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RESEARCH ARTICLE

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Histone deacetylase 1 and 3 inhibitors alleviate colon inflammation by inhibiting Th17 cell differentiation

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Abstract

Background: The etiology of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is not completely clear, but its pathogenesis is closely related to T helper 17 (Th17) cells. Several histone deacetylase (HDAC) inhibitors have been shown to exert potent anti-inflammatory effects and modulate Th17 cell polarization. Owing to the large variety and broad expression of HDACs, finding specific therapeutic targets for IBD is of clinical importance.

Methods: The proportions of Th17 cells and interleukin (IL)-17A produced between patients with UC and healthy volunteers were compared. The differentiation of human peripheral blood mononuclear cells (PBMCs) into Th17 cells was induced in vitro. Differentiated Th17 cells were treated with RGFP109 (RG), a selective inhibitor of HDAC1 and 3, to observe its effects on these cells. Subsequently, colitis was induced in mice and treated with RG. The proportion of Th17 cells, the severity of colitis in mice, and colon histopathology and immunohistochemistry were evaluated respectively.

Results: The proportion of Th17 cells and IL-17A production was significantly increased in patients with UC than in healthy individuals. RG inhibited the differentiation of human PBMCs into Th17 cells and reduced IL-17A secretion in vitro. RG-treated colitis mice had a lower Th17 ratio, mild colon inflammation, and decreased expression of HDAC1 and 3 in the colon.

Conclusions: HDAC1 and 3 inhibitors can modulate the differentiation of inflammatory Th17 cells, downregulate IL-17A levels, and exert anti-inflammatory effects in experimental colitis mice, indicating that HDAC1 and 3 may be potential therapeutic targets for patients with IBD.

1 | INTRODUCTION

Inflammatory bowel diseases (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), are chronic recurrent bowel inflammation disorders. The etiology of IBD is related to the abnormal intestinal immune imbalance caused by the interaction of multiple factors, such as the environment, genetics, and intestinal microecology.[1,2] The incidence of IBD is increasing rapidly worldwide, especially in Asian countries, and mostly affects young adults.[3,4] Most patients with IBD need to take many medications for

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a long time or even a lifetime; however, none of these drugs are curable or have side effects.[5] Therefore, it is necessary to develop new drugs that target specific pathways in patients with IBD.

The gastrointestinal tract is the largest immune organ in the human body, where naïve intestinal T cells undergoing antigen stimulation differentiate into different T-cell subtypes. The differentiation of T helper 17 (Th17) cells is induced by a combination of transforming growth factor-β (TGF-β) and interleukin (IL)-6. Inflammatory factors (such as IL-1βand IL-23) secreted by activated dendritic cells and macrophages residing in the laminae of the intestine can further promote the differentiation of Th17 cells.[6,7] Activated Th17 cells secrete various cytokines, of which IL-17A plays a major pro-inflammatory role.[8] Numerous studies have shown a close association between IBD and Th17 cell levels.[9,10] These studies have found that the proportion of Th17 cells and the secretion of Th17 cell-related cytokines were significantly higher in patients with IBD than in normal individuals and were proportional to the disease activity index and endoscopic and histological scores.[11] Another study showed that germ-free mice that received IBD gut flora developed an elevation in Th17 cell numbers.[12] Importantly, genome-wide association studies (GWAS) in IBD have identified at least 24 single nucleotide polymorphism (SNP) loci associated with Th17 cell-associated regulatory networks and signaling pathways.[13] These results suggest that targeting Th17 cells may have a therapeutic effect on IBD.

