# Microbiota Metabolite Butyrate Differentially Regulates Th1 and Th17 Cells' Differentiation and Function in Induction of Colitis

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**Background:** How the gut microbiota regulates intestinal homeostasis is not completely clear. Gut microbiota metabolite short-chain fatty acids (SCFAs) have been reported to regulate T-cell differentiation. However, the mechanisms underlying SCFA regulation of T-cell differentiation and function remain to be investigated.

**Methods:** CBir1, an immunodominant microbiota antigen, transgenic T cells were treated with butyrate under various T-cell polarization conditions to investigate butyrate regulation of T-cell differentiation and the mechanism involved. Transfer of butyrate-treated CBir T cells into $Rag1^{-/-}$ mice was performed to study the in vivo role of such T cells in inducing colitis.

**Results:** Although butyrate promoted Th1 cell development by promoting IFN-γ and T-bet expression, it inhibited Th17 cell development by suppressing IL-17, Rorα, and Rorγt expression. Interestingly, butyrate upregulated IL-10 production in T cells both under Th1 and Th17 cell conditions. Furthermore, butyrate induced T-cell B-lymphocyte-induced maturation protein 1 (Blimp1) expression, and deficiency of Blimp1 in T cells impaired the butyrate upregulation of IL-10 production, indicating that butyrate promotes T-cell IL-10 production at least partially through Blimp1. $Rag1^{-/-}$ mice transferred with butyrate-treated T cells demonstrated less severe colitis, compared with transfer of untreated T cells, and administration of anti-IL-10R antibody exacerbated colitis development in $Rag^{-/-}$ mice that had received butyrate-treated T cells. Mechanistically, the effects of butyrate on the development of Th1 cells was through inhibition of histone deacetylase but was independent of GPR43.

**Conclusions:** These data indicate that butyrate controls the capacity of T cells in the induction of colitis by differentially regulating Th1 and Th17 cell differentiation and promoting IL-10 production, providing insights into butyrate as a potential therapeutic for the treatment of inflammatory bowel disease.

**Key Words:** butyrate, Th1 cells, Th17 cells, IL-10, colitis

## INTRODUCTION

The intestines harbor trillions of bacteria that are indispensable for maintaining intestinal homeostasis. Gut microbiota metabolites regulate host immunity and maintain homeostasis as a result of long-term coevolution of the host and the microbiota.[1] In recent years, emerging evidence has indicated that gut microbiota metabolite short-chain fatty acids (SCFAs) appear to mediate host–microbe interactions.[2, 3] Among SCFAs, butyrate acts as an effective molecule in immune regulation and provides an energy source for colonic epithelial cells.[4] Moreover, butyrate is readily absorbed in the colonocyte and transported into blood circulation mainly in 3 different ways, that are, simple diffusion, carrier-mediated transportation, and binding to G protein–coupled receptor (GPCR), which mediates a significant portion of butyrate function.[3, 5] It has been shown that a lower concentration of butyrate could promote interleukin (IL) 10-expressing $Foxp3^{+}$ regulatory T cells, through binding to GPR43 and inhibition of histone deacetylase (HDAC),[6] whereas high concentration of butyrate induces transcription factor T-bet expression.[7] Butyrate and the other 2 most abundant SCFAs, acetate and propionate, can also regulate the differentiation of effector T cells, which are primarily dependent on inhibition of HDAC activity but in a GPR43-independent manner.[8] However, whether butyrate differentially regulates the differentiation of Th1 and Th17 cells and the underlying mechanisms remains unclear.

Recent studies have indicated that butyrate is involved in the regulation of a number of pathological processes. Butyrate has been shown to maintain gut immune homeostasis by inhibiting inflammation[9] and promoting tissue repair.[1] Moreover, butyrate treatment is able to ameliorate experimental

Received for publications September 23, 2018; Editorial Decision February 14, 2019.

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Conflicts of interest: The authors declare no competing interests.

Supported by: This work was supported by NIH grants DK098370, DK105585, and DK112436.

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doi: 10.1093/ibd/izz046
Published online 27 March 2019

autoimmune encephalomyelitis and reduce axonal damage in the central nervous system.[10] It has been shown that butyrate enhances phagocytic capacity of dendritic cells in the lung[11] and induces T-cell-mediated hydronephrosis and ureteritis.[12] Thus, the effects of butyrate in regulating immune cells, such as T cells, in different tissues appear very complex.

In this report, we demonstrate that butyrate differentially regulated Th1 and Th17 cell development in the intestines. Although butyrate promoted Th1 cell development by upregulating T-bet expression, it inhibited Th17 cell development by inhibiting Rorγt and other Th17-associated transcription factors. Importantly, butyrate induced IL-10 production in T cells during Th1 and Th17 cell differentiation. Functionally, butyrate inhibited the capability of T cells in the induction of colitis, thus contributing to the maintenance of intestinal homeostasis.

## METHODS

### Mice

C57BL/6 (B6), B6.129-Prdm1tm1Clme/J (Blimp-1$^{fl/fl}$) mice, and Rag1$^{-/-}$ mice were obtained from the Jackson Laboratory. GPR43$^{-/-}$ mice were obtained from Bristol-Myers Squibb. GPR43$^{-/-}$ CBir1 transgenic (Tg) mice were generated by crossing CBir1 Tg mice with GPR43$^{-/-}$ mice. All mice were bred and maintained in the UTMB animal care facility. Animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) of UTMB.

### Antibodies and Reagents

Anti-CD3, anti-CD28, anti-IL-4, anti-IFNγ, and anti-IL-10 receptor antibodies were obtained from Bio X Cell (West Lebanon, NH, USA). GolgiStop was obtained from BD Biosciences (San Diego, CA, USA). Fluorochrome-conjugated anti-mouse CD4, CD25, CTAL4, ICOS, GITR, IFN-γ, and IL-17 antibodies were obtained from Biolegend (San Diego, CA, USA). Anti-Foxp3 antibody was purchased from Invitrogen (Carlsbad, CA, USA). Foxp3 staining buffer sets, a Live/Dead Fixable Dead Cell Stain Kit, and a Celltrace carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit were purchased from Invitrogen (Carlsbad, CA, USA). Recombinant IL-12, TGFβ1, and IL-6 were obtained from Biolegend (San Diego, CA, USA). Butyrate was obtained from Sigma-Aldrich (St. Louis, MO, USA).

### Primary T-Cell Isolation and Culture

Anti-Mouse CD4 Magnetic Particles (BD Biosciences) was used to isolate the naïve CD4$^+$ T cells from the spleen of CBir1 Tg mice.[13] For Th1 cell cultures, T cells were activated with CBir1 peptides and irradiated splenic antigen-presenting cells (APCs) under the treatment of 10 ng/mL of IL-12. For Th17 cell cultures, CD4$^+$ T cells, activated with CBir1 antigen and irradiated APCs, were stimulated with 20 ng/mL of IL-6, 10 ng/mL of TGF-β1, 10 μg/mL of anti-IL-4, and 10 μg/mL of anti-IFNγ; 5 ng/mL of TGF-β1 was added for T-cell culture for Treg polarization. Butyrate (0.5 mM) was used to treat T cells.

