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ARTICLE

Procaine hydrochloride improves DSS-induced colitis via inhibition of IL-6/STAT3 and FOXP3 methylation

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ABSTRACT: Backgrounds: Disruption of immune barrier function is considered a hallmark feature of inflammatory bowel disease. In the present study, the potential effects of the methyltransferase inhibitor procaine hydrochloride on intestinal barrier integrity, as well as T cell differentiation in the context of inflammatory bowel disease were explored. **Methods:** Using an experimental model of dextran sulfate sodium-induced ulcerative colitis, mice received daily treatment with either sulfasalazine (100 mg/kg) or procaine hydrochloride (45 mg/kg), or saline (10 μL/g) as a negative control, for 7 consecutive days. Subsequent analysis included disease activity index score, histopathology, expression levels of intestinal epithelial tight junctions and apoptotic protein, production of inflammatory cytokines, frequencies of lymphocyte populations in the spleen by flow cytometry and methylation levels of Forkhead box protein 3 (FOXP3). **Results:** Procaine alleviated dextran sulfate sodium-induced colitis, with a reduced disease activity index ($p < 0.05$) and histopathological score ($p < 0.001$). Procaine treatment resulted in a 1.96-fold increase in splenic $FOXP3^+$ $CD25^+$ Tregs ($p < 0.001$). Administration of procaine significantly enhanced tight junction expression ($p < 0.01$), decreased the expression of BAX and cleaved caspase-3 protein expression, while increasing that of the anti-apoptotic protein BCL-2. Procaine also significantly suppressed IL-6/signal transducer and activator of transcription factor 3 (STAT3) signaling pathway. In addition, procaine amplified $FOXP3^+$ Tregs by inhibiting FOXP3 methylation. **Conclusions:** The protective potential of procaine hydrochloride against intestinal barrier injury in colitis may be attributed to increased abundance of Tregs and the augmentation of their anti-inflammatory properties. The potential mechanisms may involve modulation of IL-6/STAT3 signaling pathway and inhibition of FOXP3 methylation.

KEYWORDS: Ulcerative colitis, intestinal mucosal barrier, procaine hydrochloride, Tregs, interleukin-6/signal transducer and activator of transcription factor 3 signaling pathway, forkhead box protein 3

1 Introduction

Inflammatory bowel disease (IBD) represents a persistent inflammatory condition of intestinal system, typically associated with intestinal impairment, gut dysbiosis, and altered systemic biochemical indices [1,2]. It is mainly described as ulcerative colitis (UC) and Crohn's disease [3]. At the turn of the 21st century, IBD became a worldwide health concern. The epidemiology of IBD exhibits considerable geographic heterogeneity, reflecting ongoing temporal changes in its epidemiological characteristics. In recently industrialized countries undergoing progressive westernization, the incidence rates of the disease continue to rise [4].

The pathogenesis of IBD is not yet fully understood, however, the gut barrier integrity has been recognized as a critical contributor in its development [5,6]. It has been reported that the intact structure of the intestinal epithelial barrier is an essential evaluation index for the pathogenesis of IBD [7]. The intestinal epithelial barrier serves as primary defense against adverse environmental factors, primarily formed by intestinal epithelial tight junctions (TJs) between epithelial cells [8]. TJs, consisting of the transmembrane proteins (occludins and claudins) and accessory proteins, zonula occludens (ZO), prevent the paracellular translocation of pathogens and harmful antigens across epithelial cells [9]. ZO, occludins and claudins are considered key integral membrane proteins that participate in the structural integrity of

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TJs by binding to the actin cytoskeleton [10]. Therefore, research focusing on regulation of intestinal mucosal barrier function holds importance for treatment of IBD.

The maintenance of intestinal mucosal homeostasis involves participation of specific subsets of T cells, among which Tregs contribute substantially to the progression and development of intestinal inflammation [11,12]. The sustained transcription factor FOXP3 in Tregs plays a central role in intestinal immune regulation, primarily via upregulation of anti-inflammatory cytokines including TGF-β and repression of effector T helper cell responses [13]. DNA methylation is an important epigenetic modification occurring at CpG dinucleotides throughout the genome. Methylation occurring at CpG sites in gene promoter regions impedes the interaction between transcription factors and DNA, thereby repressing gene expression, whereas promoter demethylation facilitates transcription factor binding and promotes gene transcription [14,15]. Consequently, the methylation level of the *FOXP3* promoter region in Tregs influences their immunoregulatory capacity in treating IBD. Procaine hydrochloride is reported to exhibit strong demethylation activity [16,17]. Sulfasalazine (SASP), a conventional drug routinely used in the management of IBD, is related to a variety of adverse effects, including gastrointestinal intolerance, hypersensitivity reactions, hyperbilirubinemia, and hepatic or renal toxicity, and is therefore contraindicated in patients with severe organ dysfunction [18]. In contrast, procaine hydrochloride is a commonly used local anesthetic with minimal side effects and low cost, although it may cause nausea, dizziness, drowsiness, agitation, or allergic reactions in susceptible individuals. Adjustments in dosage, administration route, or dosing intervals based on further experimental and clinical data may help mitigate these adverse effects. Therefore, the present study hypothesized that procaine hydrochloride can enhance Treg cell expansion through epigenetic regulation, thus restoring the intestinal barrier function in IBD.

A dextran sulfate sodium-induced ulcerative colitis model was used to assess the effect of procaine hydrochloride in maintaining intestinal barrier integrity and the epigenetic regulation of FOXP3 methylation in Tregs. The results support a possible clinical application of procaine hydrochloride in the treatment of UC.

2 Materials and Methods

2.1 Animal Experiments

Six-week-old male BALB/c mice weighing 15–20 g were obtained from Shanghai JieSiJie Laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed under conditions with a 12-h light/dark cycle, a temperature of 23 ± 2°C, and relative humidity maintained at approximately 50%. Standard chow and drinking water were supplied ad libitum throughout the entire experimental period. All animal-related procedures were in line with the Guidelines for the Ethical Review of Laboratory Animal Welfare (People's Republic of China National Standard GB/T 35892–2018) and were approved by the Ethics Committee of Minhang Hospital, Fudan University, Shanghai, China (Approval No. 2021JS Minhang Hospital-042). Mice (n = 24) were randomly divided into four groups (n = 6): Negative control (NC) group, colitis model group, colitis model + sulfasalazine (SASP, Merck KGaA, Darmstadt, Germany, Cat# S0883) group and colitis model + procaine hydrochloride (MedChemExpress, Shanghai, China, Cat# HY-B0546) group. The colitis model was induced by supplementing 3% (w/v) DSS (Merck KGaA, Cat# 67578) in the drinking water of mice for 7 consecutive days. From day 8 onwards, the mice were switched to normal drinking water and received either an intraperitoneal injection of saline (10 µL/g) or procaine hydrochloride (procaine, 45 mg/kg) for 7 consecutive days. SASP (100 mg/kg) were administered daily via oral gavage for 1 week every day. All mice received a single injection per day. The vehicle in all groups is physiological saline. The dosage selection was determined based on a review of the literature [19,20]. No unexpected mortalities occurred during this study. From the initiation of DSS administration, body weights, external features and clinical signs were monitored and recorded on a daily basis. Sodium pentobarbital (150 mg/kg; Shanghai Yika Biotechnology Co., Ltd., Shanghai, China, Cat# P0225) was administered intraperitoneally to euthanize the mice. After dissection of the mice, the length of colon from cecum to rectum was recorded. The colon and spleen were collected and stored at −80°C.

2.2 Disease Activity Index (DAI) Score

The body weight, stool consistency, and rectal bleeding were measured every day. Fecal blood was evaluated visually based on the presence of gross bleeding. The DAI is considered a standard measure for assessing the severity and prognosis of colitis, which was calculated using a well-established system [21] (*table 1*). The DAI was measured by averaging the scores for body weight loss, stool consistency, and rectal bleeding through the following formula: DAI = (weight loss score + stool consistency score + rectal bleeding score)/3.