Protein post-translational modifications (PTM) involve the addition of functional groups, such as methylation, acetylation, and phosphorylation, changing the properties and functions of a protein.[14,15] Acetylation mainly occurs in the ε-amino group of lysine in a process catalyzed by two enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs). HDACs can remove acetyl groups from lysine residues in a protein, while HATs do the opposite. HDAC inhibitors regulate the reversible acetylation process and are currently undergoing clinical trials as anticancer drugs.[16] However, emerging evidence suggests that HDAC inhibitors may also have anti-inflammatory and immunomodulatory effects, showing promising therapeutic effects in multiple sclerosis,[17] rheumatoid arthritis (RA),[18] and IBD. Numerous studies have provided evidence for the anti-inflammatory role of HDAC inhibitors in experimental colitis models.[19-21] One study also found that pan-HDAC inhibitors inhibited the polarization of Th17 cells in vitro and at the lamina propria inflammation sites *in vivo*.[22] HDACs comprise four major groups, with a total of 18 subtypes. Studies have found that HDAC 1[23] and HDAC 3[24] are important for maintaining intestinal homeostasis. Previously, our research group found that HDAC1 was highly expressed in the colitis mucosa of patients with IBD and experimental mice[25] and that experimental colitis was ameliorated by treatment with RGFP966, an HDAC3-specific inhibitor.[26]

Therefore, in this study, we hypothesized that selective inhibition of HDAC1 and 3 using RGFP109 (RG) could ameliorate colitis in a mouse model of colitis. To further investigate whether RG inhibits Th17 cell levels, we first observed the effect of RG on Th17 cell differentiation in vitro, aiming to introduce a new approach for treating IBD.

2 | MATERIALS AND METHODS

2.1 | Patients

All laboratory mouse and human samples were reviewed and approved by the Ethical Committee and Animal Welfare Committee of the Drum Tower Hospital, Nanjing University. Informed consent was obtained from patients with UC and healthy volunteers. Patients with UC were recruited from the Drum Tower Hospital, and their diagnoses were based on clinical, endoscopic, and histological criteria.

2.2 | Cell isolation and differentiation

Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood of healthy volunteers (median age: 30.6 years; range: 24–43 years) using a lymphocyte separation medium (TBD, LTS1077) and were cultured in RPMI-1640 medium (Corning, 10-040-CV) containing 10% fetal bovine serum (FBS) (Biological Industries, 04–001-1ACS, Israel). Adherent cells were removed, and 1×10^6 suspended lymphocytes were seeded into each well of 96-well round-bottom well plates coated with 10 μg/ml anti-CD3 and 4 μg/ml anti-CD28 antibodies (eBioscience) the day before. Several stimulating factors (3 ng/ml TGF-β [R&D], 40 ng/ml IL-6 [R&D], 30 ng/ml IL-23 (R&D), 20 ng/ml TNF-α [PeproTech], and 10 ng/ml IL-1β [PeproTech]) were added to each well to induce Th17 cell polarization. These differentiated Th17 cells were then treated with 2 μM and 10 μM RG and cultured for 5 days. The proportion of Th17 cells in each group was then measured using flow cytometry analysis (FACS). The cell supernatants were used for cytokine detection.

2.3 | Animal experiments and colitis induction

Twenty C57BL/6J mice (male, 6–8 weeks old) with an average body weight of 20 g were obtained from the Animal Center of the Drum Tower Hospital. They were randomly divided into four groups of five mice each (control, colitis, RG, and medium). All mice were raised freely in specific pathogen-free (SPF) conditions in a room with a temperature of 22 ± 1°C with a 12 h light/dark cycle. The control group only received normal drinking water. In contrast, the other three groups were administered 3.5% dextran sulfate sodium (DSS, MP Biomedicals) to induce colitis for the first 5 days and were then allowed to drink normal drinking water for the next 2 days. For 7 days, the control and colitis groups were injected with 100 μl of 0.9% physiological salt solution; the RG group was injected every day with 100 μl RG-containing solution

(RG dissolved in 2% DMSO, 40% PEG-300, 2% Tween-80, and 56% ddH_2O) at a dose of 5 mg/kg; and the medium group was injected with the same volume and concentration of the solution medium without RG. The mice were weighed, and the characteristics of their stools were observed at a fixed time each day. After 7 days, the mice were anesthetized, and their peripheral blood was isolated and centrifuged to collect the serum. After sterilizing the anatomical apparatus, the spleens of mice were removed in a relatively sterile environment and lymphocytes were extracted for FACS detection. The colon tissues of the mice were also isolated, the distance from the anus to the cecum was measured, and the distal colon tissue was fixed in 4% paraformaldehyde for pathological and immunohistochemical examinations.

