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ORIGINAL ARTICLE

H.pylori Infection Alleviates Acute and Chronic Colitis with the Expansion of Regulatory B Cells in Mice

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Abstract— Epidemiological studies showed that there was an inverse relationship between *Helicobacter pylori* (*H. pylori*) infection and the incidence of inflammatory bowel diseases (IBD). Our previous research indicated that the regulatory immune responses induced by *H. pylori* infection were not limited to gastric mucosa, and the balance of intestinal mucosal immunity was influenced. In this study, mice were infected with *H. pylori SS1*, and then colitis was induced by 3% dextran sulphate sodium (DSS), to investigate the role of the regulatory B cells in the effects of *H. pylori* infection on acute and chronic colitis. In acute and chronic colitis groups, DAI and colonic histological scores reduced significantly and colon length shorted less, the proinflammatory cytokines mRNA expression downregulated in colonic mucosa, and the percentages of $CD19^+IL\text{-}10^+$Breg cells were higher in the *H. pylori*/DSS co-treated groups compared with the DSS-treated groups. Our study suggests that *H. pylori* infection can alleviate the acute and chronic colitis induced by DSS, and $CD19^+IL\text{-}10^+$Breg cells may play a critical role in the alleviation of acute and chronic colitis following *H. pylori* infection.

KEY WORDS: Regulatory B cells; *Helicobacter pylori*; Inflammatory bowel diseases; Regulatory T cells.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is the predominant bacterium colonized in the stomach, and associated with several diseases including gastritis, peptic ulcer, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphomas [1]. *H. pylori* infection cannot be cleared effectively even though it can elicit a series of immune responses. The immunological escape is considered to be mediated by the immunosuppressive regulatory cells. Foxp3 expression can be induced by *H. pylori* infection, and there is no or less Foxp3 mRNA expression in the stomach without *H. pylori* infection [2, 3]. Gastritis caused by *H. pylori* is relieved by increased CD4 + CD25 + Foxp3+ marked regulatory T cells (Treg), which inhibit the Th1 and Th17 cells through the secretion of anti-inflammatory cytokines (IL-10,TGF-β) and the manner of cell to cell contact [4, 5].

Our previous study showed that, besides the $CD4^+CD25^+Foxp3^+$Treg cells, CD19 + IL-10+ marked regulatory B cells (Breg) also expanded significantly after *H. pylori* infection. The regulatory immune responses induced by *H. pylori* infection were not limited to the stomach [6]. In addition to Treg cells and Breg cells, regulatory dendritic cells are also associated with the immunological escape of *H. pylori* colonization [7, 8].

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In recent years, more and more studies showed that *H. pylori* infection was negatively related to certain autoimmune diseases, such as inflammatory bowel disease (IBD), asthma, and eczema [9–13]. IBD is a common kind of autoimmune disease, consisting of ulcerative colitis (UC) and Crohn's disease (CD), which are characterized as chronic and recurrent intestinal inflammation. The etiology and pathogenesis are undetermined, inappropriate activation of the mucosal immune system plays an important role in the process of IBD [14, 15]. Peter D.R. Higgins and colleagues [16] showed that *H. pylori* infection can reduce the Th17 immune response in cecum caused by Salmonella typhimurium infection, and increase the IL-10 expression in mesenteric lymph nodes (MLN). Jay Luther and colleagues [17] detected *H. pylori* DNA in intestine of *H. pylori*-positive patients, which can inhibit the secretion of pro-inflammatory cytokine (IL-12,IFN-I) from intestinal DC cells *via* TLR-9 pathway, alleviate the intestinal inflammatory responses induced by dextran sulphate sodium (DSS). It was also reported that *H. pylori* infection changed the balance of Th17/Treg cells and skewed to the Treg cell responses in intestinal DC cells, and then affected the intestinal mucosal immune responses [7, 18].

Numerous studies have shown that, experimental treatment of colitis induced by DSS was primarily performed by elevating the $CD4^+CD25^+Foxp3^+$Treg cells, reducing the Th17 cells, increasing IL-10 expression, and decreasing IL-1β, IL-17 expression [15, 19]. Ansary MM. et al. found that apoptic cells could alleviate the chronic colitis induced by DSS through enhancing the function of Breg cells [20].