2.3 Hematoxylin and Eosin (H&E) Staining

Colon tissues were fixed with 4% paraformaldehyde and then embedded in paraffin before being sectioned at a thickness of 5 µm. The resulting sections were further processed for hematoxylin and eosin (HE) staining. (Yeasen, Shanghai, China, Cat# 60524ES60). Histological evaluation was performed using a grading system [22] (*table 2*). Morphological changes were evaluated and photographed with a light microscope (Nikon ECLIPSE E100, Tokyo, Japan, 20× and 40×).

2.4 Immunofluorescence

Colon tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 µm thickness. The sections were deparaffinized in xylene twice for 10 min each and rehydrated by a graded ethanol series for 5 min each, followed by rinsing in distilled water.

Table 1
Disease activity index score

Score	Index		
	Weight loss	Stool consistency	Rectal bleeding
0	0	Normal	Normal
1	>0%~5%	Soft pellets not adhering to the anusSoft formed	Small spots of blood stool; dry anal region
2	>5%~10%	Very soft pellets adhering to the anus	Large spots of blood in stool; blood appears through the anal orifice
3	>10%~15%	Liquid stool in long streams; wet anus	Deep red stool; blood spreads largely around the anus
4	>15%	Diarrhea	Gross bleeding

Table 2
Histological evaluation of the H&E-stained colon sections

Score	The morphological changes
0	No inflammation
1	Low infiltration of leukocytes
2	Moderate leukocyte infiltration
3	High leukocyte infiltration, moderate fibrosis, high vascular density, thickened colonic wall, moderate loss of goblet cells, focal loss of crypts
4	Transmural infiltration, extensive loss of goblet cells, widespread fibrosis and diffuse loss of crypts

Antigen retrieval was achieved via heating the sections in citrate buffer for 15–20 min. Following cooling to room temperature, the sections were rinsed three times with PBS. The sections were then permeabilized with 0.1%–0.3% Triton X-100 in PBS for 10–15 min at room temperature and blocked with 5% bovine serum albumin (BSA) for 30 min. Thereafter, the sections were incubated overnight at 4°C with primary antibodies against ZO-1 (Affinity, Changzhou, China, Cat# AF5145, 1:200), claudin-1 (Affinity, Changzhou, China, Cat# DF6919, 1:200), and occludin (Affinity, Changzhou, China, Cat# DF7504, 1:200). After washing with PBS, the sections were incubated with fluorescent secondary antibodies (1:1000, GeneCopoeia, Rockville, MD, USA; cat. no. L147A) for 1 h at room temperature while protected from light, followed by nuclear counterstaining with DAPI for 5 min. After washing with PBS, the sections were mounted with anti-fade mounting medium and observed under a fluorescence microscope (Nikon Eclipse C1, Tokyo, Japan).

2.5 Flow Cytometry

The spleens were harvested, mechanically dissociated, and passed through a 200-μm nylon mesh filter to obtain single-cell suspensions. The suspensions were centrifuged at 251.55× *g* for 5 min at 4°C after which the supernatants were discarded. Red blood cells were lysed with 2 mL 0.83% NH_4Cl for 2 min, and lysis reaction was terminated by the addition of 15 mL PBS. Cells were subsequently resuspended in PBS at a density of 1×10^6 cells/mL and blocked with 2.5% BSA at room temperature for 1 h. Regulatory T cells were stained using the Mouse Regulatory T Cell Staining Kit (Multisciences, Hangzhou, China, KTR201) with 5 μ anti-CD4-FITC, 5 μ anti-CD25-APC, and 5 μ anti-FOXP3-PE antibodies (Multisciences, Hangzhou, China). Samples were acquired on a flow cytometer (BD LSRFortessa™, BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (version 7.6.1, FlowJo LLC, Ashland, OR, USA).

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

Colonic mucosa tissue was harvested for ELISA and western blotting. Next, ~10 mg of colonic mucosa from every group was obtained and homogenized in 1 mL Phosphate Buffered Saline (PBS, containing 1 μg aprotinin (Shanghai Qcbio Science & Technologies Co., Ltd.; cat. no. 20105ES08) and 1 μg leupeptin pepstatin A (Maokangbio Co., Ltd.; cat. no. 103476-89-7). The homogenates were centrifuged at 16,000× *g* for 20 min at 4°C and then 500 μL of supernatants were collected for ELISA. The levels of cytokines, including IL-10 (cat. no. 1212002; Dayou Bio Ltd., Guangzhou, China), TGF-β (cat. no. 1217102; Dayou Bio Ltd.), IL-6 (cat. no. 1110602; Dayou Bio Ltd.) and IL-17 (cat. no. 1111702; Dayou Bio Ltd.), were measured according to the manufacturer's instructions.

2.7 Western Blotting

Colon tissues were lysed in Radio Immunoprecipitation Assay (RIPA, Solarbio, Beijing, China, cat. no. R0020) buffer with Phenylmethanesulfonyl fluoride (Solarbio, cat. no. P8340), and the concentrations of extracted proteins were measured by Bicinchoninic Acid Assay (BCA assay, Beyotime, Shanghai, China, cat. no. P0012). Proteins were isolated with 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, transferred to Polyvinylidene Fluoride membranes (Thermo Fisher Scientific, Waltham, MA,

USA, cat. no. 88520), blocked with QuickBlock solution at room temperature for 20 min, and incubated with primary antibodies overnight at 4°C. Membranes were then incubated with Horseradish Peroxidase-conjugated secondary antibodies (1:1000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, cat. no. 111-005-003) for 2 h at room temperature. Bands were visualized using an Enhanced Chemiluminescence kit (Yeasen, cat. no. 36208ES60) and analyzed with ImageJ 8.0. Antibody information is provided in *table A1*.

2.8 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

Colon sections were prepared and deparaffinized as described previously. Subsequently, in accordance with the manufacturer's instructions provided with the TUNEL assay kit (cat. no. A111-01; Vazyme Biotech Co., Ltd., Nanjing, China), the 3′-OH ends generated by DNA fragmentation were labeled with fluorescein-12-deoxyuridine triphosphate (FITC-12-dUTP) using Terminal deoxynucleotidyltransferase. The FITC-12-dUTP-labeled DNA can be observed directly with a fluorescence microscope (Nikon Eclipse C1, Tokyo, Japan) and three random colonic regions were captured for each section. Briefly, tissue sections were deparaffinized, rehydrated, and the sections were then permeabilized with proteinase K (20 µg/mL) for 20 min at room temperature, and then incubated with TUNEL reaction mixture at 37°C in the dark. Following incubation, sections were rinsed with PBS to remove excess reaction solution. Cells were incubated with 100 µL DAPI solution (2 µg/mL) at room temperature for 5 min for nuclear counterstaining, followed by three washes with PBS. The TUNEL-positive cells were quantitatively analyzed using ImageJ 8.0.

2.9 Reverse-Transcription Quantitative PCR (RT-qPCR)

Total RNA was isolated from colon tissues by Trizol (Invitrogen, Waltham, MA, USA) and reverse-transcribed to cDNA. RT-qPCR was conducted using SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) with specific primers (*table A2*). The reaction conditions were: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. β-actin was used as an internal control for normalization. Gene expression changes were presented as fold changes in expression ($2^{\Delta\Delta Ct}$) relative to the baseline, which was normalized to β-actin as the reference gene.

2.10 Methylation-Specific PCR (MSP)

Genomic DNA was isolated from murine spleenic mononuclear cells using a DNA extraction kit (A&D Technology, Beijing, China) and bisulfite-modified using a DNA Modification Kit (A&D Technology, cat. no. A-P-1054). FOXP3 methylation status was analyzed by MSP with primers listed in *table A3*. PCR products were separated on 2% agarose gels and visualized under UV light. The total reaction volume was 25 µL, consisting of 1 µL of modified DNA, 0.5 µL of forward primer, 0.5 µL of reverse primer, 10.5 µL of nuclease-free water, and 12.5 µL of Premix Taq (Zymoresearch, Irvine, CA, USA, cat. no. E2055). The PCR amplification conditions were set as follows: initial denaturation at 95°C for 15 min; followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 90 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min.

