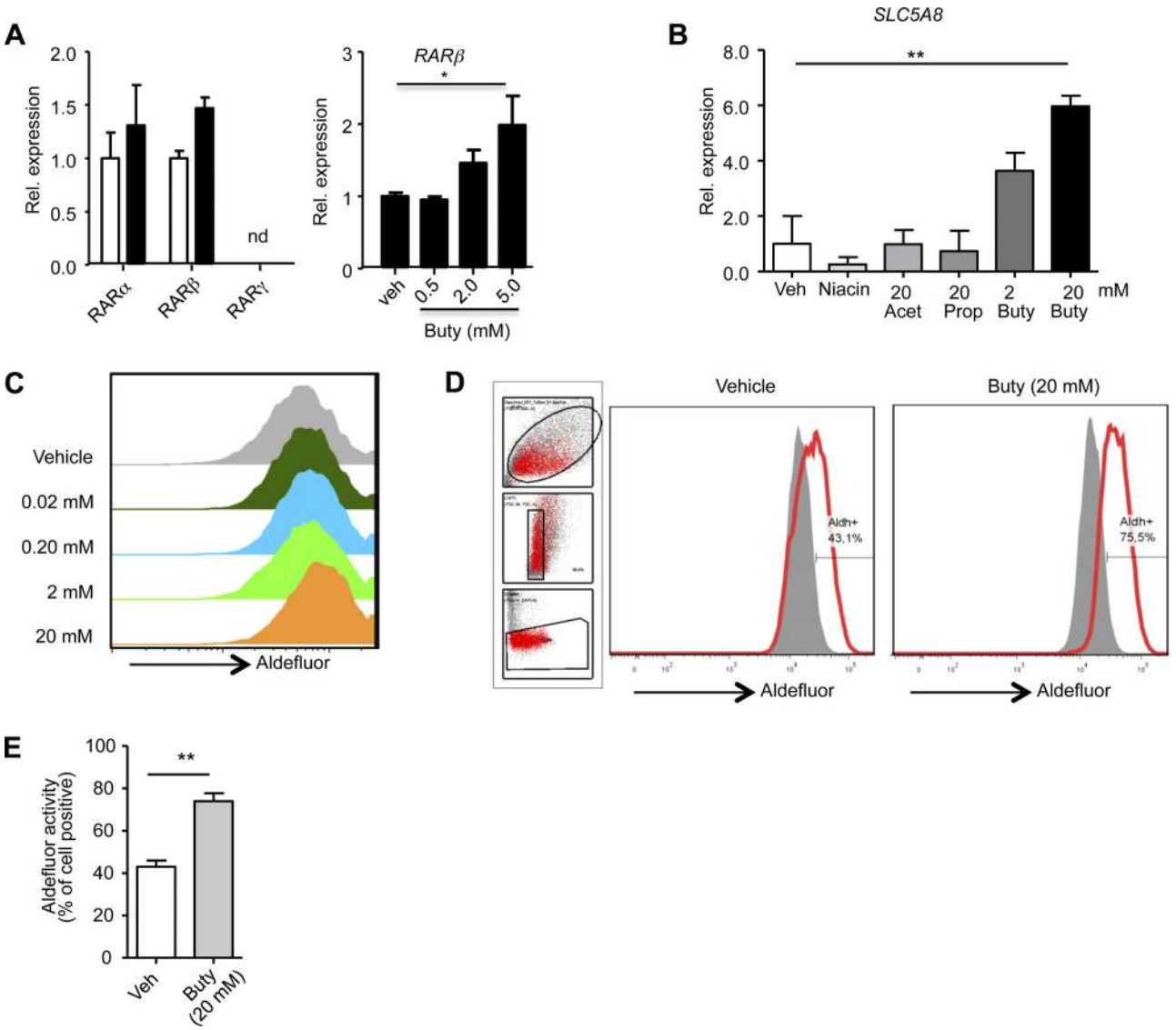


Fig. 3. *A*: expression of retinoic acid receptor (RAR) isoforms in butyrate-treated murine enteroids. *Left*: 2 mM butyrate incubations for 24 h (black bars) and vehicle control (white bars); *right*: *rarβ* expression shown toward increasing butyrate concentrations. *B*: butyrate incubation enhances the expression of the butyrate membrane transporter *slc5a8*. *C* and *D*: Aldefluor activity measured in butyrate (0–20 mM)-treated CMT93 cells. Gray shading, diethylaminobenzaldehyde control. Gating strategy of Adlefluor assay in CMT93 cells is shown (*D*, *left*). *E*: data from *C* and *D* are quantified. Data shown in graphs are means ± SE, $n = 3$–5 for each concentration tested. *$P < 0.05$; **$P < 0.01$.