### Flow Cytometry

After stimulating with PMA and ionomycin, followed by Golgistop, T cells were incubated with live/dye and anti-CD4 antibody, followed by fixation and permeabilization. Subsequently, the cells were stained with different antibodies as indicated in text. Finally, samples were assessed using LSRII/Fortessa and analyzed by FlowJo software.

### Real-time Quantitative Reverse Transcription Polymerase Chain Reaction

Trizol (Life Technologies, Carlsbad, CA, USA) was used to extract total RNA, and sebusequently cDNA was synthesized. T-bet, Prdm1, Rorα, Rorγt, Prdm1, Gata3, Batf, and gapdh expression was measured using TaqMan Gene Expression Assays (Bio-Rad, Hercules, CA, USA), and all the primers and probes were predesigned and purchased from ThermoFisher.

### T-Cell Suppression Assay

$2 \times 10^5$ CFSE-labled CBir1 Tg-naïve CD4$^+$ T cells, activated with $2 \times 10^5$ irradiated splenic APCs and CBir1 flagellin, were co-cultured with or without $2 \times 10^5$ control Treg cells or butyrate-treated Treg cells. The cells were collected, and cell proliferation was analyzed by flow cytometry (FACS) on day 3.

### Induction of Colitis

CBir1 Tg CD4$^+$ T cells were cultured under Th1 and Th17 conditions with or without butyrate (0.5 mM) for 5 days; $1 \times 10^6$ T cells, cultured under various conditions, were transferred into Rag1$^{-/-}$ mice through tail vein injection. According to study designs, the mice were treated with anti-IL-10R antibody or control IgG intraperitoneally twice a week.

### Preparation of Lamina Propria Cells

After washing, intestinal tissues were incubated with 0.5 mM of EDTA to remove the epithelial cells. Subsequently, 5 mg/mL of DNase I and 0.5 g/mL of collagenase intravenously were used to digest tissues. Finally, lamina propria cells were purified using Percoll (40%/75%).

### Ex Vivo Colon Organ Culture and Enzyme-Linked Immunosorbent Assay

Two pieces of biopsies from the ascending colon were collected and cultured in RPMI 1640 with 10% FBS, HEPES, sodium pyruvate, 2-ME, and penicillin-streptomycin in an incubator for 24 hours. Supernatants were collected, and the cytokines were measured by enzyme-linked immunosorbent assay (ELISA; Biolegend, San Diego, CA, USA).

## Histopathological Assessment

As described previously,[14] at necropsy, the colon and cecum were dissected, and Swiss rolls were performed. Samples were fixed, embedded, and sliced. Hematoxylin and eosin staining was then performed for analysis of colitis severity by using histological scoring based on a modified scoring system.

## Statistical Analysis

All the statistical analysis in this study was performed using Prism 6.0 (San Diego, CA, USA). The Student *t* test was used to analyze the difference between 2 groups, and one-way analysis of variance (ANOVA) was used when groups >=3 For comparing pathology scores, the nonparametric Mann-Whitney *U* test was performed. Data are shown as mean ± SD, and a *P* value of <0.05 was considered statistically significant.

# RESULTS

## Butyrate Promotes Th1 Cell But Inhibits Th17 Cell Development

Butyrate has recently been reported to facilitate Treg cell differentiation and possibly affect the development of other T-cell subsets.[6, 8, 15, 16] To investigate the mechanisms by which butyrate regulates the differentiation of microbiota antigen-specific Th1 and Th17 cells, we cultured $CD4^+$ T cells from the spleen of CBir1 (an immunodominant microbiota antigen)[17] Tg mice with or without butyrate under Th1 polarization conditions or Th17 polarization conditions. On day 5, cytokine levels in T cells were analyzed by FACS. Consistent with previous reports,[8] butyrate promoted IFN-γ production under Th1 conditions (Fig. 1A). However, butyrate inhibited IL-17 production under Th17 conditions (Fig. 1B). Interestingly, butyrate increased T-cell IL-10 production under both Th1 and Th17 conditions (Fig. 1A and B). We also collected the culture supernatants and measured IL-10, IFN-γ, and IL-17 by ELISA. Butyrate promoted both IL-10 and IFN-γ production under Th1 conditions, whereas it inhibited IL-17 but promoted IL-10 under Th17 conditions (Fig. 1C and D). Moreover, we obtained similar results using $CD4^+$ T cells from B6 mice activated with anti-CD3/CD28 mAb (Supplementary Figure 1A and B). Next, CBir1 Tg $CD4^+$ T cells were cultured with or without butyrate under Treg conditions. We confirmed that butyrate facilitated Treg differentiation in vitro (Supplementary Figure 2A), which was consistent with a previous study.[8] To determine whether butyrate could affect Treg immunophenotypes in addition to Foxp3, we also measured GITR, ICOS, CTLA4, and CD25 in T cells and found that T cells treated with butyrate expressed higher levels of ICOS and CTLA4, but not GITR and CD25, compared with control T cells under Treg conditions (Supplementary Figure 2B). Next, suppressive assay was performed to check whether butyrate affects the regulatory functions of Treg cells. As shown in Supplementary Figure 2C, the suppressive capacity of butyrate-treated Tregs was enhanced compared with control Treg cells.

To determine whether butyrate regulates Th1 and Th17 cell differentiation in vivo, naïve $CD4^+$ T cells from CBir1 Tg mice were isolated and transferred into $Rag1^{-/-}$ mice through intravenous injection. The mice then received drinking water along or butyrate in drinking water. Ten days later, the mice were sacrificed, and T-cell cytokine production in the spleen, mesenteric lymph node, and intestinal lamina propria (LP) of recipient mice was analyzed by FACS. Feeding with butyrate increased the percentages of IFN-$\gamma^+$ and IL-$10^+$ T cells, but significantly decreased IL-$17^+$ T cells in LP (Fig. 1E). Of note, although it has been reported previously that feeding SCFAs, including butyrate, promoted $Foxp3^+$ Treg cells in intestines, we did not observe an increase of Foxp3 expression in our experimental settings (Fig. 1E). Taken together, these data suggest that butyrate differentially regulates Th1 and Th17 cell development.

## Butyrate Promotes T-Cell Expression of T-bet Under Th1 Conditions, Whereas It Inhibits Th17-Associated Transcription Factors Under Th17 Polarized Conditions

T-bet (encoded by Tbx21) serves as a master transcription factor for Th1 cell development.[18, 19] To determine whether butyrate promotes Th1 cell development through regulation of T-bet, we treated CBir1 Tg $CD4^+$ T cells with or without butyrate under Th1 conditions and examined T-bet expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Consistent with previous studies,[8] butyrate promoted T-cell expression of T-bet (Fig. 2A).

We then investigated whether butyrate inhibits transcription factors associated with Th17 cells, including Rorγt, Rorα, Runx1, and Batf.[20–22] CBir1 Tg $CD4^+$ T cells were treated with or without butyrate under Th17 conditions, and the expression of different transcription factors was determined by qRT-PCR. As shown in Figure 2B, butyrate inhibited the expression of Rorγt, Rorα, Runx1, and Batf.