2.11 Cell Culture

The spleen from BALB/c mice was collected, mononuclear cells were isolated and purified following the same procedures described above. The mitomycin-treated mononuclear cells from the mouse spleens were used as stimulating cells and the untreated mononuclear cells from the mouse spleens served as responding cells for one-way mixed lymphocyte cultures. The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C with 5% CO_2 for 3 days. The cells were subsequently treated with procaine hydrochloride at concentrations of 1.0, 2.5, and 5.0 µM for 24 h, and a control group without any drug treatment was included. Flow cytometry analysis was performed to evaluate the cellular responses. All cultured cell lines were regularly examined for mycoplasma contamination, and none tested positive. Cell line identity was verified by short tandem repeat (STR) analysis.

2.12 Statistical Analysis

All experiments were performed in triplicate. The data were expressed as the mean ± Standard Deviation (SD). Statistical analysis was carried out using ANOVA followed by Tukey's post hoc test in GraphPad Prism version 8.2.1 (GraphPad Software, La Jolla, CA, USA). Differences were considered statistically significant at $^{*}p < 0.05$.

3 Results

3.1 Procaine Hydrochloride Alleviates DSS-Induced Colitis in Mice

Experimental colitis was induced by administering 3% DSS in the drinking water for 7 days, after which the therapeutic efficacy of procaine hydrochloride was assessed (*figure 1A*). After 7 days, the mouse groups were administered daily intraperitoneal injections of either saline (the DSS group) or procaine hydrochloride for 1 week. SASP were administered by oral gavage for 1 week. Both SASP and procaine hydrochloride were able to reverse colon shortening, a key characteristic of DSS-induced colitis (*figure 1B,C*). During the establishment of experimental colitis, mice of DSS-treated group exhibited marked reduction in body weight, reaching a reduction of 23.5% ($p < 0.001$). However, in comparison with the DSS group, mice treated with procaine hydrochloride exhibited a gradual recovery in body weight (*figure 1D*). A significant reduction in DAI score was observed in procaine group relative to

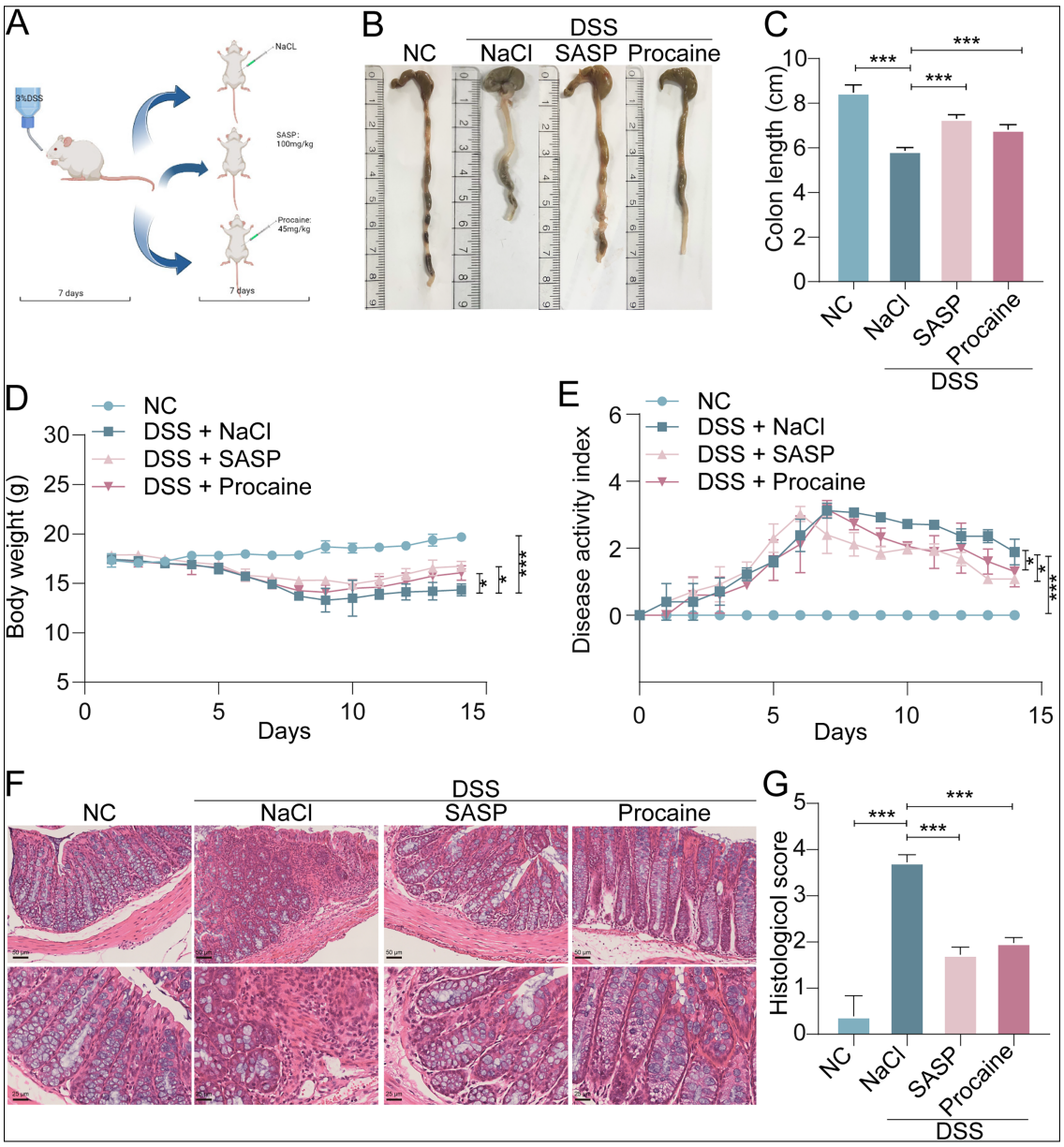

Figure 1: Procaine alleviated dextran sulfate sodium (DSS)-induced colitis. (**A**) The colitis model treatment groups. (**B**) Morphological changes in colons under different treatment regimens ($n = 6$). (**C**) Colon length in mice ($n = 6$). (**D**) Body weight in mice ($n = 6$). (**E**) Disease activity index (DAI) in mice ($n = 6$). (**F**) Hematoxylin and Eosin (H&E) staining of colonic tissue in mice (magnification, ×20/×40). (**G**) Histopathological scores on the basis of H&E staining of colon tissue sections from different groups. Data are presented as mean ± SD. *$p < 0.05$ and ***$p < 0.001$ indicate significant differences. NC: negative control; SASP: sulfasalazine.

the DSS group, reflecting attenuation of DSS-induced disease manifestations, involving weight loss, diarrhea and rectal bleeding ($p < 0.05$, *figure 1E*). Compared with the NC group, colon sections from DSS-treated mice exhibited severe inflammatory cell infiltration, decreased goblet cell numbers, and disrupted crypt architecture, whereas these histopathological changes were significantly ameliorated following procaine treatment, as reflected by lower histopathological scores ($p < 0.001$, *figure 1F,G*).