2.4 | Disease activity index (DAI) of the established mouse model of DSS-induced colitis

The disease activity index (DAI) scoring system was used to score the mice for percentage weight loss, stool consistency, and stool bleeding. These parameters could quantify the severity of colitis induced in the mouse model and to observe the drug efficacy. The specific grading criteria for DAI were body weight loss (0% = 0; 1%–5% = 1; 5%–10% = 2; 10%–15% = 3; 15%–20% = 4), stool consistency (normal = 0; loose stools = 2; watery diarrhea = 4), and stool bleeding (normal = 0; slight bleeding = 2; severe bleeding = 4).[27]

2.5 | Flow cytometry analysis (FACS) and enzyme-linked immunosorbent assay (ELISA)

First, the cells were resuspended with RPMI-1640 medium containing 10% FBS and adjusted to a density of 1×10^6 cells per sample before being subjected to subsequent analyses. Mouse spleen lymphocytes were extracted via grinding and filtering through cell screens on a sterile operating table. Before adding antibodies, all the cells were treated with a cell-stimulating cocktail (a solution comprising phorbol 12-myristate 13-acetate [PMA], ionomycin, brefeldin A, and monensin) (eBioscience,00–4975-03) for 4–6 h in an incubator (37°C, 5% CO_2). Afterward, the cells were fixed and permeabilized using a Fixation/Permeabilization Kit (BD Biosciences). For cell surface staining, anti-human CD3-FITC and anti-CD8-PerCP antibodies were used for human specimens, while anti-mouse CD3-FITC and anti-mouse CD4-APC antibodies were used for mouse specimens. Intracellular staining was also performed using anti-human IL17A-APC and anti-mouse IL17A-PE antibodies. All antibodies used for FACS analysis were purchased from eBioscience. After staining, all cells were subjected to flow cytometry (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). Th17 cells were identified as $CD3^+$ and $CD8^-$ $IL\text{-}17A^+$ for human specimens and $CD3^+$ and $CD4^+$ $IL\text{-}17A^+$ for mouse specimens. Apoptosis was also measured via FACS using an Annexin V FITC Apoptosis Detection Kit (BD Biosciences). IL-17A levels were determined using an ELISA kit (eBioscience) according to the manufacturer's instructions.

2.6 | Colon histopathological grading and immunohistochemistry

The fixed colon tissues of the mice were embedded in paraffin and sectioned for hematoxylin–eosin staining. The histopathology scores were provided by an experienced pathologist, and the degree of inflammation was graded semi-quantitatively from 0 to 4 (0 = no signs of inflammation to 4 = presence of transmural infiltrations).[28] As for colon immunohistochemistry, paraffin sections were subjected to conventional dewaxing, dehydration, and antigen retrieval. Next, the sections were incubated with primary antibodies against HDAC 1 and 3 (Proteintech) at 4°C for 24 h. After incubation with the corresponding secondary antibody for 30 min at 37°C, the sections were treated using a DAB kit for chromogenic development. The sections were scored according to the staining intensity and staining range.[29]

2.7 | Statistical analysis

Data analysis was performed using GraphPad Prism version 5.0 software (La Jolla). Student's *t* test was used for comparisons between groups, while other data were analyzed using one-way analysis of variance (ANOVA). All data are presented as the mean ± SEM, and statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Patients with UC have a high proportion of Th17 cells and a high level of IL-17A in their peripheral blood

An imbalance of the immune system is an important aspect of the pathogenesis of IBD. Th17 cells, characterized by the secretion of IL-17A, are a newly discovered subset of T cells mainly found to have a pro-inflammatory effect. Many studies have shown that Th17 cells play a crucial role in inflammatory diseases, particularly IBD. For instance, it can damage the integrity of the gut barrier and cause a cascade of inflammation.[30] To verify whether there is a difference in the proportion of Th17 cells between patients with UC and healthy individuals, we collected peripheral blood from 10 healthy subjects and six patients with UC. Consistent with our hypothesis and the current knowledge, patients with UC had a higher proportion of Th17 cells than healthy subjects (3.23 ± 0.45% vs. 1.20 ± 0.12%, $p < 0.01$). Moreover, the secretion of IL-17A in the peripheral blood of patients with UC was also significantly higher than that of healthy subjects (291.33 ± 27.04 pg/ml vs. 114.50 ± 21.24 pg/ml, $p < 0.01$) (Figure 1A,B).