Induction of colitis by application of DSS in drinking water is a widely used and well-characterized model of colitis in mice [21]. In our study, we use a rodent model, which was established acute and chronic colitis induced by DSS with or without *H. pylori* pre-infection, to investigate the effects of *H. pylori* infection on acute and chronic colitis, and the role of $CD19^+IL\text{-}10^+$Breg cells in the process.

MATERIALS AND METHODS

Animals

Female 6–8 weeks old C57BL/6 mice were purchased from Beijing Vitalriver Laboratory Animal Technology Company Limited, China. All mice were housed in specific pathogen-free room in microisolator cages in the Experimental Animal Center of Chinese Center for Disease Control and Prevention.

H. pylori Infection

All mice fasted overnight were intragastrically inoculated five times with 300 μl phosphate buffer containing 1×10^9CFU/ml *H. pylori SS1* every other day. Age-matched control mice were inoculated with physiological saline without *H. pylori SS1*. Urease rapid test and Warthin-Starry were utilized to confirm the colonization of *H. pylori SS1*.

DSS-Induced Colitis

Six weeks after *H. pylori* inoculated, 3% DSS (MP Biomedicals, CA, USA) was dissolved in sterile phosphate buffer and *ad libitum* for 7 days followed by substitution of phosphate buffer for another 7 days (one cycle) to induce acute colitis, repeating the cycle three times to induce chronic colitis. All mice were assessed daily for body weight, diarrhea, and bloody stool. The extent of the colitis was assessed by disease activity index (DAI). The DAI was calculated using the average of the total score: loss in body weight (0, none; 1, 1–5%; 2, 5–10%; 3, 10–15%; 4, > 15%); stool consistency (1, none; 2, loose stools; 4, diarrhea), and bloody stool (0, normal; 2, slight bleeding; 4, gross bleeding). There were five to six mice in each group.

Flow Cytometry

FITC-, APC-, PE- , and PeyCP-cy5.5-conjugated anti-mouse CD4, CD25, Foxp3 (eBioscience, San Diego, CA, USA) and CD19, IL-10 (Biolegend, San Diego, CA) antibodies were used to stain the regulatory B cells and regulatory T cells. Briefly, after stimulating the cells with the cell stimulation cocktail (plus protein transport inhibitors) (eBioscience) for 5 h, $CD19^+IL\text{-}10^+$Breg cells were stained according to the manufacturer's instruction of the Intracellular Fixation & Permeabilization Buffer set (Biolegend), including cell surface staining, Fixation & Permeabilization, and intracellular staining. $CD4^+CD25^+Foxp3^+$Treg cells were stained according to the manufacturer's instruction of Foxp3-staining buffer set (eBioscience). All samples were collected and analyzed by BD FACS Calibur flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ, USA). The data were analyzed by FlowJo 7.6 (FlowJo, Ashland, Oregon, USA).

Histology and Immunohistochemistry

Sections of 4 μm thick were cut from formalin-fixed and paraffin-embedded stomach and colon tissue and stained with hematoxylin and eosin (H&E) or Warthin-Starry. Histological scores were assessed according to L.A.Dieleman [14]. Antigen retrieval was performed by heating the sections for 15 min in citric acid solution (pH 6.0). Sections were incubated with rabbit anti-mouse Foxp3 antibody (1:200; Abcam, HongKong SAR, China) overnight at 4 °C after being blocked with sheep serum solution. Foxp3+ cells were quantified based on the mean number of stained cells in the lamina propria of two sections, including five different fields per section and excluding lymphoid follicles.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

To detect the levels of the anti-inflammatory cytokines (IL-10,TGF-β) and pro-inflammatory cytokines (IFN-γ,TNF-α,IL-17A) in the colonic mucosa, total RNA of colon was extracted using Trizol reagent (Ambion, Carlsbad, CA, USA) and reverse-transcribed to first-strand complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo, Vilnius, Lithuania). RT-PCR was performed with a StepOne Plus Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, and the primers were listed in Table 1.