3.2 *Procaine-Mediated Amelioration of Colitis is Associated with an Expansion of Tregs*

In comparison with the DSS group, mice receiving procaine treatment exhibited a 1.96-fold elevation in the proportion of FOXP3-expressing $CD25^+$ T cells in spleen ($p < 0.001$), while SASP treatment significantly increased the proportion by 1.22-fold ($p < 0.05$) (*figure 2A,B*). Cytokine in colon tissue homogenates showed a significant increase in levels of IL-6 ($p < 0.001$) and IL-17 ($p < 0.01$) in DSS-treated mice, as compared with NC group, which were reduced to different extents following treatment with SASP and procaine. Moreover, SASP treatment significantly increased IL-10 ($p < 0.001$) and TGF-β ($p < 0.001$) levels in colon tissue, while procaine treatment also markedly elevated IL-10 ($p < 0.001$) and TGF-β ($p < 0.001$) (*figure 2C–F*).

3.3 *Procaine Improves DSS-Induced Epithelial Barrier Damage*

The protective effects of procaine against DSS-induced epithelial barrier injury were detected by analyzing

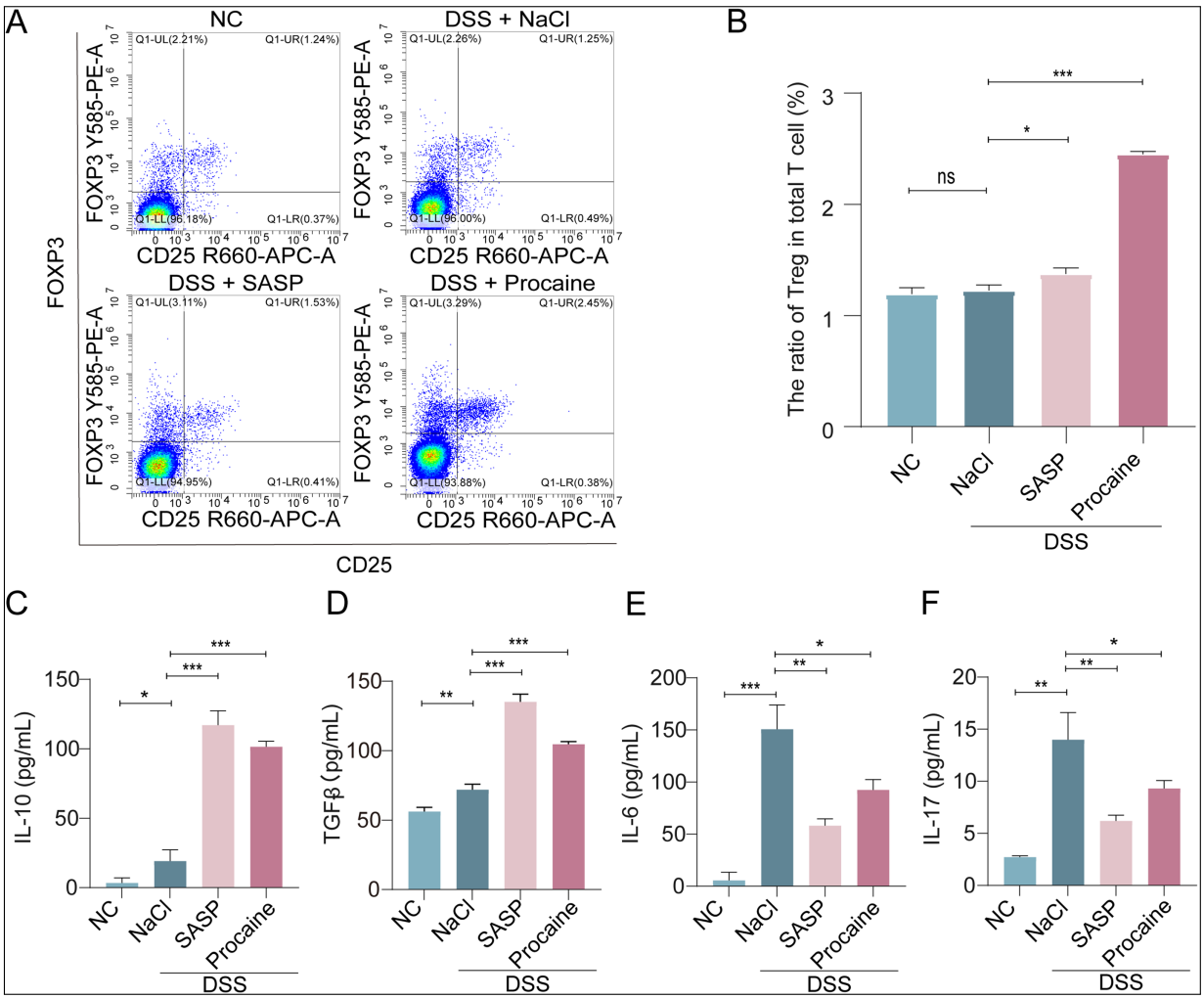

Figure 2: Procaine enhanced anti-inflammatory effects through the expansion of Tregs. (**A**) Flow cytometry analysis of Treg cell proportions in different groups. (**B**) The percentage of Treg cells from mice. (**C**–**F**) The level of IL-10 (**C**), TGF-β (**D**), IL-6 (**E**) and IL-17 (**F**) in the colonic tissue. All experiments were independently replicated three times, and the values are presented as mean ± SD ($n = 3$). ns, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NC: negative control; DSS: dextran sulfate sodium; SASP: sulfasalazine; IL: interleukin; TGF-β: transforming growth factor-β.

TJs within the intestinal epithelium. As anticipated, the DSS-treated mice exhibited decreased expression of ZO-1, occludin, and claudin-1 in colonic tissues (*figure 3A–C*), suggesting severe disruption of epithelial barrier by DSS. Treatment with procaine increased expression of TJs, revealing its reparative effect on intestinal epithelial barrier damage. Western blotting analysis further confirmed the above observations, revealing significantly decreased occludin and claudin-1 protein expression in DSS-treated mice, whereas procaine treatment substantially restored their expression levels ($p < 0.01$; *figure 3D*).

3.4 Procaine Attenuates DSS-Induced Epithelial Cell Apoptosis

Inflammation is a key contributor to cell apoptosis and is closely linked to colonic epithelial barrier function. The effects of procaine treatment on intestinal epithelial cell apoptosis were detected in colon tissues using TUNEL staining (*figure 4A*). DSS-induced colitis significantly increased apoptosis levels which were markedly reduced after treatment with SASP ($p < 0.05$) or procaine ($p < 0.05$, *figure 4B*). These changes were further confirmed by altered expression of apoptotic proteins in colon tissue, with increased expression of pro-apoptotic proteins BAX and cleaved-caspase-3, as well as decreased anti-apoptotic protein BCL-2 in DSS group. Procaine reversed these DSS-induced changes, leading to reduced pro-apoptotic proteins together with enhanced BCL-2 expression (*figure 4C,D*).

3.5 Procaine Hydrochloride Regulates T Cell Differentiation into Tregs via the IL-6/STAT3 Signaling Pathway

The frequency of Treg in colon of DSS and procaine-treated mice was assessed by determination of *FOXP3* and *TGF*-β mRNA levels by RT-qPCR analysis. As shown in *figure 5A,B*, transcripts of both *FOXP3* and *TGF*-β were elevated in procaine and DSS-treated mice, as compared to those in mice treated with DSS alone. These results were further validated by Western blotting showing a significant increase in FOXP3 ($p < 0.01$) and TGF-β ($p < 0.01$) protein levels in colonic tissue, after procaine treatment of DSS-treated mice as compared to those in DSS group (*figure 5C,D*). It has been reported that DSS treatment in mice leads to