## Blimp1 Mediates Butyrate Regulation of T-Cell IL-10 Production

Next, we determined the mechanisms by which butyrate induces IL-10 in T cells during Th1 and Th17 differentiation. The role of B-lymphocyte-induced maturation protein 1 (Blimp-1) has been demonstrated in regulating T-cell IL-10 production.[23] We first generated T cells by culturing naïve $CD4^+$ T cells with irradiated APCs and CBir1 peptide with or without butyrate for 48 hours. We found that butyrate-treated $CD4^+$ T cells expressed higher levels of Blimp1 (Fig. 3A). Next, we measured Blimp1 expression in T cells treated with butyrate under Th1 or Th17 conditions. As shown in Figure 3B, butyrate upregulated T-cell Blimp1 expression under both Th1 and Th17 conditions.

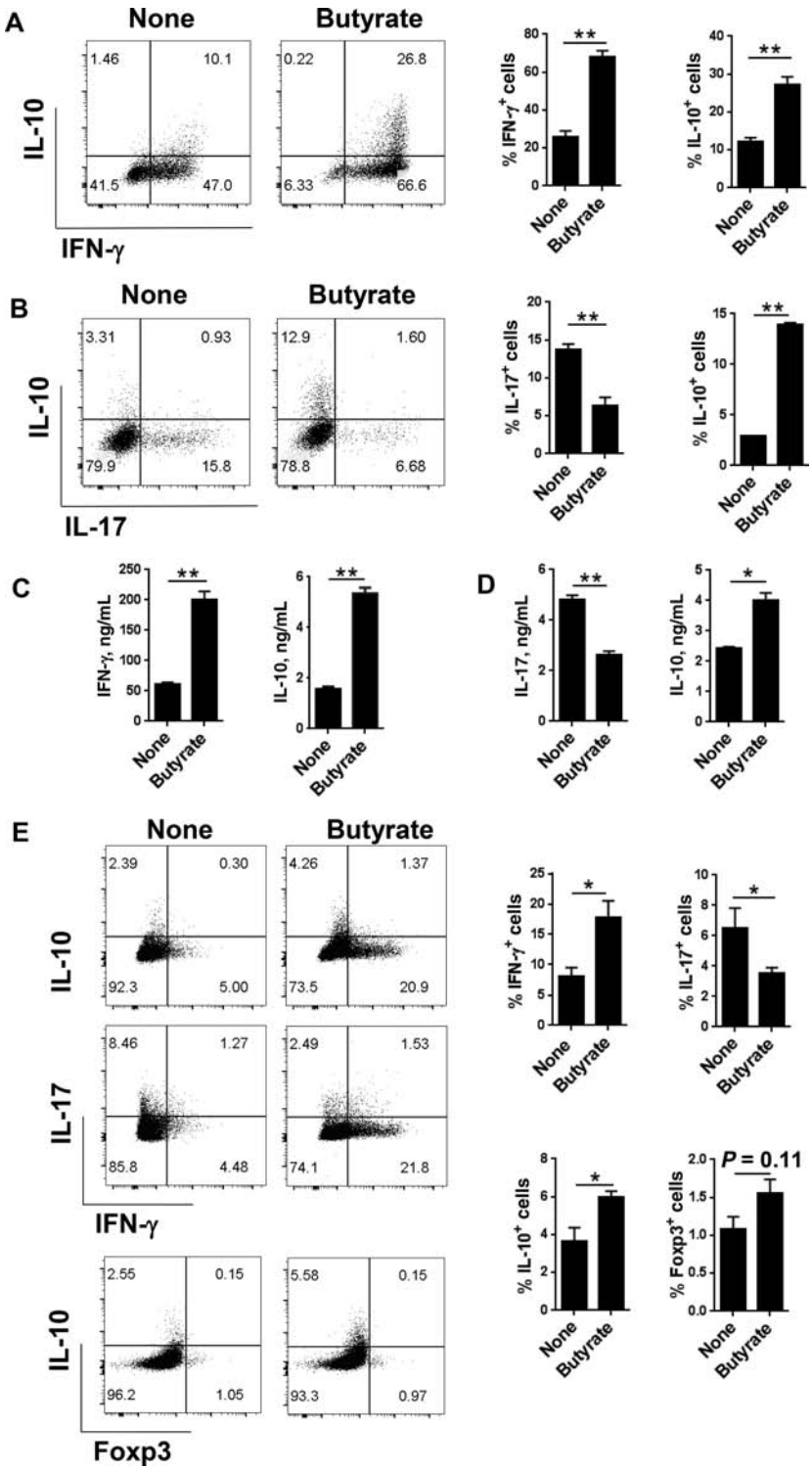


FIGURE 1. Butyrate differentially regulated the Th1 and Th17 cells' differentiation. A and B, CBir1 Tg-naïve CD4+ T cells were cultured with irradiated APCs and CBir1 peptide in the presence or absence of butyrate (0.5 mM) under Th1 (IL-12) (A) and Th17 (TGF-β and IL-6) (B) conditions for 5 days. The expression of IL-10, IFN-γ, and IL-17 was examined by flow cytometry. C and D, The IFN-γ, IL-17, and IL-10 production in Th1 (C) and Th17 (D) cell culture supernatants was measured by ELISA. E, CBir1 Tg CD4+ T cells were injected IV into groups of Rag1$^{-/-}$ mice, and the mice were fed with or without butyrate (300 mM) in drinking water. Lamina propria CD4+ T-cell cytokine production was determined by flow cytometry 10 days after T-cell transfer. Plot numbers represented the percentage of CD4+ T cells in the respective quadrants. Results were shown as mean ± SD. One representative of 3 experiments was performed. Student *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$.

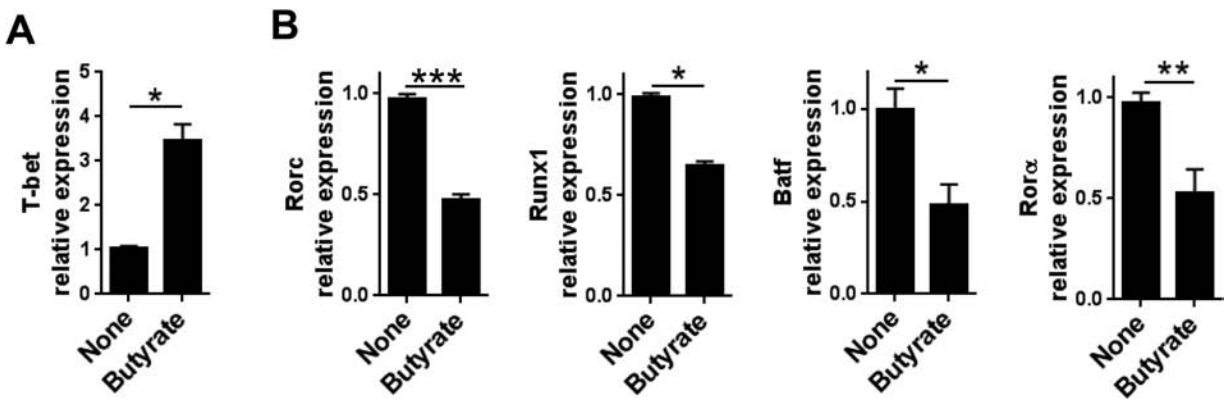


FIGURE 2. Butyrate differentially regulated T-cell gene expression under Th1 and Th17 conditions. CBir1 Tg-naïve CD4+ T cells were cultured under Th1 and Th17 conditions with or without butyrate (0.5 mM) as indicated. A and B, The gene expression of T-bet under Th1 conditions (A) and Rorα, Rorγt, Runx1, and Batf under Th17 conditions (B) was examined by real-time PCR and normalized against gapdh. The results are shown as mean ± SD. One representative of 3 experiments was performed. Student *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