Statistical Analysis

All data were shown as mean ± SEM. Statistical analysis was performed with Statistical Package for Social Science version 17.0 software (SPSS Inc. Chicage.IL). Student's *t* test or one-way ANOVA was utilized to analyze values between different groups. $P < 0.05$ was considered statistically significant. The graphs were plotted by GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

RESULTS

H. pylori Infection-Alleviated Acute Colitis Induced by DSS

To investigate the effects of *H. pylori* infection on acute colitis induced by DSS, we treated wild type C57BL/6 mice, which infected with *H. pylori* 6 weeks ago, with 3% DSS for 7 days followed by PBS for 7 days to induce acute colitis model. The severity of colitis was evaluated by measuring DAI scores.

In acute colitis groups, body weight loss was initially observed on day 4 in DSS-treated mice. However, *H. pylori*/DSS cotreated mice began to display significant body weight loss on day 6. DSS-treated mice showed more weight loss between day 4 to day 8 compared with *H. pylori*/DSS cotreated mice (Fig. 1a). DAI scores in DSS-treated mice were significantly increased on day 3 and higher than *H. pylori*/ DSS cotreated mice between day 3 to 8 (Fig. 1b). The length of colon was measured when the mice were sacrificed. It is equally a parameter of the colitis. The colon length of DSS-treated mice shortened more than *H. pylori*/DSS cotreated mice (Fig. 1d). To further assess colitis severity, the degree of colitis was assessed histopathologically. DSS treatment induced epithelial injury, crypt damage, lamina propria edema and thickened, and mononuclear cells infiltration transmural (Fig. 1c). These changes were more severe in DSS-treated mice and the histology scores were significantly higher than *H. pylori*/DSS cotreated mice (Fig. 1e).

Table 1. Primers for real-time polymerase chain reaction

Primers	Forward 5′ → 3′	Reverse 5′ → 3′
GAPDH	GACATTGTTGCCATCAACGACC	CCCGTTGATGACCAGCTTCC
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
TGF-β	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC
Foxp3	TACCACAATATGCGACCC	CTCAAATTCATCTACGGTCC
TNF-α	TCCAGGCGGTGCCTATGT	CACCCCGAAGTTCAGTAGACAGA
IFN-γ	CAGCAACAGCAAGGCGAAA	CTGGACCTGTGGGTTGTTGAC
IL-17A	CTCCAGAAGGCCCTCAGACTAC	GAGCTTCCCAGATCACAGAGG