3.2 | RG inhibits the differentiation of Th17 cells in vitro

Several studies have confirmed that when naïve CD4+ T cells are stimulated by a specific set of cytokines, they could differentiate into pathogenic Th17 cells. An increase in the number of Th17 cells is often associated with inflammation. HDAC inhibitors have potent anti-inflammatory effects, and the regulatory effects of broad-spectrum HDAC inhibitors on Th17 cells have been reported. The roles of HDAC1 and HDAC3 in intestinal mucosal homeostasis have been identified. Therefore, selective HDAC1 and HDAC3 inhibitors were chosen in this experiment to validate their effects on Th17 cells. First, Th17 cell differentiation was induced in vitro, and differentiated Th17 cells were treated with or without RG. The results showed that the related cytokines significantly induced Th17 cell differentiation compared with the control group (6.10±0.23% vs. 1.18±0.16%). Exposure to RG at higher concentrations (10 μM; 1.49±0.05%) significantly inhibited differentiation lower concentrations (2 μM; 3.18%±0.20%) (Figure 2A). There was no significant difference in apoptosis levels among the groups (Figure 2B). These results suggest that the reduction in Th17 cell number is not associated with apoptosis. Meanwhile, the levels of IL-17A (mainly secreted by Th17 cells) in the cell supernatant of the RG group also decreased remarkably (control, 77.67±2.08 pg/ml; polarization, 223.00±2.65 pg/ml; 2 μM RG, 134.33±4.04 pg/ml; 10 μM RG 85.00±3.00 pg/ml) (Figure 2C).

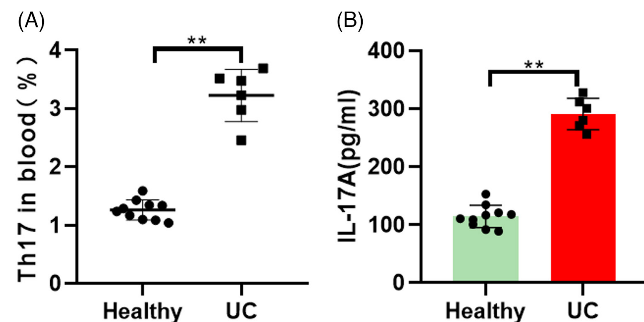

FIGURE 1 Higher Th17 cell ratio and IL-17A secretion in UC patients. (A) FACS analysis proportion of Th17 cells between healthy individuals and UC patients. Each spot represents one sample. Data are expressed as the mean±SEM. $n = 16$. (B) Serum IL-17A levels were measured by ELISA in healthy subjects and UC patients. Data are expressed as the mean±SEM. $n = 16$. Asterisks (*) indicate significant differences (*$p<0.05$; **$p<0.01$)

3.3 | RG exerts anti-inflammatory effects on a DSS-induced colitis mouse model

Here, we found that RG inhibited Th17 cell differentiation through an in vitro cell assay. As Th17 cells and the accumulation of Th17 cell-related cytokines play an important role in IBD disease activity and mucosal damage,[31] we conducted an in vivo experiment in a mouse model of colitis to explore whether RG could inhibit Th17 cells and relieve colonic inflammation. Interestingly, the control mice steadily gained weight and showed no significant stool