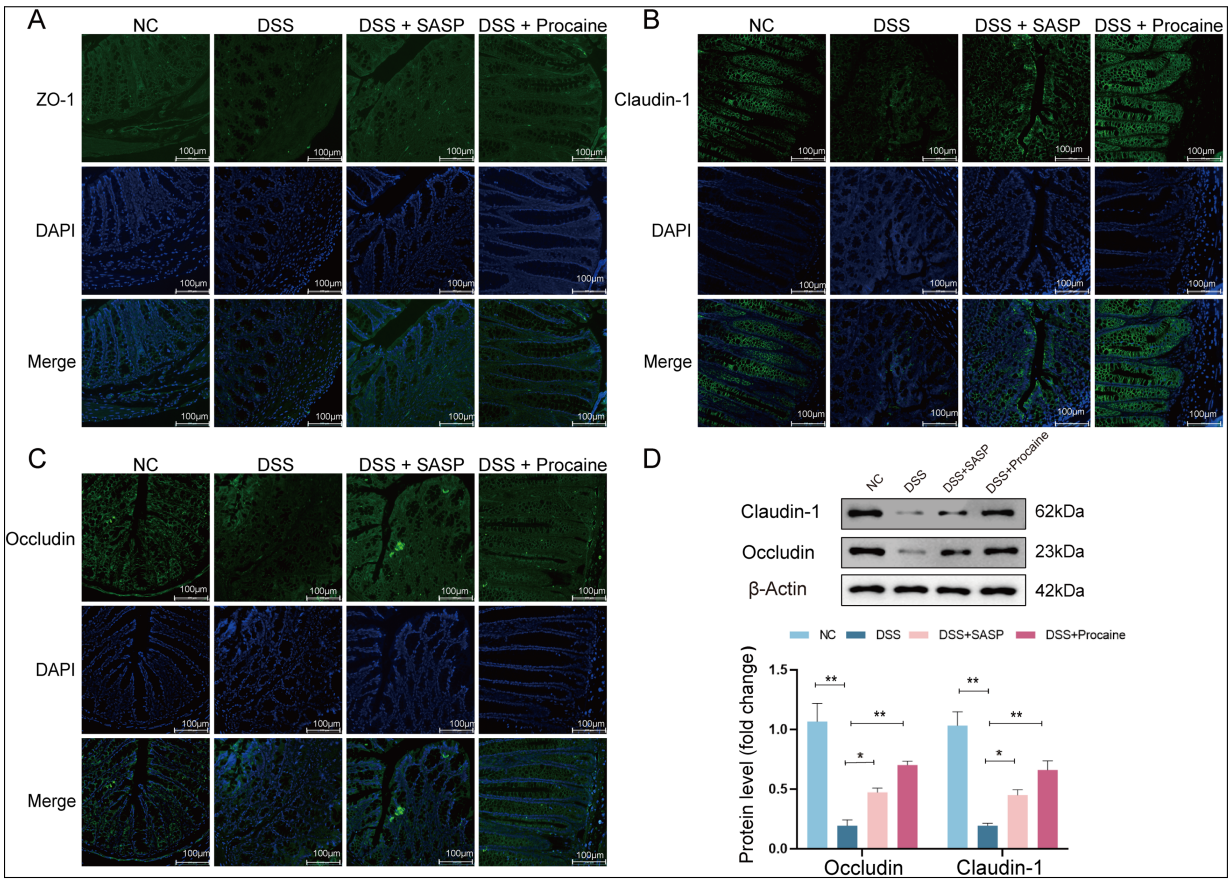

Figure 3: Procaine protected intestinal barrier function in dextran sulfate sodium (DSS)-induced colitis. (**A**–**C**) ZO-1 (**A**), claudin-1 (**B**) and occludin (**C**) immunofluorescence staining in negative control and colitis murine colons (magnification, ×20). (**D**) Western blotting analysis of claudin-1 and occludin expression in colonic tissues of mice. All experiments were independently repeated three times ($n = 3$). *$p < 0.05$, **$p < 0.01$, NC: negative control; SASP: sulfasalazine; DAPI: 4′,6-diamidino-2-phenylindole.

increased IL-6 expression in colonic tissue, leading to the phosphorylation of downstream STAT3 proteins and the promotion of Th17 differentiation. Indeed, the Western blotting results demonstrated elevated IL-6 protein expression in colon tissues after DSS stimulation, accompanied by increased expression levels and phosphorylation of STAT3. Treatment with procaine effectively inhibited activation of the IL-6/STAT3 signaling cascade and facilitated the expansion of regulatory T cells (*figure 5E,F*).

3.6 Procaine Amplifies Expansion of FOXP3$^+$ Tregs by Inhibiting FOXP3 Methylation

To determine whether the expansion of Tregs was associated with DNA methylation, freshly isolated T cells were cultured *in vitro* with procaine hydrochloride, followed by the measurement of FOXP3$^+$ T cells by flow cytometry. Cells were cultured for 24 h with procaine at concentrations ranging from 0 to 5 µM, which led to a dose-dependent increase in the proportion of FOXP3$^+$ T cells (*figure 6A*). Furthermore, only the highest concentration (5.0 µM) significantly increased FOXP3 protein expression ($p < 0.05$; *figure 6B,C*). MSP demonstrated a significant decrease in FOXP3 promoter methylation in T cells cultured with 2.5 or 5 µM procaine, highlighting the demethylating effect of procaine on the FOXP3 promoter ($p < 0.05$, *figure 6D,E*).

4 Discussion

The intestinal mucosal barrier is recognized as a key contributor to the development and progression of colitis [23,24]. It is largely dependent on intercellular TJs [25,26], which are responsible for cell-cell adhesion, epithelial permeability and paracellular diffusion [27]. The structure of tight junctions (TJs) is formed by transmembrane proteins, primarily occludin and claudin family proteins, in association with adaptor proteins such as zonula occludens (ZO). Increased intestinal TJ permeability facilitates invasion of harmful substances into tissues, which in turn elevates the susceptibility to intestinal infection and inflammatory lesions [28]. Our findings demonstrated that DSS-induced colitis caused substantial disruption of epithelial barrier, concurrent with reduced tight junction-associated proteins ZO-1, claudin-1, and occludin. Administration of SASP significantly reversed DSS-induced reduction in the expression of these junctional proteins, and procaine hydrochloride exhibited similar therapeutic effects by repairing the damaged epithelial barrier. However, one point that needs to be addressed is that ZO-1 protein was not successfully detected by Western blotting in our study, possibly due to the technical difficulties associated with its relatively high molecular weight, which can compromise protein transfer efficiency and band resolution. Nevertheless, immunofluorescence staining