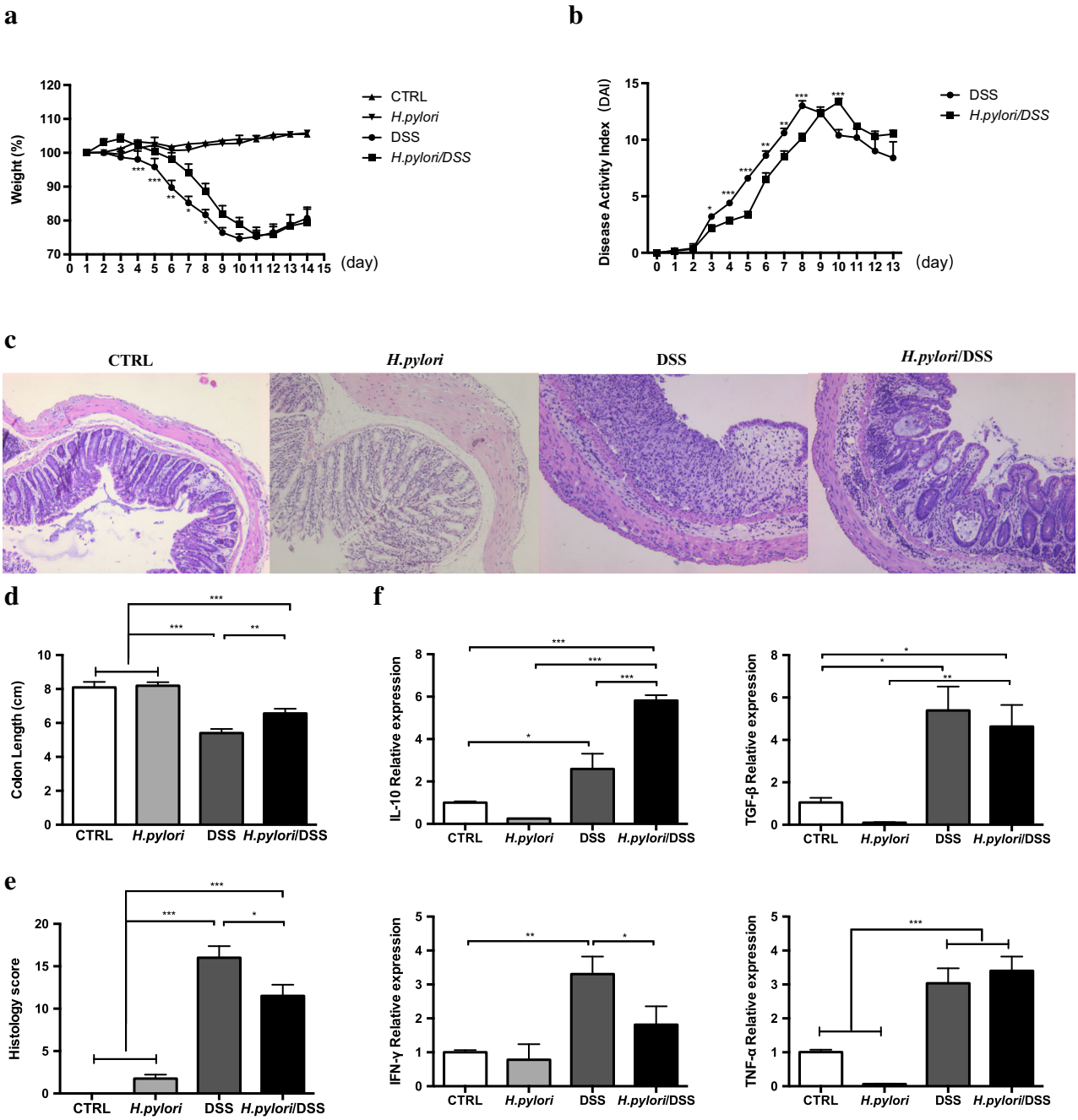

Fig. 1. *H. pylori* infection alleviated acute colitis induced by DSS. **a** Body weight. **b** Disease activation index (DAI). **c** H&E staining of colon.(× 100). **d** Colon length. **e** Histology score. **f** Relative expression of anti- and pro-inflammatory cytokines in colon of acute colitis mice.(*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; NS, no significant.)

Gene expression of cytokine reflects the immune and inflammatory states. We used quantitative real-time PCR to examine the expression of pro- and anti-inflammatory cytokine in colon. IL-10 and TGF-β

mRNA expression levels upregulated in DSS-treated mice and IL-10 mRNA was significantly lower than *H. pylori*/DSS cotreated mice (Fig. 1f). Both IFN-γ and TNF-α mRNA expression levels upregulated significantly in DSS-treated mice, while IFN-γ mRNA expression level was lower significantly in *H. pylori*/DSS cotreated mice (Fig. 1f). Together, the findings above demonstrated that *H. pylori* infection can alleviate the acute colitis induced by DSS.

CD19$^+$IL-10$^+$Breg Cells and CD4$^+$CD25$^+$Foxp3$^+$Treg Cells Expanded Notably in ***H.pylori***/DSS Cotreated Acute Colitis Mice

To find out whether CD19$^+$IL-10$^+$Breg cells play a role in the process of *H. pylori* infection affect DSS-induced acute colitis, the percentages of CD19$^+$IL-10$^+$Breg cells were detected in peripheral blood mononuclear cells (PBMC), spleen, MLN, and Peyer's Patches (PP).

In acute colitis groups, CD19$^+$IL-10$^+$Breg cells were trend to expand in PBMC, spleen, MLN, and PP in *H. pylori*-infected mice, and DSS treatment induced the expansion of CD19$^+$IL-10$^+$Breg cells, while the extent of expansion of CD19$^+$IL-10$^+$Breg cells in *H. pylori*/DSS cotreated mice was notably higher than the other three groups (Fig. 2b).