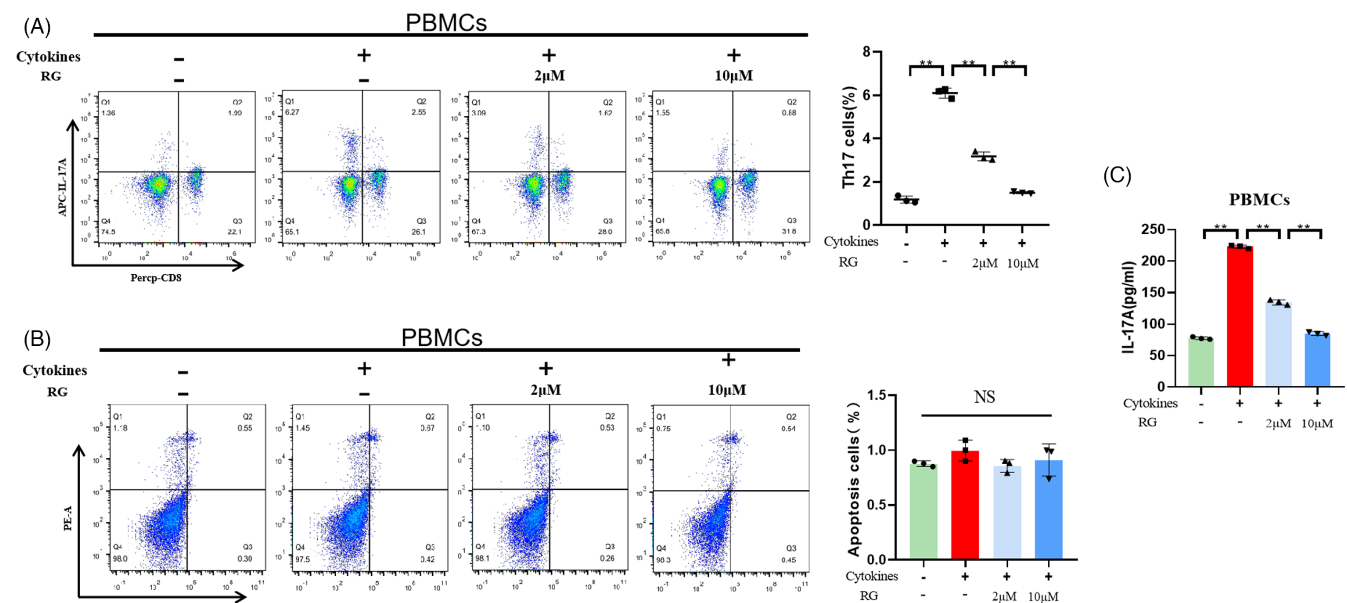

FIGURE 2 RG inhibits the differentiation of Th17 cells in vitro. Human PBMCs were stimulated by specific cytokines to form Th17 polarization conditions and co-cultured with or without 2 μM and 10 μM RG. FACS was used to analyze the proportion of Th17 cells (A) and apoptosis (B) in each group. The proportion of Th17 cells is shown in the UL quadrant, that is, $CD8^-$ IL-17A $^+$ cells after CD3+cells selection. The rate of apoptosis was analyzed by adding the UR and LR quadrants together (total Annexin V+ cells). (C) IL-17A was detected in cell supernatant by ELISA. Data are expressed as the mean±SEM. (*$p<0.05$; **$p<0.01$; NS not significant)

abnormalities. The DSS-induced colitis model and medium groups showed significant weight loss, severe diarrhea, and even bloody stools (Figure 3A,B). After dissection, mice in the control group had normal colons without edema or exudation. The colons of mice in the colitis and medium groups were significantly shortened and swollen; marked blood and loose stools were likewise observed in the colonic lumen (Figure 3C,D). Histopathological examination showed inflammatory cell infiltration and obvious damage to the epithelium and glands (Figure 3E,F). RG intervention significantly improved these symptoms in mice, decreasing the DAI and histopathological scores. To validate whether RG improves colitis in mice by inhibiting Th17 cells, we examined the proportion of Th17 cells in the mouse spleens. We found that RG treatment reduced the proportion of Th17 cells compared with the control group (Control group, 0.26±0.03%; Colitis group, 0.97±0.08%; RG group, 0.46±0.03%; Medium group, 0.90±0.7%). IL-17A secretion in the peripheral blood was also decreased (Control group, 7.80±0.84 pg/ml; Colitis group, 36.40±3.05 pg/ml; RG group, 11.60±1.14 pg/ml; Medium group, 35.40±1.82 pg/ml) (Figure 3G,H). These findings suggested that RG treatment can improve DSS-induced colitis in mice by inhibiting Th17 cells.