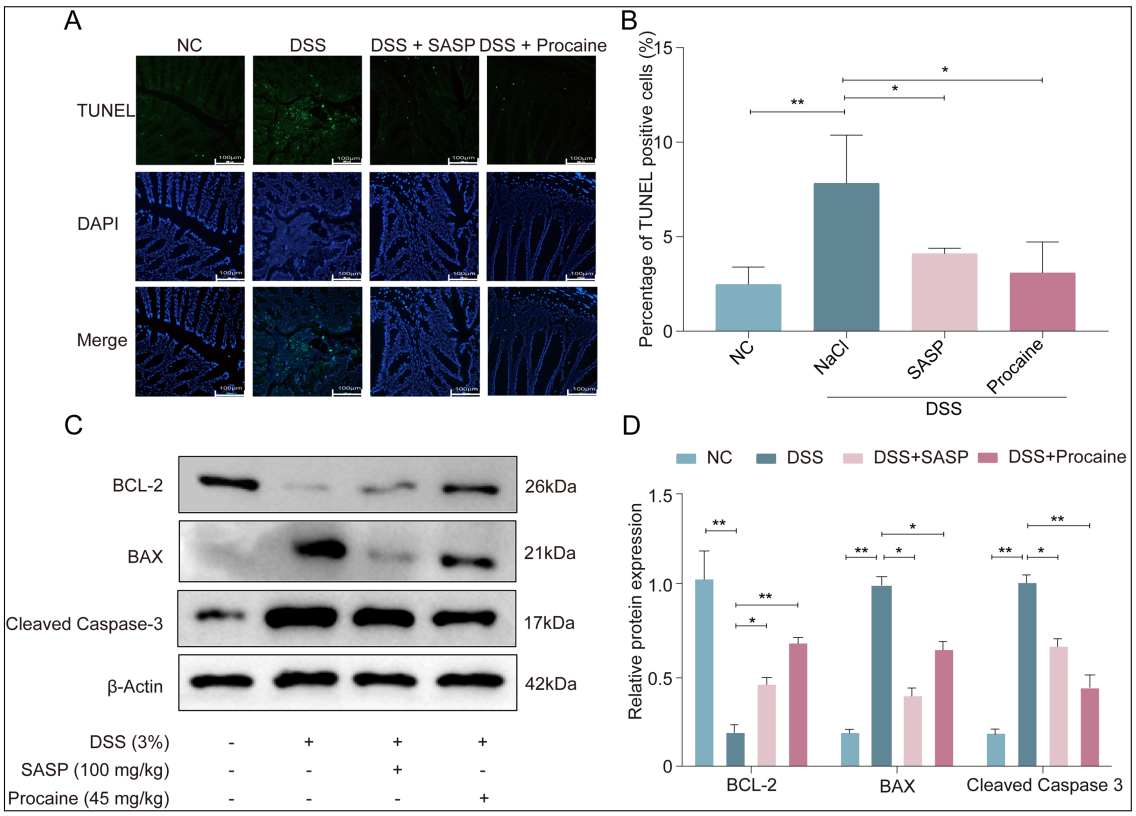

Figure 4: Procaine attenuated apoptosis levels in colonic tissue. (**A**) TUNEL staining of colon tissue sections to evaluate cellular apoptosis levels. (**B**) The percentage of apoptotic cells. (**C**) Apoptosis-related proteins were detected by western blotting. (**D**) Relative changes in apoptotic protein expression. All experiments were independently repeated three times ($n = 3$). Data are presented as mean ± SD. *$p < 0.05$ and **$p < 0.01$ indicate significant differences. NC: negative control; DSS: dextran sulfate sodium; SASP: sulfasalazine. TUNEL: terminal deoxynucleotidyl transferase dUTP nick end-labeling; DAPI: 4′,6-diamidino-2-phenylindole; BCL-2: B-cell lymphoma/leukemia 2; BAX: Bcl-2-associated X protein.

confirmed the alterations in ZO-1 expression, supporting the conclusion that procaine hydrochloride improved intestinal barrier function. Epithelial cell apoptosis exacerbates the inflammatory progression of colitis [29]. Therefore, to further elucidate the effects of procaine hydrochloride on experimental colitis, epithelial cell apoptosis in colonic sections was evaluated. Notably, procaine hydrochloride significantly inhibited colon epithelial cell apoptosis. Consistent with the above observations, reduced expression of BAX and cleaved caspase-3, together with increased BCL-2 expression in colonic tissues, further demonstrated the anti-apoptotic effect of procaine hydrochloride. Collectively, these findings indicated that procaine hydrochloride mitigated inflammation by preserving integrity of intestinal epithelial barrier and attenuating epithelial cell apoptosis.

DSS-induced colitis is accompanied by severe oxidative stress and lipid peroxidation [30]. During inflammatory bowel disease (IBD), the colonic microenvironment involves a complex spectrum of regulated cell death. Various distinct cell death modalities, such as ferroptosis, necroptosis, and pyroptosis, make unique contributions to pathogenesis of IBD [31,32]. Recent studies have demonstrated that procaine exhibits regulatory activity in cell apoptosis. Evidence from Chandra Sekhar Bhol and colleagues revealed that procaine elevates PAX9 expression through inhibiting DNA methylation in oral squamous cell carcinoma, thereby triggering apoptosis and autophagy, and controlling stemness and differentiation [33]. Similarly, previous studies have demonstrated that procaine exerts antitumor effects in human tongue squamous cell carcinoma by triggering cell cycle arrest, apoptosis, and autophagy [34]. In our experiments, procaine hydrochloride alleviated DSS-induced colitis by inhibiting epithelial cell apoptosis. Moreover, procaine exhibited antioxidant effects in both *in vivo* and *in vitro* models, suggesting that procaine hydrochloride may also alleviate ulcerative colitis through these mechanisms [35]. These findings prompt the use of specific inhibitors (e.g., Ferrostatin-1 to inhibit ferroptosis, necroptosis-1 to inhibit necroptosis) or the detection of relevant markers in future studies to clarify whether procaine additionally suppresses these cell death pathways.

Naïve T cells have the potential to undergo differentiation into regulatory T cells or T helper 17 (Th17) cells in response to distinct immunological stimuli [36]. Tregs preserve immune homeostasis and limit inflammatory responses through production of anti-inflammatory cytokines, whereas T helper 17 (Th17) cells are strongly implicated in the pathogenesis of autoimmune diseases, including autoimmune myocarditis, arthritis, and myelitis [37,38]. The present results corroborate our previous finding that agents targeting DNA methylation pathways, including decitabine, facilitate restoration of epithelial barrier integrity, which appears to be accompanied by enhanced differentiation of naïve T cells into splenic