It is considered that, CD4$^+$CD25$^+$Foxp3$^+$Treg cells are important in the development of IBD. In our study, we also detected the percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in PBMC, spleen, MLN, and PP to examine the changes of Treg cells. The expansion of CD4$^+$CD25$^+$Foxp3$^+$Treg cells was observed in MLN and PP of *H. pylori*-infection mice and in MLN of DSS-treated mice, and the percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in spleen, MLN, and PP were significantly higher in *H. pylori*/DSS cotreated mice (Fig. 2d). We also detected the Foxp3 + Treg cells in colonic mucosa by immunohistochemistry. The results showed that Foxp3 + Treg cells increased significantly in *H. pylori* infection and DSS-treated mice, and the extent of increase was more notable in *H. pylori*/DSS cotreated mice (Fig. 2e). Collectively, the results above showed that CD19$^+$IL-10$^+$Breg cells increased in DSS-treated acute colitis, CD19$^+$IL-10$^+$Breg cells and CD4$^+$CD25$^+$Foxp3$^+$Treg cells expanded more remarkably in *H. pylori*/DSS cotreated mice.

***H. pylori* Infection Can Also Alleviate Chronic Colitis Induced by DSS**

Chronic colitis was induced by repeating three cycles of 3% DSS and PBS to investigate the effects of *H. pylori* pretreatment.

In chronic colitis groups, the clinical performance of chronic colitis changed in keeping with the component in drinking water. The colon length of *H. pylori*/ DSS cotreated mice shortened less than DSS-treated mice (Fig. 3a), and colonic histology was significantly slighter than DSS-treated mice (Fig. 3b). Histopathologically, performance was more severe in DSS-treated mice than *H. pylori*/ DSS cotreated group (Fig. 3c).

The mRNA expression of IL-10,TGF-β, and Foxp3 in colon were significantly higher in *H. pylori*/DSS cotreated mice comparing with DSS-treated mice, and the pro-inflammatory cytokine, including IFN-γ,TNF-α, and IL-17A mRNA expression upregulated in DSS-treated mice, which were significantly higher than *H. pylori*/DSS cotreated mice (Fig. 3d). Thus, the anti- and pro-inflammatory cytokine expression was consistent with the colonic histopathology, *H. pylori* infection can alleviate chronic colitis induced by DSS.

CD19$^+$IL-10$^+$Breg Cells Increased while CD4$^+$CD25$^+$Foxp3$^+$Treg Cells Decreased in ***H. pylori***/DSS Cotreated Chronic Colitis Mice

In chronic colitis groups, CD19$^+$IL-10$^+$Breg cells in spleen, MLN, and PP expanded significantly in *H. pylori* infection and DSS-treated mice, and the percentages of CD19$^+$IL-10$^+$Breg cells in spleen, MLN, and PP were significantly higher in *H. pylori*/DSS cotreated mice (Fig. 4a). The results were similar to the acute colitis groups.

While, the changes of CD4$^+$CD25$^+$Foxp3$^+$Treg cells were confusing, the percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in PBMC, spleen, and PP were increased significantly, which was consistent with the acute colitis groups in DSS-treated mice. However, the percentages of CD4$^+$CD25$^+$Foxp3$^+$Treg cells in tissues mentioned above were lower notably in *H. pylori*/DSS cotreated mice (Fig. 4b). The results of immunohistochemistry showed that there was no significant differences between the DSS-treated mice and *H. pylori*/DSS cotreated mice (Fig. 4c). Together, *H. pylori* infection induced the further expansion of CD19$^+$IL-