3.4 | RG inhibits the expression of HDAC1 and 3 in the mouse colon

To validate whether RG works by inhibiting the expression of HDACs 1 and 3, we examined the expression of HDACs 1 and 3 in the murine colon. We found that RG treatment significantly reduced the expression of HDACs 1 and 3 compared with colitis mice (Figure 4). These results indicate that RG decreased the expression of HDACs 1 and 3 in the colon of mice, inhibited Th17 cell differentiation, and improved colon inflammation.

4 | DISCUSSION

The clinical characteristics of IBD include alternating phases of relapse and remission, and long-term chronic stimulation of intestinal inflammation, increasing the risk of developing colorectal cancer (CRC).[32,33] At present, the etiology of IBD remains elusive; however, it is clear that abnormal immune responses play a considerable role in its pathogenesis.[34] Th17 cells, which are an IL-17A-expressing subset of CD4+ T cells, have been reported to play a vital role in various

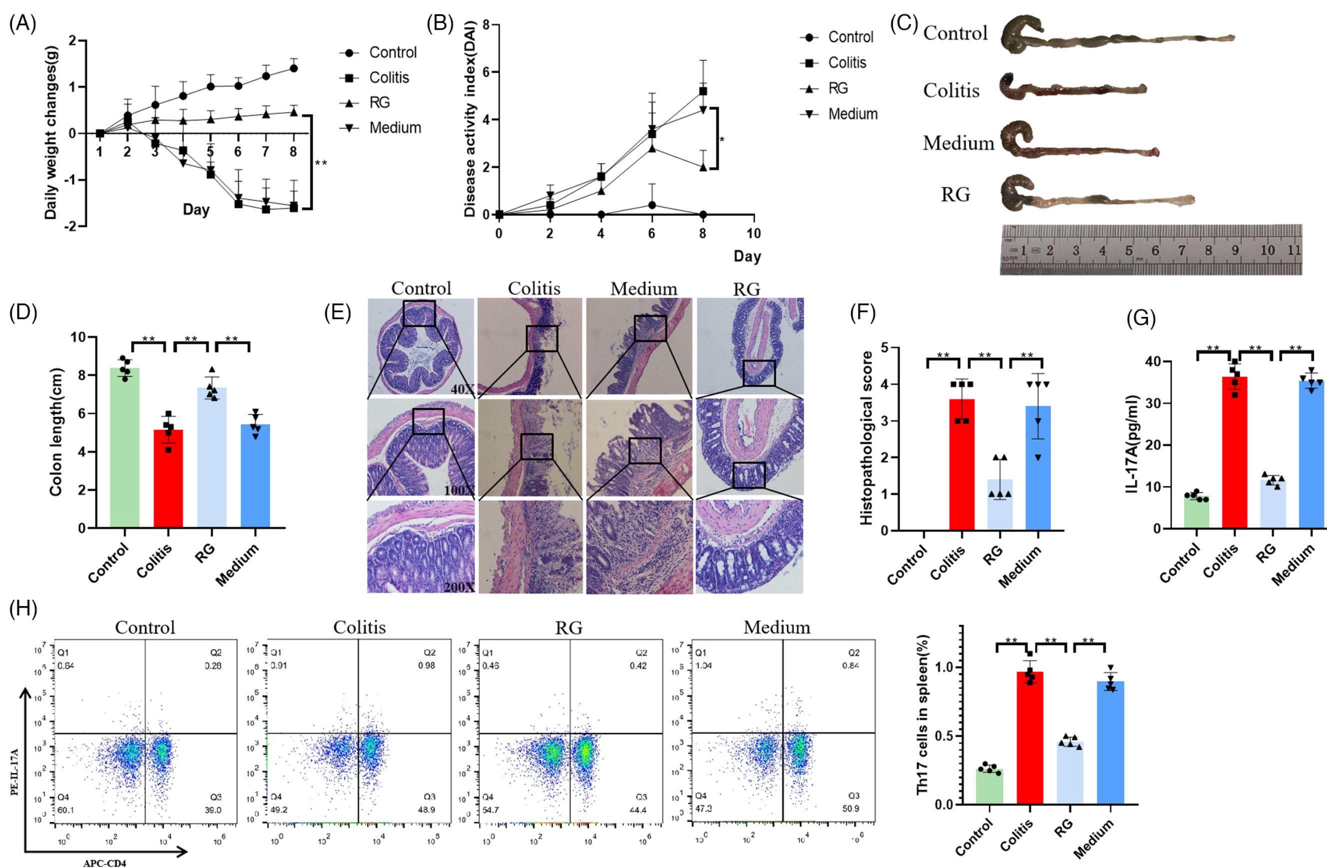

FIGURE 3 RG ameliorates DSS-induced colitis. Except the control group, every C57BL/6 mice in the other 3 groups were free to drink 3.5% DSS solution. Mice in RG group received 5 mg/kg of RG intraperitoneally for 5 days, and other mice were injected with equal volume of normal saline or medium. (A) Changes in body weight of mice, (B) Disease Activity Index (DAI), (C-D) colon morphology and length, (E) colon HE