markers *villin* and *muc2*, observed at 24 h after incubation (Fig. 5*B*). In line, elevated intensity of MUC2 and villin staining was observed after butyrate exposure, whereas Ki67 and lysozyme staining seemed unaffected (Fig. 5*C*). When analyzing the expression of genes related to stem cell activity or Wnt signaling, we did not observe significant changes in *Lgr5*, *ascl2*, or *olfm4* or Wnt target gene *cdkn1*, with the exception of *CD44*, upon treatment with butyrate (Fig. 5*D*), indicating that butyrate is not likely to enhance stemness in enteroid cultures.

DISCUSSION

Metabolites, such as SCFA, are produced in the gut lumen by anaerobic bacterial fermentation of carbohydrates and are accessible to host epithelial cells to influence cellular physiology. Microbiome sequencing studies and metabolic analyses demonstrate an altered microbiome composition in a variety of inflammatory disorders with a relative lower composition of SCFA-producing bacteria in patients compared with healthy individuals (4, 13). The butyrate concentrations found in human colons of healthy individuals vary from 12 to 94 mM (30), in line with the concentrations used in our in vitro study. Notably, in inflammatory bowel disease patients, a marked reduction in SCFA, including butyrate, is well documented (7), although SCFA concentrations probably represent "signatures" rather than being directly related to specific bacterial strain abundances (17).

Besides its role as a major energy source for intestinal epithelial cells, butyrate affects other cellular processes, including the inhibition of HDAC enzymes (16). Here, we show that butyrate enhances the biosynthesis of RA by epithelial cells via HDAC inhibition. RA is generated from retinol (vitamin A) in two steps—the first step involving oxidation of