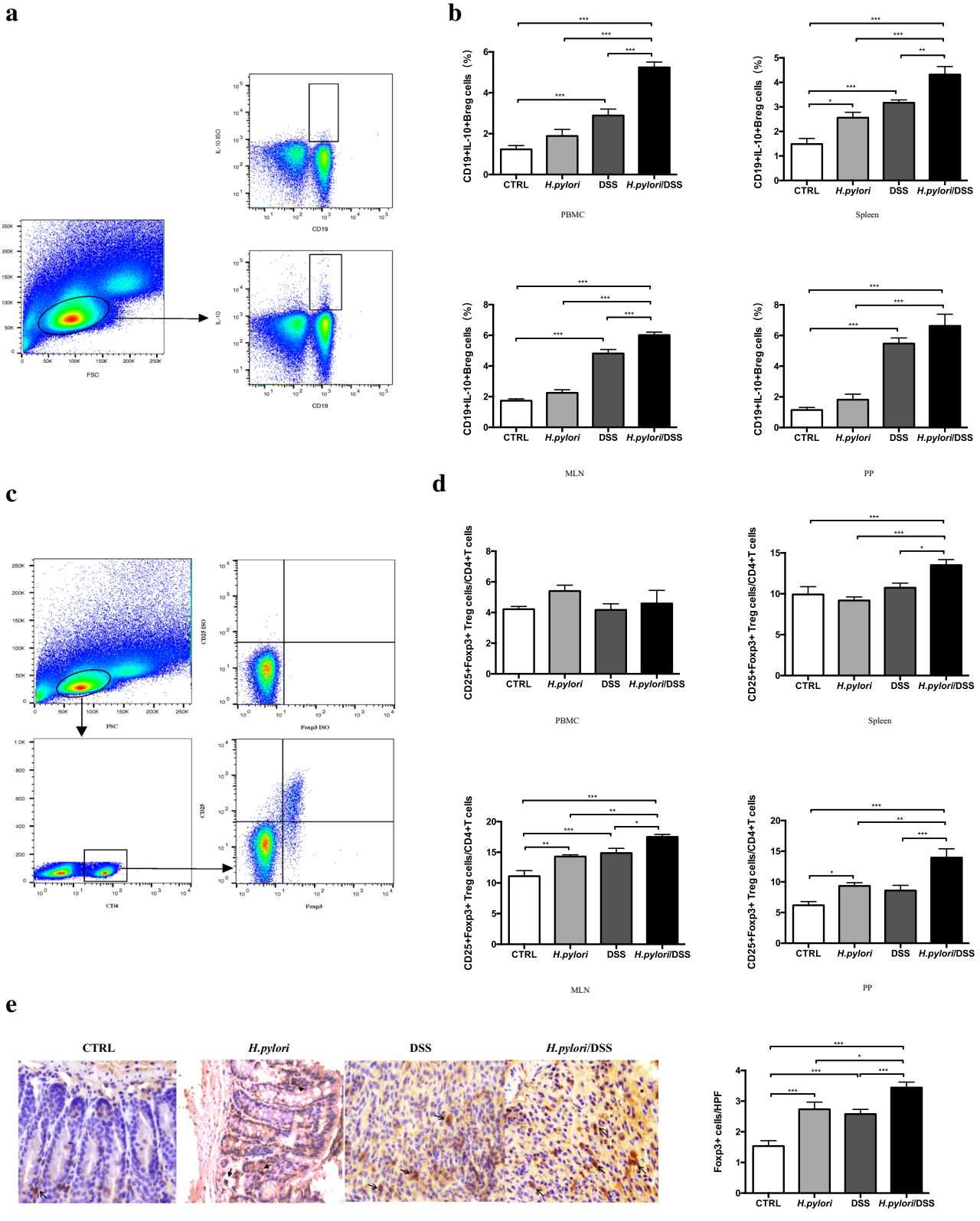
a
b
c
d
e
FSC
CD19
IL-10
IL-10 ISO
CD19+IL-10+Breg cells (%)
CTRL
H.pylori
DSS
H.pylori/DSS
PBMC
Spleen
MLN
PP
CD4
CD25
Foxp3
Foxp3 ISO
CD25 ISO
CD25+Foxp3+ Treg cells/CD4+T cells
Foxp3+ cells/HPF

Fig. 2 $CD19^+IL\text{-}10^+$Breg cells and $CD4^+CD25^+Foxp3^+$Treg cells in acute colitis mice. **a** The gating strategy of $CD19^+IL\text{-}10^+$Breg cells in FlowJo. The sample was from spleen of control group (CTRL). **b** The percentages of $CD19^+IL\text{-}10^+$Breg cells in PBMC, spleen, MLN and PP were detected by flow cytometry. **c** The gating strategy of $CD4^+CD25^+Foxp3^+$Treg cells in FlowJo. The sample was from spleen of control group (CTRL). **d** $CD4^+CD25^+Foxp3^+$Treg cells in PBMC, spleen, MLN, and PP were detected by flow cytometry. **e** Foxp3 + Treg cells in colon were stained *via* immunohistochemistry.(*$P < 0.05$; **$P < 001$; ***$P < 0.005$; NS, no significant.)