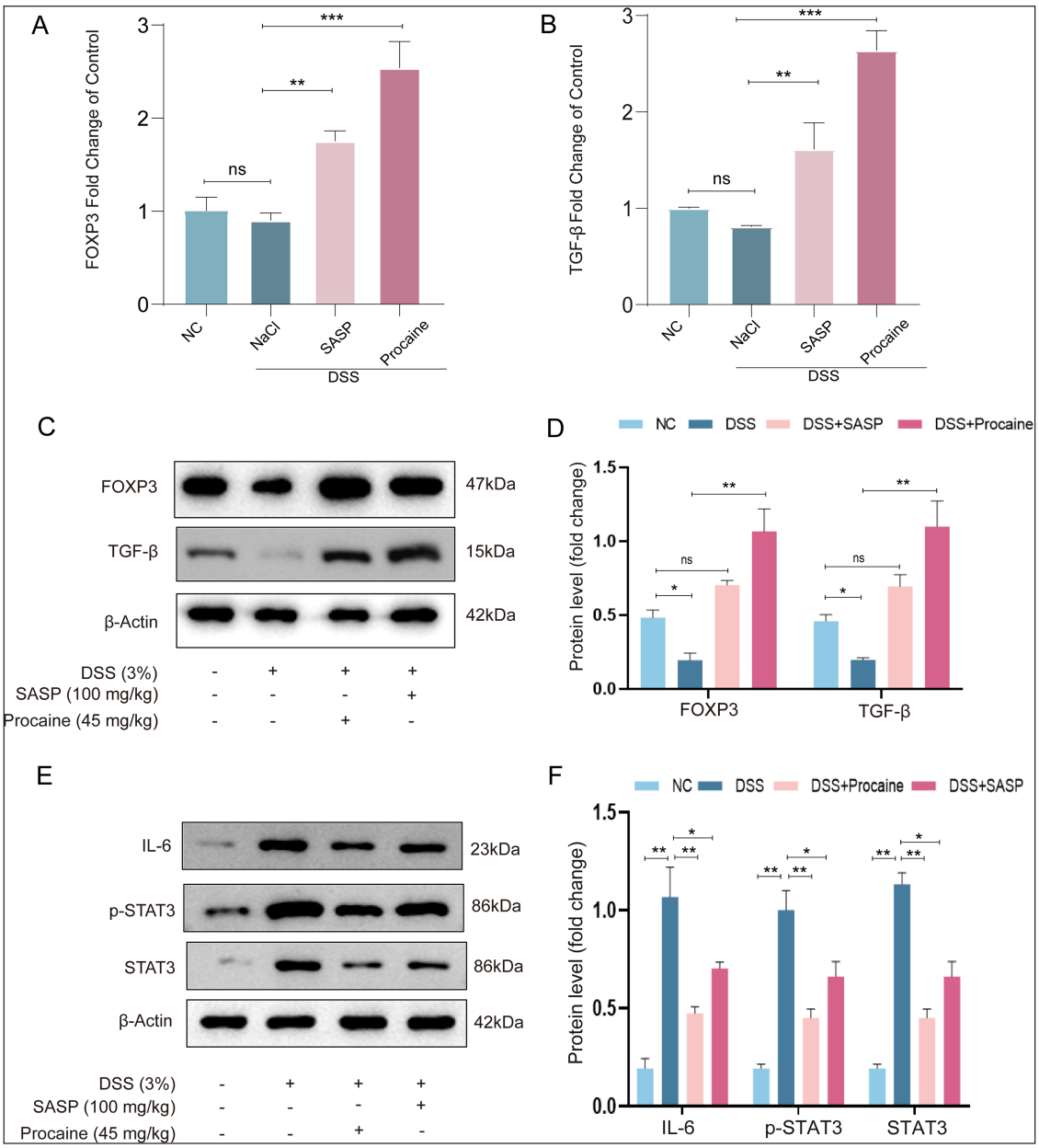

Figure 5: Procaine induced the formation of Tregs through inhibiting IL-6/STAT3 signaling. (**A**,**B**) The mRNA expression of FOXP3 and TGF-β in colonic tissues was detected by RT-qPCR. (**C**,**D**) Protein expression of FOXP3 and TGF-β in colonic tissue was assessed by western blotting analysis, followed by quantification using ImageJ. (**E**,**F**) Protein expression of IL-6, STAT3 and p-STAT3 in colonic tissue was examined by western blotting analysis, followed by quantification using ImageJ. All experiments were independently repeated three times and the data are presented as mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ represent significant differences, and ns denotes no significant difference. NC: negative control; DSS: dextran sulfate sodium; SASP: sulfasalazine; p: phosphorylated; FOXP3: forkhead box protein 3; STAT3: signal transducer and activator of transcription factor 3; TGF-β: transforming growth factor-β; IL-6: interleukin-6.

Tregs in mice [19]. The IL-6/STAT3 signaling cascade is closely involved in the differentiation of Th17 cells and Tregs, and restoration of the Treg/Th17 balance has emerged as a potential therapeutic strategy for the management of chronic inflammatory diseases. During the onset of colitis, immune cells in intestinal tract become overly activated and secrete considerable amounts of inflammatory cytokines, including IL-6 [39]. IL-6 triggers activation of downstream JAK/STAT signalling pathway, leading to the phosphorylation of STAT3, thereby promoting the induction of differentiation of Th17 cells in intestinal tract, further promoting intestinal damage [40]. Compared with NC group, mice in DSS group exhibited markedly elevated IL-6 expression and enhanced STAT3 phosphorylation in colonic tissues. Treatment with procaine hydrochloride significantly suppressed IL-6 expression and STAT3 activation, while simultaneously increasing the proportion of Tregs in colitis mice. These findings suggest that procaine hydrochloride may promote Treg expansion through modulation of IL-6/STAT3 signaling pathway. *In vitro*, the present research investigated the inhibitory effects of procaine hydrochloride, which acts as a DNA methyltransferase inhibitor on DNA methylation. At present, DNA methylation is currently the most well-understood and important form of epigenetic modification [41]. DNA methylation is

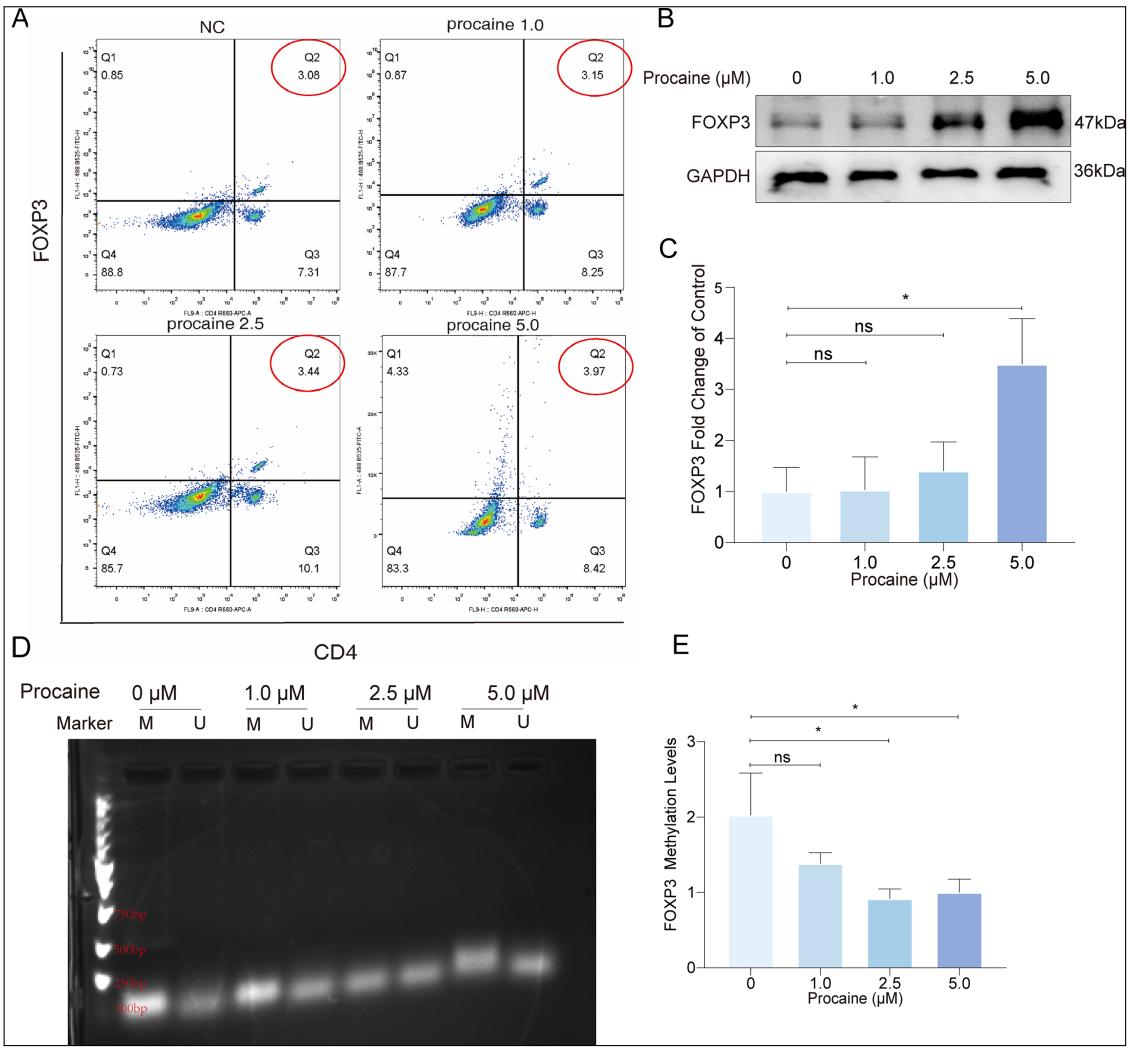

Figure 6: Procaine inhibited *FOXP3* promoter methylation in Tregs. (**A**) Administration of procaine hydrochloride promoted expansion of Tregs. (**B**) Western blotting of the Tregs-specific protein FOXP3 after procaine treatment. (**C**) Quantitative analysis of intracellular FOXP3 expression using ImageJ software. (**D**,**E**) MSP analysis of FOXP3 promoter methylation levels, analyzed by ImageJ software. The experiments were independently repeated three times ($n = 3$). *$p < 0.05$ represents significant differences, and ns denotes no significant difference. NC: negative control; MSP: methylation-specific PCR; FOXP3: forkhead box protein 3.

generally associated with transcriptional silencing of specific genes, whereas demethylation promotes their reactivation and subsequent expression. In this respect, procaine hydrochloride inhibited the methylation of the FOXP3 promoter *in vitro*, resulting in the induction of stable FOXP3 expression which was associated with an enhanced proportion of Tregs (*figure 7*).