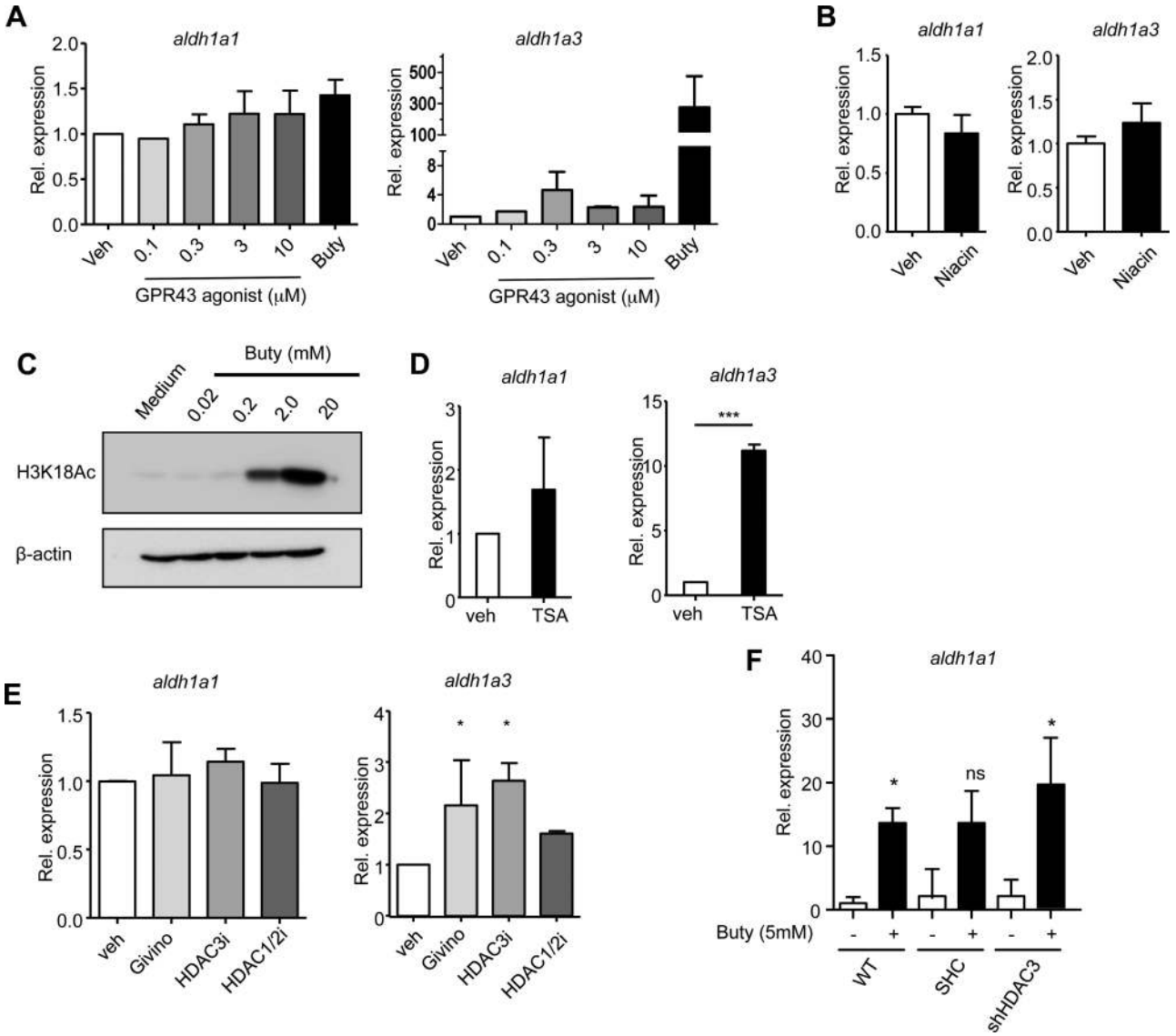
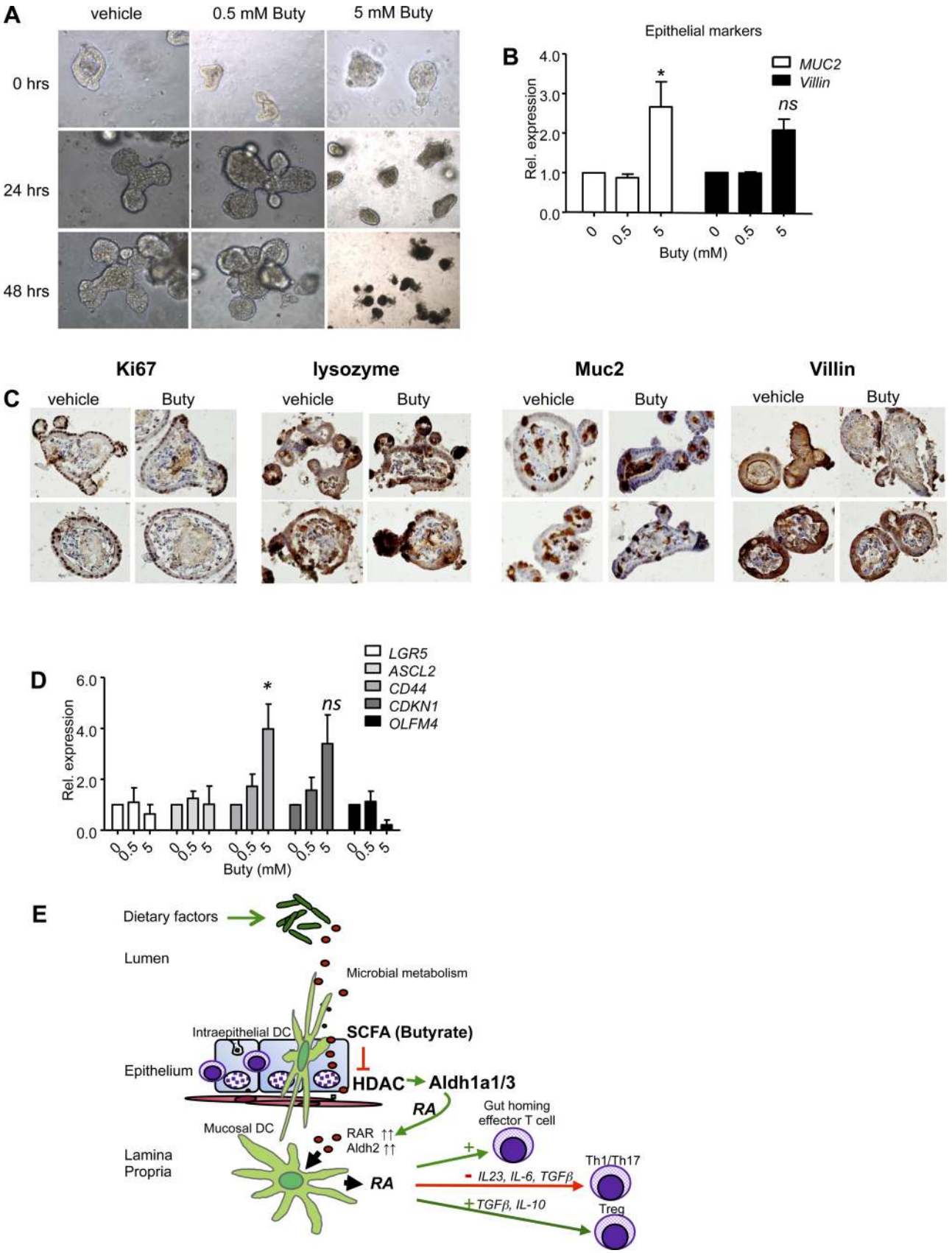