10^+Breg cells and the decrease of $CD4^+CD25^+Foxp3^+$Treg cells in chronic colitis.

DISCUSSION

H. pylori infection is a world-wide popular question due to its contribution to the development of gastric cancer and peptic ulcer, and the infection will be lifelong without eradication. It was pointed out that the prevalence of IBD was lower in *H. pylori*-positive patients, and the objective of this study was to verify that *H. pylori* infection can ameliorate colitis and investigate the function of Breg cells in the process. Given on the symptoms and histological characters and the cytokine expression in colon, the results support that *H. pylori* infection can alleviate acute and chronic colitis induced by DSS, which was in line with epidemiologic studies [9, 22].

Breg cells were considered as converted from different B cells stages in spleen firstly. Splenic B10 cells, as reported previously, expanded significantly during DSS-induced acute colitis [23–26]. It was revised that the conversion occurred in the inflammatory sites in recent years [27]. Our previous study had shown that $CD19^+IL\text{-}10^+$Breg cells, ahead of $CD4^+CD25^+Foxp3^+$Treg cells, expanded significantly in spleen, MLN, and gastrointestinal mucosa after *H. pylori* infection. Taking the aforementioned studies into consideration, we detected the $CD19^+IL\text{-}10^+$Breg cells in different tissues including PBMC, spleen, MLN, and PP in the rodent model. Our data showed that the percentages of $CD19^+IL\text{-}10^+$Breg cells both increased in acute and chronic colitis, and the extent of increase was more striking in acute and chronic colitis following *H. pylori* infection.

Breg cells can inhibit excessively activated immune response through the production of regulatory cytokines such as IL-10 and TGF-β. They can also cooperate with DC cells to promote the differentiation of Treg cells, leading to the inhibition of effective T cells responses [28]. So, we also detected the $CD4^+CD25^+Foxp3^+$Treg cells in different tissues. In acute colitis groups, Treg cells increased mainly in the MLN in DSS-treated mice, and the percentages of Treg cells in *H. pylori*/DSS cotreated mice were significantly higher. This was also demonstrated that Breg cells expanded earlier than Treg cells. In chronic colitis groups, Treg cells continued to be expanded in PBMC, spleen, and PP. However, It was confusing that the percentages of Treg cells decreased dramatically in *H. pylori*/DSS cotreated mice. Other regulatory cells including immature DC cells and macrophages were involved in chronic colitis [29, 30], and their amount and function may be changed after *H. pylori* infection and made a contribution to the decrease of Treg cells.

There was an interesting discovery during the study that the numbers of PP in the small intestine reduced and the volume diminished in DSS-treated mice. The numbers and volume of PPs recovered partly in *H. pylori*/DSS cotreated mice. Shigenori Nagai and colleagues thought that DC cells in PP can recognize *H. pylori* antigen, initiate naive $CD4^+$T cells differentiate into Th1 cells, which can migrate to the stomach and cause gastritis, *H. pylori* infection can activate systemic immune responses through PP [31, 32]. Spahn TW.et al. reported that DSS can induce more severe colitis in PP-deficient mice compared with wild type mice, suggesting that PP may protect the mice from the DSS [33]. In summary, *H. pylori* may affect the acute and chronic colitis through regulating the function of PP, and it needs to be confirmed and investigated through further experiments.

Overreaction of immune responses is considered to play a crucial role in the process of IBD, and we focused on the regulatory immune responses in this study. Th1/Th17/Treg cells axis was the main target in numerous researches. Verifying the dynamic change of Th1/Th17 cells in our model may be helpful to understand the results and to study the mechanism underlying the association between *H. pylori* infection and colitis.