Multiple studies have indicated that procaine exerts therapeutic effects in cancer by acting as a non-specific inhibitor of DNA methyltransferases [20,33,42]. However, such systemic administration may induce widespread transcriptomic alterations beyond the FOXP3 and IL-6/STAT3 axes, potentially leading to unintended activation of off-target genes. This could result in a series of adverse effects, including the activation of proto-oncogenes, disruption of cellular metabolic networks, and alterations in normal cell states [43]. Precise gene regulation will therefore be an important direction for future research. Going forward, by deeply mining public databases such as GEO to integrate multi-omics information, utilizing spatial multi-omics technologies to precisely localize tissue heterogeneity, combining precise tracking at the single-cell level, and supplementing with AI-driven prediction platforms, the integration of these approaches will systematically construct an "off-target panoramic map" ranging from molecular sequences to the tissue environment.

Taken together, the present study shows that procaine hydrochloride ameliorates DSS-induced colonic injury and inflammation through maintenance of intestinal barrier integrity and suppression of apoptosis in colon tissue. These therapeutic effects may involve the modulation of IL-6/STAT3 signaling pathway, inhibition of FOXP3 methylation and expansion of Tregs that, in addition to exerting anti-inflammatory activity, are also involved in tissue repair and thus might play a role in maintenance of epithelial barrier integrity [44]. Importantly, sulfasalazine (SASP) was included in the present study as a clinically established reference treatment for DSS-induced colitis. Our results demonstrated that procaine hydrochloride exerted therapeutic effects generally comparable to those of SASP in alleviating colonic inflammation, epithelial barrier disruption,

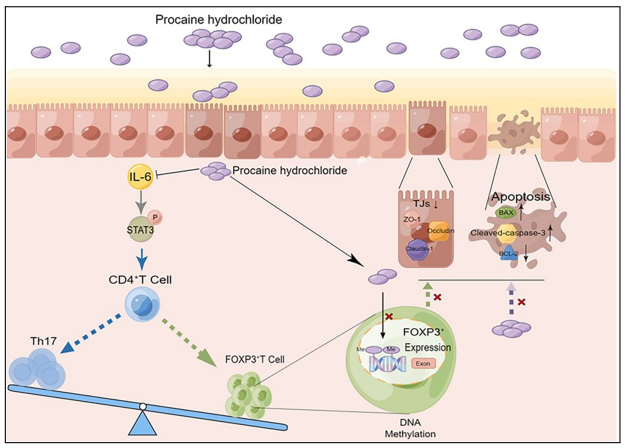

Figure 7: Graphical summary of the mechanism by which procaine hydrochloride amplifies Tregs proportion and modulates the barrier function through inhibiting IL6/STAT3 and FOXP3 methylation in DSS-induced colitis. IL-6: interleukin-6; STAT3: signal transducer and activator of transcription factor 3; CD: Cluster of Differentiation; Th17: T helper cell 17; FOXP3: forkhead box protein 3; TJs: tight junctions; ZO-1: zonula occludens-1; BAX: Bcl-2-associated X protein; BCL-2: B-cell lymphoma/leukemia 2.

and immune imbalance. It should be noted that the primary objective of this study was not to demonstrate head-to-head superiority over SASP, but rather to establish procaine's non-inferiority while exploring its unique epigenetic properties. Consequently, the *in vitro* methylation experiments in the later stage of this study were designed as mechanistic validation assays focused specifically on procaine's ability to inhibit FOXP3 promoter methylation and promote Treg expansion. Since SASP acts through traditional anti-inflammatory pathways and is not recognized as an epigenetic demethylating agent, it was excluded from these specific mechanistic assays. Given these distinct pathways, procaine offers unique translational advantages. While its efficacy matches that of conventional treatments, procaine may circumvent the severe adverse effects frequently associated with SASP, such as hypersensitivity and hepatotoxicity, offering a safer alternative for intolerant patients. Furthermore, their non-overlapping mechanisms suggest a strong potential for future synergistic combination therapies.

Although the present study yielded promising results, several limitations remain and warrant further investigation in future research. A major limitation in our study is that the conclusions are based predominantly on preclinical animal models of experimentally induced colitis. Given the substantial interspecies differences in drug metabolism and physiological responses between animals and humans, additional studies involving compound optimization, pharmacokinetic assessment, and rigorous clinical validation are required to facilitate clinical translation. It should be noted that the current study concentrated primarily on the immunomodulatory mechanistic basis of procaine via Treg expansion. Whether Procaine exerts a direct protective effect on intestinal epithelial cells independent of immune microenvironment remains to be investigated using isolated epithelial cell lines in future studies. Additionally, procaine overdose can cause adverse effects on the central nervous system. Based on a review of the literature, we consider that intraperitoneal administration of procaine hydrochloride at 45 mg/kg in mice is a relatively safe regimen that generally does not induce central nervous system adverse reactions such as convulsions [20,45,46]. Future studies should focus on optimizing oral administration strategies to better reflect clinical therapeutic conditions, while systematically investigating the long-term safety and biological effects of procaine in diverse human cell types, including its potential neurotoxicity, such as convulsions and seizures, and other adverse reactions including anaphylactic shock and cardiovascular toxicity. Such investigations would enhance the clinical translational value of the study. Furthermore, increasing evidence has indicated that the gut microbiota is closely associated with initiation and progression of IBD [47–49]. However, the potential relationship between procaine and the gut microbiota remains unclear. Future studies are needed to further investigate the interactions between procaine hydrochloride and the gut microbiota. In addition to its known biological activities, procaine has been implicated in the regulation of various cell death pathways. However, whether procaine modulates inflammatory bowel disease via ferroptosis, necroptosis, or other forms of regulated cell death remains unknown and deserves further mechanistic investigation.

5 Conclusions

Taken together, our findings indicate that procaine hydrochloride, a local anesthetic with a long history of clinical application, may represent a safe and potential therapeutic option for inflammatory bowel disease.

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Availability of Data and Materials: Not applicable.

Ethics Approval: Ethics approval was obtained from the Ethics Committee of Minhang Hospital, Fudan University, Shanghai, China (Approval no. 2021JS Minhang Hospital-042) and experiments were performed according to the Guidelines for the Ethical Review of Laboratory Animal Welfare (People's Republic of China National Standard GB/T 35892–2018).

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1
Antibody information

Antibodies	Identifier	Source	Dilution ratio
ZO-1	AF5145	Affinity	1:1000
Claudin-1	DF6919	Affinity	1:1000
Occludin	DF7504	Affinity	1:1000
BCL-2	AF6139	Affinity	1:1000
BAX	AF0120	Affinity	1:1000
Cleaved-caspase-3	AF7022	Affinity	1:1000
FOXP3	AF6544	Affinity	1:1000
TGF-β	AF1027	Affinity	1:1000
β-actin	AF7018	Affinity	1:10,000
IL-6	ab229381	Abcam	1:1000
p-STAT3	ab32143	Abcam	1:1000
STAT3	ab68153	Abcam	1:1000
GAPDH	AF7021	Affinity	1:3000

Table A2
RT-qPCR primer sequences

Gene		Primer sequences
FOXP3	For	CAGCACATTCCCAGAGTTCCTC
	Rev	GCGTGTGAACCAGTGGTAGATC
TGF-β	For	GCGGACTACTATGCTAAAGAGG
	Rev	CCGAATGTCTGACGTATTGAAG
β-Actin	For	ATGGGTCAGAAGGATTCCTATGTG
	Rev	CTTCATGAGGTAGTCAGTCAGGTC

Table A3
MSP primer sequences

Gene		Primer sequences
FOXP3-Meth	For	AGAGGTTTAAAAACTGGGAGATTTC
	Rev	ATTAACTCGCTACAACCATTATCGT
FOXP3-Unmeth	For	AGAGGTTTAAAAAGTGGGAGATTTT
	Rev	TTAACTCACTACAACCATTATCATC

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