staining and (F) pathological scoring, (G) IL-17A levels in peripheral blood, (H) proportion of Th17 cells in spleen were assessed respectively. The proportion of Th17 cells is shown in the UR quadrant, that is, $CD4^+$ IL-$17A^+$ cells after CD3+cells selection. Data are expressed as the mean±SEM. (*$p<0.05$; **$p<0.01$)

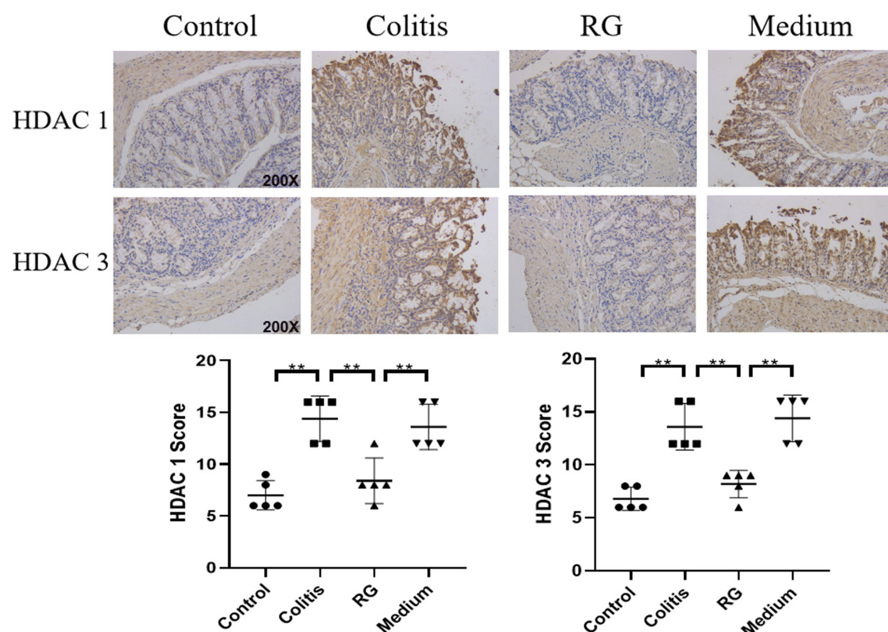

FIGURE 4 RG inhibits HDAC 1 and 3 expression in mouse colon. Immunohistochemical images and scoring of HDAC 1 and 3 expression levels in the mice colon, magnifications are ×200

inflammatory and autoimmune diseases, including IBD. Th17 has also been shown to be deeply involved in the pathogenesis of CRC.[35,36] Phase 3 clinical trials of monoclonal antibodies targeting IL-17A have confirmed that IL-17A is a highly effective therapeutic target for plaque psoriasis;[37] however, targeting IL-17A alone is insufficient to improve clinical endpoints for other autoimmune diseases, such as RA and Crohn's disease. Given that Th17 cells produce and modulate other pro-inflammatory factors besides IL-17A, targeting the Th17 cell lineage may be superior to blocking a single effector cytokine.[38] In this study, the proportion of Th17 cells in the peripheral blood of patients with UC was higher than that of normal subjects, and both IBD and Th17 cells were correlated with CRC occurrence. Therefore, the inhibition of Th17 cells may provide a compelling prospect for the treatment of IBD.