To investigate whether Blimp1 is involved in butyrate regulation of IL-10 by T cells, wild-type (WT; $CD4^{Cre}Prdm1^{+/fl}$) and CD4-specific $Prdm1^{-/-}$ (CKO: $CD4^{Cre}Prdm1^{fl/fl}$)-naïve $CD4^+$ T cells were cultured with or without butyrate under Th1 and Th17 conditions. Although there was no significant difference of the induction of IFN-γ and IL-17 by butyrate in WT and CKO T cells, deficiency of Prdm1 impaired IL-10 production, which was induced by butyrate in WT T cells under both Th1 (Fig. 3C) and Th17 conditions (Fig. 3D). We further confirmed the results by measuring IL-10 in culture supernatants (Fig. 3E). Collectively, these data indicate that increased Blimp-1 is required for IL-10 production induced by butyrate in T cells under Th1 and Th17 conditions.

## Butyrate-Treated T Cells Under Th1 and Th17 Conditions Induce Less Severe Colitis

As butyrate differentially regulates development of Th1 and Th17 cells, which is crucial in pathogenesis of colitis, we then investigated whether butyrate regulates capability of microbiota antigen-specific T cells in inducing colitis in vivo. CBir1 Tg $CD4^+$ T cells were treated with or without butyrate under Th1 or Th17 conditions and transferred into $Rag1^{-/-}$ mice through intravenous injection. We have shown previously that CBir1 Tg Th1 and Th17 cells induced colitis when transferred into $Rag1^{-/-}$ mice.[24] Four to 6 weeks later, these mice were sacrificed, and the histopathology of the colon and cecum was examined. $Rag1^{-/-}$ recipient mice that received butyrate-treated T cells under Th1 conditions developed milder colitis than the $Rag^{-/-}$ mice that received control T cells, as demonstrated by histopathology (Fig. 4A and B). $CD4^+$ T-cell cytokine levels in LP were also measured by FACS. Compared with $Rag1^{-/-}$ mice that received control T cells, $Rag1^{-/-}$ mice that received butyrate-treated T cells under Th1 conditions showed lower levels of IFN-γ-producing $CD4^+$ T cells but increased IL-10-producing $CD4^+$ T cells (Fig. 4C). In addition, colonic tissue produced more IL-10, but less pro-inflammatory cytokines (eg, TNFα, IFN-γ, and IL-17), in $Rag1^{-/-}$ recipient mice with butyrate-treated T cells under Th1 conditions (Fig. 4D). Similarly, $Rag1^{-/-}$ mice with butyrate-treated T cells under Th17 conditions demonstrated lower-severity colitis compared with $Rag1^{-/-}$ mice receiving control T cells (Fig. 5A and B). Consistently, there were fewer IFN-γ-producing and IL-17-producing T cells, but more IL-10-producing T cells in the LP of the $Rag1^{-/-}$ mice that received butyrate-treated T cells under Th17 conditions (Fig. 5C). Furthermore, increased IL-10 production and decreased pro-inflammatory cytokine expression (eg, IFN-γ, IL-17, and IL-6) were found in colonic organ cultures in the $Rag1^{-/-}$ recipients with butyrate-treated T cells under Th17 conditions (Fig. 5D).

These data indicate that butyrate inhibits the potential of microbiota-specific effector T cells during their differentiation in the induction of colitis.

## Butyrate Induction of IL-10 in T Cells Under Th1 Conditions Contributes to Less Severe Colitis

To investigate whether the butyrate-induced IL-10 in T cells during Th1 differentiation contributes to alleviating colitis, we treated CBir1 Tg $CD4^+$ T cells with or without butyrate under Th1 conditions and transferred them into $Rag1^{-/-}$ mice through intravenous injection. Additionally, a group of mice that receved butyrate-treated T cells under Th1 conditions were treated with anti-IL-10R antibody intraperitoneally twice a week, whereas the other groups of mice were administrated with IgG as controls. When the mice were sacrificed 6 weeks later, we found that administration of anti-IL-10R antibody worsened the colitis severity in $Rag1^{-/-}$ mice that received butyrate-treated T cells under Th1 conditions compared with IgG-treated $Rag1^{-/-}$ mice that received butyrate-treated T cells under Th1 conditions (Fig. 6A and B). Moreover, treatment with anti-IL-10R antibody increased IFN-$\gamma^+$ T cells but decreased IL-$10^+$ T cells