Fig. 4. Inhibition of histone deacetylases 3 (HDAC3) recapitulates the effect of butyrate. *A*: *aldh1a1* and *aldh1a3* expression in enteroids treated with GPR43 agonist (concentrations indicated) and butyrate (2 mM). *B*: *aldh1a1* and *aldh1a3* expression in enteroids treated with GPR109A ligand niacin. *C*: H3K18 acetylation in butyrate (0–20 mM)-treated Caco-2 cells. Beta-actin was used as loading control. *D*: *aldh1a1* (*left*) and *aldh1a3* (*right*) expression in TSA-treated enteroids. *E*: *aldh1a1* and *aldh1a3* expression in enteroids treated with Givinostat (Givino) and specific HDACi, as indicated. *F*: *aldh1a1* expression in CMT93 cells transduced with lentiviral vectors for HDAC3 or control scrambled vector (SHC). WT, wild-type. Data shown are means ± SE, n = 2–5 for each concentration tested. *$P < 0.05$; ***$P \leq 0.001$.

retinol to retinal by a retinol dehydrogenase and the second step involving further oxidation of retinal to all-trans RA by tissue-specific isoforms of aldh1a1-3. Earlier studies have implicated that in the small intestinal epithelium, a relatively large fraction of available retinol is converted to retinyl esters for hepatic storage (3). Our results indicate an important potential of butyrate to redirect the fate of dietary retinol via elevated aldh1a1 and -1a3 activity, which may help the use of available retinal for RA synthesis. Aldhs are a family of evolutionarily conserved enzymes. Nineteen isoforms are expressed in humans, of which aldh1a1, -1a2, and -1a3 function in RA cell signaling by oxidizing vitamin A metabolite retinal to RA (19). We observed that butyrate exclusively upregulates aldh1a1 or -1a3 isoforms, dependent on cell type and species origin of cells. As suggested earlier (19), a differential expression of aldh1a1 and -1a3 in primary cultures and epithelial cell

Fig. 5. Effects of butyrate on enteroid growth. *A*: enteroids incubated with increasing butyrate concentration, 24 and 48 h incubation time. *B*: expression of muc2 and villin in enteroids treated with butyrate (0–5 mM). *C*: immune labeling of enteroids treated with butyrate (0.5 mM, 24 h). The labeling of Ki67, lysozyme, muc2, and villin are shown as indicated. *D*: expression of stem cell marker genes in butyrate-treated enteroids. *E*: proposed scheme of butyrate-induced RA production in epithelia. TGFβ, transforming growth factor β; Th1/17, T helper cell 1/17; Treg, regulatory T cell. Data shown are means ± SE, n = 3–5 for each concentration tested (*B* and *D*). *$P < 0.05$.



lines likely reflects the different origin of these cell types (intestinal stem cells vs. cancerous cells) and underlying genetic differences between cell types and species. Indeed, in our experiments, butyrate enhanced either *aldh1a1* or *aldh1a3*, depending on cell type of species tested.