HDACs regulate the acetylation levels of proteins; acetylation can dramatically alter the biological properties of proteins. Histone acetylation regulates gene transcription by causing structural changes in the chromatin, while non-histone acetylation controls multiple cellular processes. Eighteen HDAC enzymes have been found in mammalian cells. On the contrary, HDAC inhibitors are roughly divided into four major categories: hydroxamic acids, cyclic peptides, benzamides, and short-chain fatty acids.[39,40] Therefore, it is necessary to clarify the role of specific HDAC inhibitors. The most common role of HDAC inhibitors is to act as apoptosis inducers in tumor cells. It is well known that many immune mechanisms are activated when fighting tumor cells; therefore, it is not surprising that HDAC inhibitors might also play a role in treating chronic inflammatory diseases.[41,42] The overexpression of HDAC1/3 was found to aggravate inflammation in an experimental osteoporosis model, and its inhibition had protective effects.[43] Moreover, HDAC1 was found to regulate IL-1β-dependent inflammatory responses in intestinal epithelial cells (IECs),[44] and inhibiting HDAC3 expression in the colon improved experimental colonic inflammation.[45] RGFP109, a selective HDAC1 and 3 inhibitor, has been shown to overcome temozolomide resistance by mediating NF-κB transactivation in glioblastoma cell lines and attenuating L-DOPA-induced dyskinesia in an animal model of Parkinson's disease.[46,47] However, its impact on IBD has not been studied. Following the results of our previous studies, HDAC1 or 3 may be a potential therapeutic target for improving colonic inflammation;[25,26] hence, we chose to investigate RGFP109 in this study. This study showed that RG inhibited the differentiation of Th17 cells and the secretion of the inflammatory factor IL-17A in vitro and in vivo, showing significant anti-inflammatory effects against DSS-induced colitis. We also detected no significant difference in apoptosis levels, indicating that RG did not reduce the number of Th17 cells by increasing apoptosis.

RORγT is a key transcription factor of Th17. Although studies have shown that the regulatory effect of HDAC inhibitors on Th17 cells is associated with histone H4 acetylation near the proximal promoter of RORγT,[48] the specific mechanism and inflammatory pathways by which RG inhibits the differentiation of Th17 cells in this experiment are not clear. Moreover, the anti-inflammatory effects of HDAC3 are controversial. Alenghat et al. found that HDAC3 expression in IECs is important for maintaining intestinal homeostasis. HDAC3-deficient mice showed impaired IEC function, a loss of Paneth cells, and a significantly increased susceptibility to intestinal injury and inflammation.[24] Another study found that HDAC3-deficient macrophages failed to activate almost half of the inflammatory gene expression program, implicating HDAC3 as an effective anti-inflammatory target.[49] This experiment found that DSS-induced colitis mice exhibited increased HDAC3 expression compared with normal mice, while the administration of an HDAC3 inhibitor decreased HDAC3 expression and showed a protective effect against colitis. It has been found that excessive inflammation leads to severe intestinal epithelial injury. Inflammatory attenuation is critical for epithelial reconstruction,

and HDAC3 is an indispensable co-inhibitory factor in attenuating the RARα-mediated NF-κB signaling pathway in IECs.[50] This may explain the results of this experiment, but a large number of studies are still needed to elucidate this.

5 | CONCLUSION

In summary, this study showed that the proportion of Th17 cells and the levels of their effector cytokine IL-17A were elevated in patients with UC compared to healthy volunteers. Moreover, the results showed that specific HDAC1 and 3 inhibitors could inhibit Th17 cell differentiation in vitro and in vivo by exerting significant anti-inflammatory effects in DSS-induced colitis mice and improving colonic inflammation. These results suggest a potential role for HDAC1 and 3 in treating colon inflammation and may provide a new approach for IBD treatment.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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