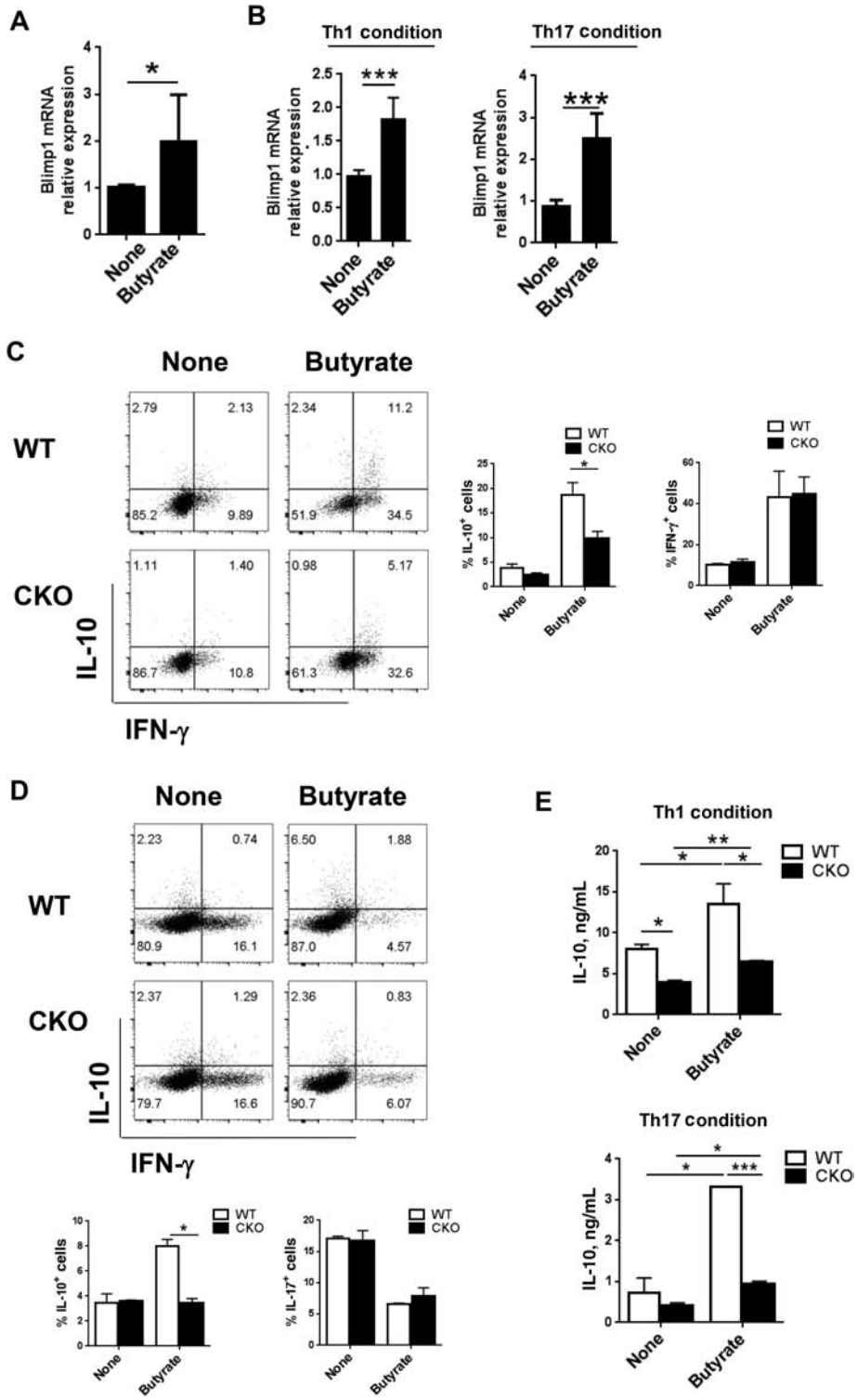


FIGURE 3. Butyrate induced IL-10 production by T cells through Blimp1. A, CBir1 Tg $CD4^+$ T cells were cultured with Cbir1 peptide and irradiated APCs in the presence or absence of butyrate (0.5 mM). The gene expression of Blimp-1 and IL-10 was determinated by real-time PCR. B, Blimp1 expression in $CD4^+$ T cells, cultured under Th1 and Th17 conditions, was examined by real-time PCR. C and D, $CD4^{Cre}Blimp1^{+/fl}$ (WT) and $CD4^{Cre}Blimp1^{fl/fl}$ (CKO) $CD4^+$ T cells were cultured with irradiated APCs and anti-CD3 (5 μg/mL) in the presence or absence of butyrate (0.5 mM) for 5 days. The expression of IL-10 and IFN-γ in Th1 cells (C) and IL-10 and IL-17 in Th17 cells (D) was determined by flow cytometry. E, The production of IL-10 in Th1 culture supernatants and Th17 culture supernatants was measured by ELISA. Results were shown as mean ± SD. One representative of 3 experiments was performed. Student *t* test, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

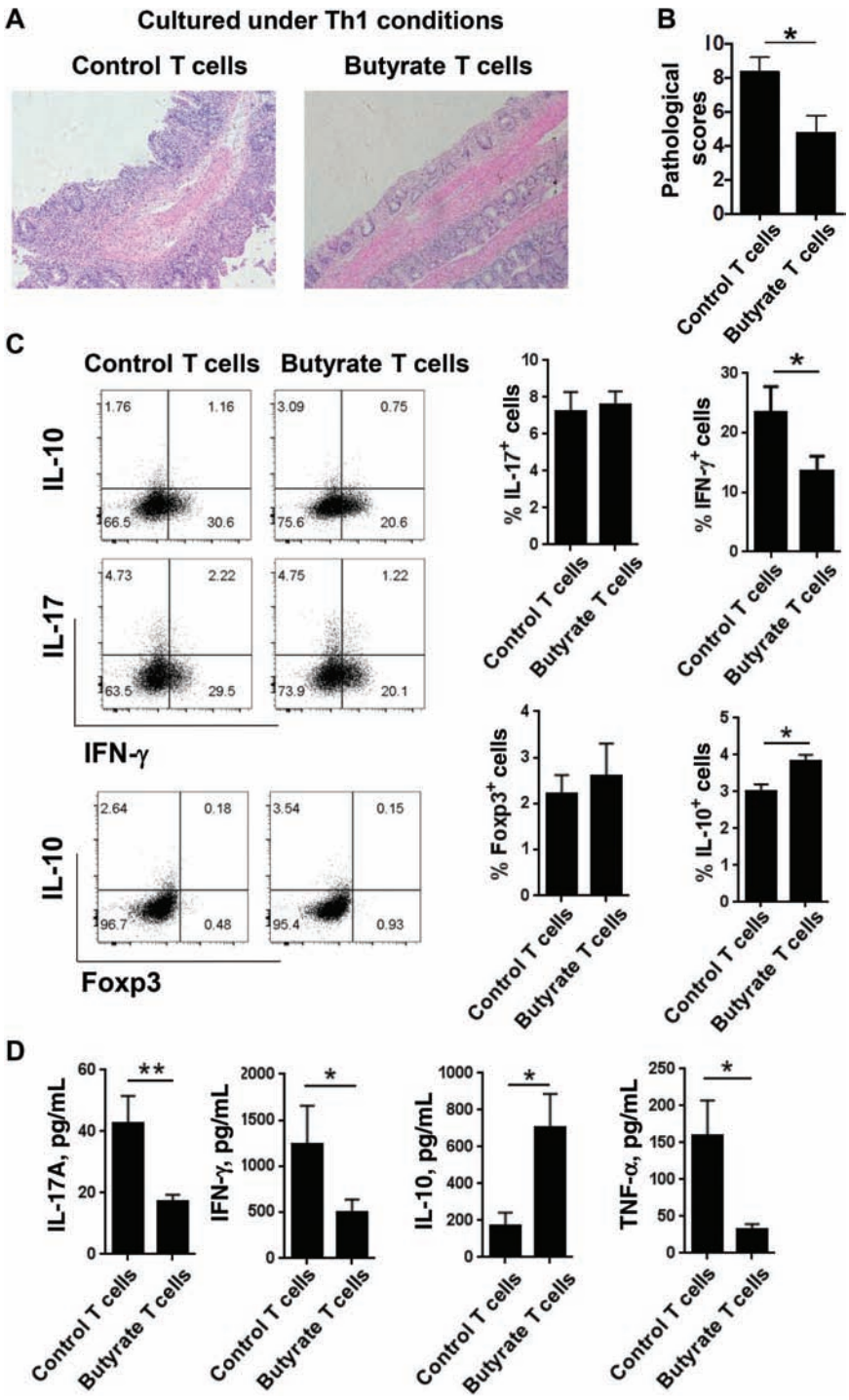


FIGURE 4. Butyrate inhibited pathogenicity of CBir1 Tg T cells cultured under Th1 conditions. CBir1 Tg T cells cultured under Th1 conditions in the presence or absence of butyrate were injected IV into groups of Rag1$^{-/-}$ mice. Mice were killed 4–6 weeks after T-cell transfer. A and B, Assessment of the severity of intestinal inflammation. Colonic histopathology was conducted using hematoxylin and eosin staining (A), and histological scores were examined and compared between the 2 groups by Mann-Whitney *U* test, $^{*}P < 0.05$. B and C, LP CD4$^{+}$ T-cell cytokine production was determined by flow cytometry. D, Colonic tissues were cultured ex vivo for 24 hours, and the relative cytokine production was measured by ELISA. Plot numbers represent the percentage of CD4$^{+}$ T cells in the respective quadrants. Results are shown as mean ± SD. One representative of 3 experiments was performed. Student *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$.