In the myeloid immune system, raldh has an important modulating role, in particular, with respect to the differentiation of DCs in the intestinal mucosa, the key APCs for activating naive T cells in gut-draining lymphoid organs [see for reviews, for instance, Iwata (15) and Mora and von Andrian (21)]. Earlier studies have suggested that RA production by mucosal DCs and macrophages (23) is imprinted by RA derived from epithelial cells that are in close apposition to mucosal DCs and macrophages by virtue of their expression of CX3CR1 (31). DCs express the three isotypes of RAR, especially RARα, and are able to respond directly to RA required for tolerizing capacity of intestinal $CD11b^+CD103^+$ DCs (32). Moreover, RA has recently been shown to enhance the differentiation of the $CD127^+$ innate lymphoid cell, type 1 to type 3, in the presence of IL-2, IL-23, and IL-1β (2).

Our findings suggest that *aldh1a1*- and *aldh1a3*-upregulated expression and activity in epithelia are the result of the HDAC-inhibiting activity of butyrate, as agonists for butyrate receptors GPR43 and GPR109A failed to mimic butyrate in this effect. Moreover, butyrate, not other SCFAs, stimulated epithelial expression of *slc58a*, in line with the recent report of the potential of butyrate to mediate acetylation of promoter regions of *slc58a* stimulating its expression (20). Notably, >60% of the absorbed butyrate is taken up intracellularly by diffusion rather than being actively imported (21).

From the SCFAs tested, only butyrate (the strongest HDACi) was effective in inducing *aldh* expression. The inhibitory effect of butyrate is strongest on the deacetylase activity of class I HDACs separately. Butyrate specifically inhibited class I HDACs with an IC_{50} between 100 and 700 μM (5). In monocytes, butyrate mediates cytokine modulation via inhibition of HDAC1, and HDAC2, rather than HDAC8 (5), whereas in our study, the activity of butyrate was recapitulated via HDAC3 inhibition in enteroids. As we did not note activity of SCFA other than butyrate on RA production, we conclude that butyrate elevates RA conversion via HDAC inhibition. The specific HDAC responsible cannot be determined for all cell systems tested. In mouse enteroids, inhibition of HDAC3 and not HDAC1/2, using small molecule inhibitors, was effective in upregulating *aldh1a3* expression, mimicking the effect of butyrate in mouse enteroids. This suggests a critical role for HDAC3 inhibition in this process. However, our data cannot definitively demonstrate a similar role for HDAC3 in the potential of butyrate to stimulate *aldh1a1* expression in CMT93 cells, as chemicals of genetic inhibition of HDAC3 did not enhance expression of aldh1a1 in these cells. Several factors may explain this effect. The first and most likely explanation is that the HDAC-related mechanism responsible for the upregulation of *aldh1a1* in CMT93 cells differs from that of *aldh1a3* in enteroid cultures. Second, knockdown of HDAC3 using viral transduction may lead to off-target effects in the transduced cells or activation of alternative routes for butyrate-induced cellular responses. Third, the inhibition of HDAC transcript expression may not be the same as the inhibition of the HDAC enzyme activity compared with what was observed by Sun et al. (27). Notably, we were not able provide the comparative demonstration of the effect of HDAC3-specific knockdown in enteroid cultures so that the demonstration of HDAC3 as a critical mediator is only based on chemical inhibition studies, whereas HDAC3 protein may still exert deacetylase-independent functions.

Besides epithelial cells, other cell types in the mucosa may be targeted by butyrate or other gut microbial metabolites that have HDAC-inhibiting capacities. For instance, HDAC inhibition via propionate was shown to induce higher levels of acetylation at the forkhead box P3 locus in T cells (1). These results were in line with earlier reports showing an increase in number and function of regulatory T cells after HDAC inhibition (28) and with reduced disease severity in HDACi-treated animals in experimental animal models of inflammatory bowel disease (12). Irrespective, our findings imply a new mechanism by which butyrate, through induction of RA synthesis, can contribute to the potential of epithelia to maintain immune homeostasis in the gut.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

R.S., T.T.L., E.A.v.T., and W.J.d.J. conception and design of research; R.S. and C.V. performed experiments; R.S., C.V., J.S., V.M., E.A.v.T., and W.J.d.J. analyzed data; J.S., V.M., G.R.v.d.B., E.A.v.T., and W.J.d.J. interpreted results of experiments; C.V., E.A.v.T., and W.J.d.J. prepared figures; R.S., E.A.v.T., and W.J.d.J. drafted manuscript; T.T.L. edited and revised manuscript; V.M., G.R.v.d.B., E.A.v.T., and W.J.d.J. approved final version of manuscript.

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