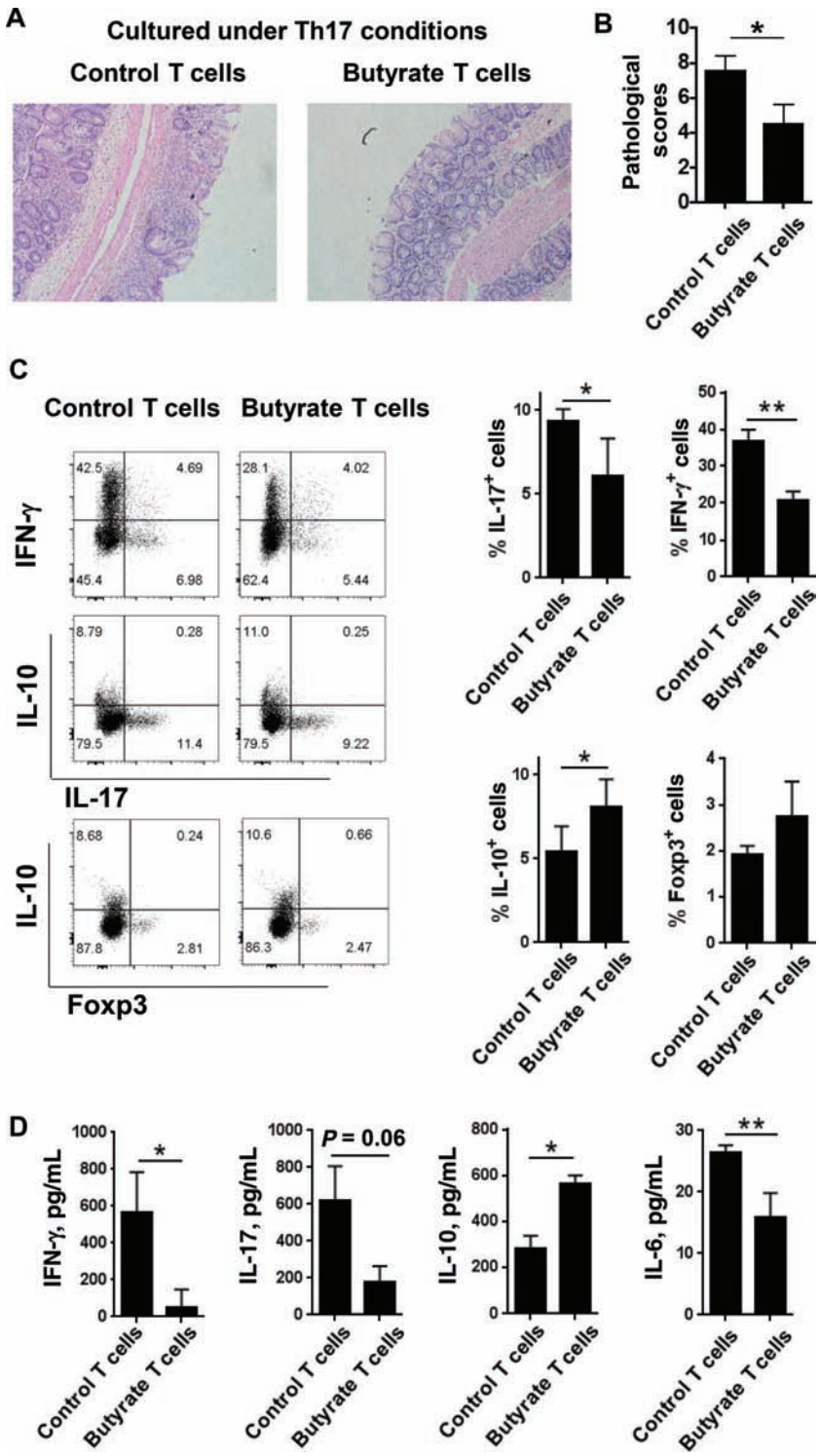


FIGURE 5. Butyrate inhibited pathogenicity of CBir1 Tg T cells cultured under Th17 conditions. CBir1 Tg T cells cultured under Th17 conditions in the presence or absence of butyrate were injected IV into Rag1$^{-/-}$ mice. Mice were killed, and the severity of intestinal inflammation was assessed 4–6 weeks after T-cell transfer. A and B, Colonic histopathology was conducted using hematoxylin and eosin staining (A), and colonic histological scores were examined and compared between the 2 groups by Mann-Whitney *U* test, $^{*}P < 0.05$. B and C, LP CD4$^{+}$ T-cell cytokine production was determined by flow cytometry. D, Colonic tissues were cultured for 24 hours, and cytokine production was measured by ELISA. Results are shown as mean ± SD. One representative of 3 experiments was performed. Student *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$.

in the intestine of Rag1$^{-/-}$ mice that received butyrate-treated T cells under Th1 conditions, whereas anti-IL-10R administration did not affect Foxp3$^{+}$ and IL-17$^{+}$ T cells in LP (Fig. 6C). Treatment of anti-IL-10R antibody increased levels of TNF-α, IFN-γ, and IL-6 but decreased production of IL-10 in the colon tissues of the Rag1$^{-/-}$ mice reconstituted with butyrate-treated T cells under Th1 conditions (Fig. 6D).

In summary, these data reveal a critical role of butyrate in the induction of IL-10 in T cells during their differentiation in alleviating intestinal inflammation in vivo.

## HDAC Inhibitory Activity, But Not GPR43, Mediates Butyrate Regulation of Th1 Cell Development

Butyrate can function through inhibition of the HDAC or activating the GPCR, such as GPR43, of intestinal cells.[25] To investigate whether GPR43 mediates butyrate regulation of Th1 and Th17 cell differentiaion, T cells were isolated from the spleen of WT CBir1 Tg mice and GPR43$^{-/-}$ CBir1 Tg mice and cultured in the presence or absence of butyrate under Th1 and Th17 polarization conditions. Deficiency of GPR43 did not affect butyrate regulation of Th1 and Th17 cells (Fig. 7A and B). To determine the role of HDAC activity in butyrate regulation of T-cell differentiation, we then treated CBir1 Tg-naïve CD4$^{+}$ T cells under Th1 conditions and Th17 conditions with butyrate or TSA, a known HDAC inhibitor, respectively. TSA treatment increased the IFN-γ and IL-10 levels in T cells under Th1 conditions but did not affect IL-17$^{+}$ and IL-10$^{+}$ T cells under Th17 conditions (Fig. 7C and D). Collectively, these data suggest that butyrate regulates Th1 cell, but not Th17 cell, differentiation through inhibition of HDAC activity in a GPR43-independent manner.

## DISCUSSION

Emerging evidence has demonstrated the crucial role of SCFAs, including butyrate, in the regulation of immune responses and maintenance of intestinal immune homeostasis. Although it has been well established that butyrate promotes Treg cell development, how it regulates Th1 and Th17 cells is still not completely understood. In the current study, we demonstrated that butyrate promoted Th1 development but inhibited Th17 cell differentiation. Furthermore, butyrate upregulated IL-10 production in CD4$^{+}$ T cells during their differentiation into Th1 and Th17 cells and inhibited the potential of those T cells in the induction of colitis.

It has been controversial how butyrate regulates Th17 cell development, as both promoting and inhibiting effects have been reported.[7, 8] In our study, using gut bacterial antigen-specific T cells, butyrate inhibited Th17 cell differentiation under Th17 polarization conditions. Furthermore, feeding butyrate suppressed the differentiation from naïve T cells into Th17 cells in the intestines, thus demonstrating that butyrate inhibits the differentiation of Th17 cells both in vitro and in vivo. This is supported by a recent report, in which it was demonstrated that administration of butyrate in vivo suppresses IL-17 levels in both the plasma and colonic mucosa.[26] We further showed that butyrate inhibited the T-cell expression of Rorγt, Rorα, Runx1, and Batf, all of which are associated with Th17 cell differentiation,[22, 27–29] indicating that butyrate inhibited Th17 cell development possibly through inhibition of Rorγt, Rorα, Runx1, and Batf expression.

IL-10 has been established as a crucial anti-inflammatory cytokine in the maintenance of intestinal homeostasis and inhibition of intestinal inflammation. In addition to Treg cells, both Th1 and Th17 cells also produce IL-10, which has been considered a self-regulating factor that controls Th1 and Th17 cell induction of autoimmune diseases to prevent uncontrolled consequences. We previously demonstrated that SCFA promoted differentiated Th1 effector cells to produce IL-10 production.[30] Our current study showed that butyrate increased IL-10 production in T cells during their differentiation into both Th1 and Th17 cells. Furthermore, adoptively transferred butyrate-treated gut microbiota antigen-specific T cells under Th1 and Th17 polarization conditions resulted in less severe colitis compared with control T cells in Rag1$^{-/-}$ mice. Interestingly, blockade of the IL-10-IL-10R pathway in vivo exacerbated colitis development in Rag1$^{-/-}$ mice receiving butyrate-treated T cells under Th1 conditions, indicating that butyrate-treated T cells during their differentiation restrict excessive intestinal inflammation at least partially through increased production of IL-10.

In this study, we found that butyrate promoted T-cell IFN-γ production in vitro and butyrate facilitated Th1 differentiation in vivo in a healthy context. However, 6 weeks post-transfer of butyrate-treated T cells under Th1 conditions when the Rag1$^{-/-}$ mice developed colitis, T cells in the intestine showed lower IFN-γ levels compared with T cells in Rag1$^{-/-}$ mice reconstituted with control T cells. Multiple reasons could account for these discrepancies. For example, many environmental factors have been changed in the intestine in the context of colitis, such as altered microbiota compositions, which affect T-cell differentiation.[31] Furthermore, the increased levels of IL-10 could suppress IFN-γ production during colitis development.[32]

Butyrate acts on cells mainly through the inhibition of HDAC and activation of GPCR, such as GPR41, GPR43, and GPR109a, the major intracellular receptor that mediates SCFAs function.[8, 16] Both HDAC inhibitory activity and GPR43 have been reported in mediating SCFAs' regulation of T cell functions.[8, 16, 33] Thus, whether GPR43 and inhibition of HDAC activity are involved in butyrate regulation of naïve T-cell differentiation remains to be determined. In this study, we found that butyrate regulated Th1 cell differentiation through inhibition of HDAC, rather than GPR43 pathways—findings that differ from the differentiated Th1 cells reported in our recent study.[30] The expression of GPR43 is very low in

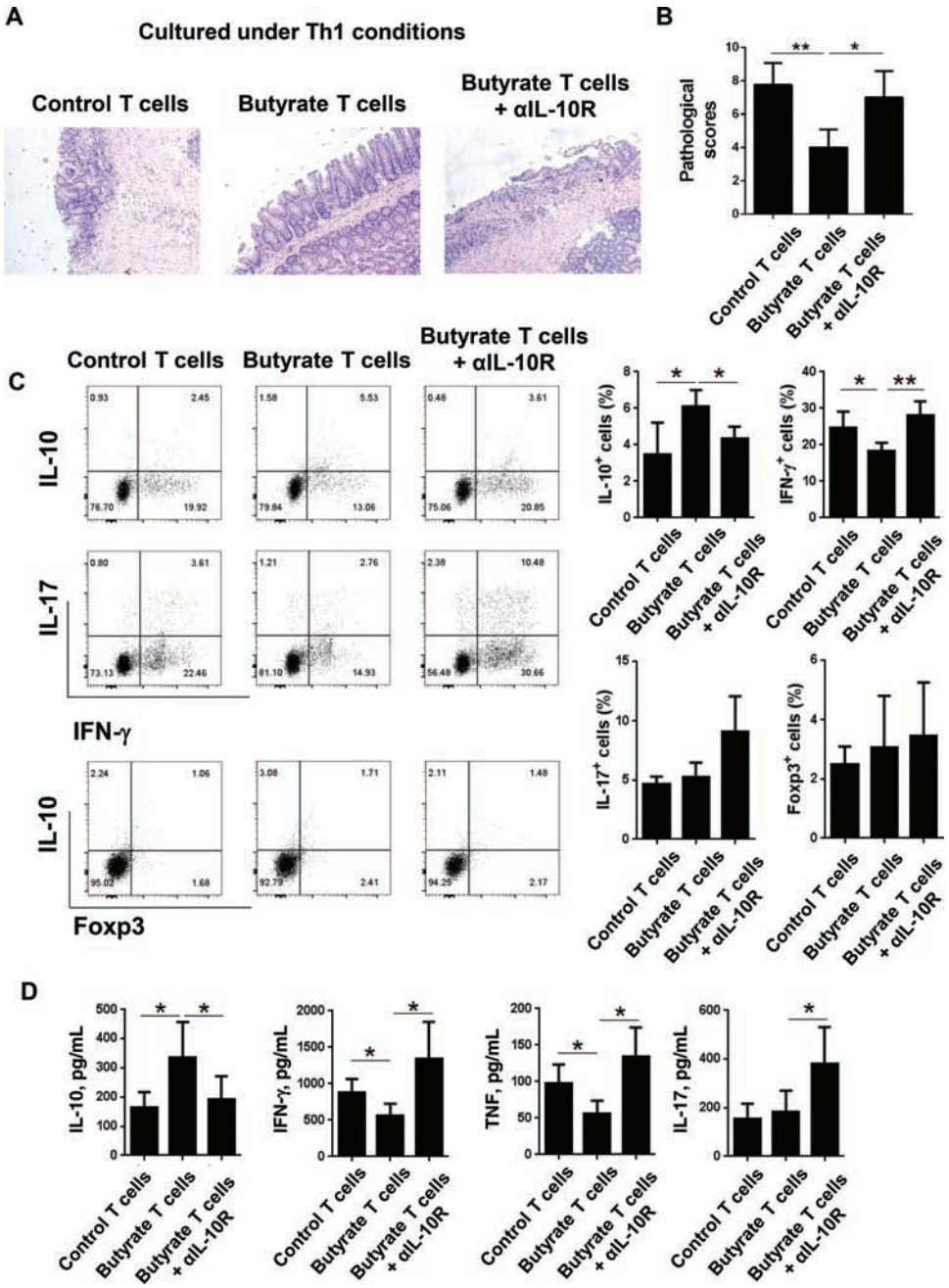


FIGURE 6. Administration of anti-IL-10R antibody worsened the colitis induced by T cells treated with butyrate. Control or butyrate-treated T cells, cultured under Th1 conditions, were injected IV into groups of Rag1$^{-/-}$ mice, respectively. A group of mice that received butyrate-treated T cells under Th1 conditions were administered with anti-IL-10R antibody intraperitoneally twice a week. The other 2 groups of mice were injected with IgG. Mice were killed 4–6 weeks after T-cell transfer. Colitis severity and histopathological scores are shown. Mann-Whitney *U* test, $^{*}P < 0.05$; $^{**}P < 0.01$. C, LP CD4$^{+}$ T-cell cytokine expression was determined by flow cytometry. D, Colonic tissues were cultured ex vivo for 24 hours, and the cytokine production was measured by ELISA. Plot numbers represent the percentage of CD4$^{+}$ T cells in the respective quadrants. Results are shown as mean ± SD. One representative of 3 experiments was performed. One-way ANOVA test, $^{*}P < 0.05$; $^{**}P < 0.01$.

naïve T cells and relatively higher in differentiated Th1 effector cells,[8, 34] which may account for the different mechanisms of butyrate on differentiated effector Th1 cells and naïve T cells during their differentiation to Th1 cells. Moreover, butyrate promoted Blimp1 expression. The deficiency of Blimp1 decreased IL-10 production induced by butyrate in T cells. Our study thus suggests that butyrate induction of IL-10 production during Th1 and Th17 cell differentiation is also

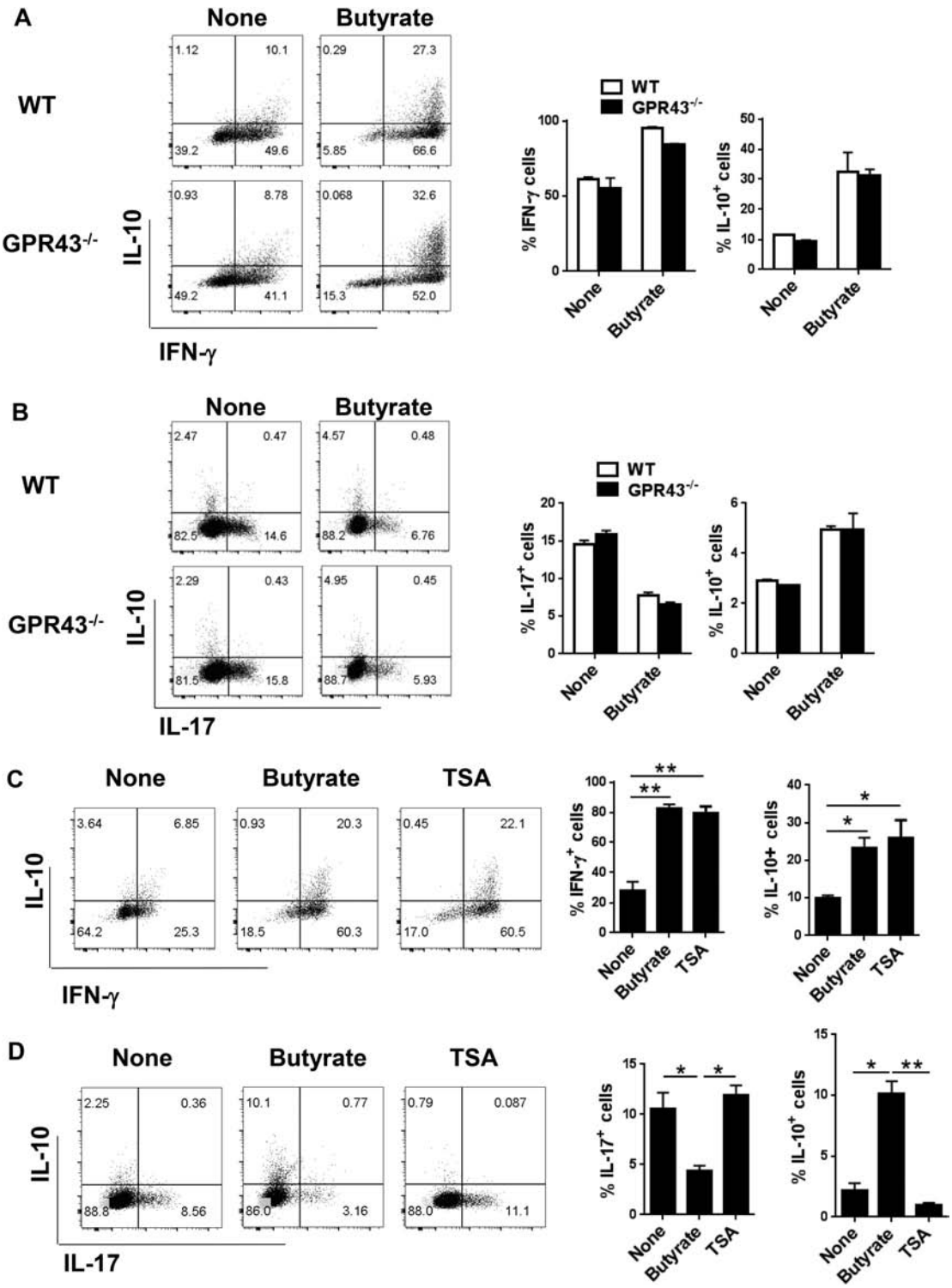


FIGURE 7. The effect of butyrate on Th1, but not Th17, differentiation is through HDAC inhibitory activity, independent of GPR43. GPR43$^{-/-}$ and WT CBir1 Tg-naïve CD4$^+$ T cells were cultured under Th1 and Th17 conditions for 5 days in the presence or absence of butyrate (0.5 mM). A and B, The expression of IFN-γ, IL-17, and IL-10 on Th1 cells (A) and Th17 cells (B) was examined by flow cytometry. Plot numbers represent the percentages of IFN-γ$^+$, IL-17$^+$, and IL-10$^+$ T cells in the respective quadrants. C and D, CBir1 Tg T cells were treated with butyrate in addition to HDAC inhibitor TSA (30 nM) under Th1 conditions (C) and Th17 conditions (D), respectively, for 5 days. Results are shown as mean ± SD. One representative of 2 experiments was performed. Student *t* test, 1-way ANOVA test, $^*P < 0.05$; $^{**}P < 0.01$.

regulated by Blimp1, which is similar to differentiated Th1 effector cells,[30] indicating that Blimp1 is crucial in the butyrate induction of IL-10, both in naïve T cells during differentiation and in differentiated Th1 effetor cells.

## SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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