In conclusion, *H. pylori* infection can alleviate acute and chronic colitis induced by DSS, and Breg

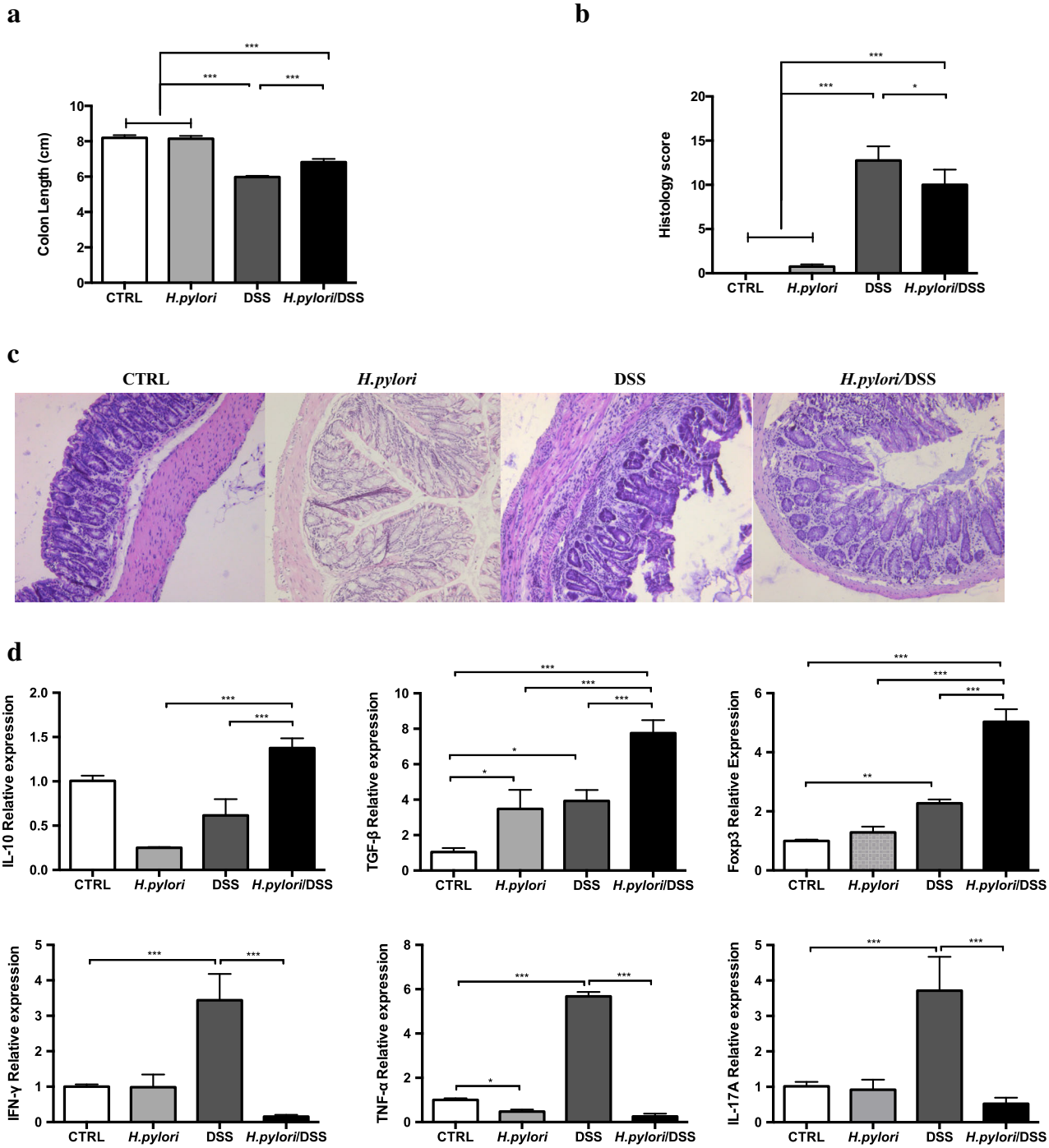

Fig. 3. *H. pylori* infection alleviated chronic colitis induced by DSS. **a** Colon length. **b** Histology scores. **c** H&E staining of colon.(× 100) **d** relative expression of anti- and pro-inflammatory cytokines in colon of chronic colitis mice. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; NS, no significant.)

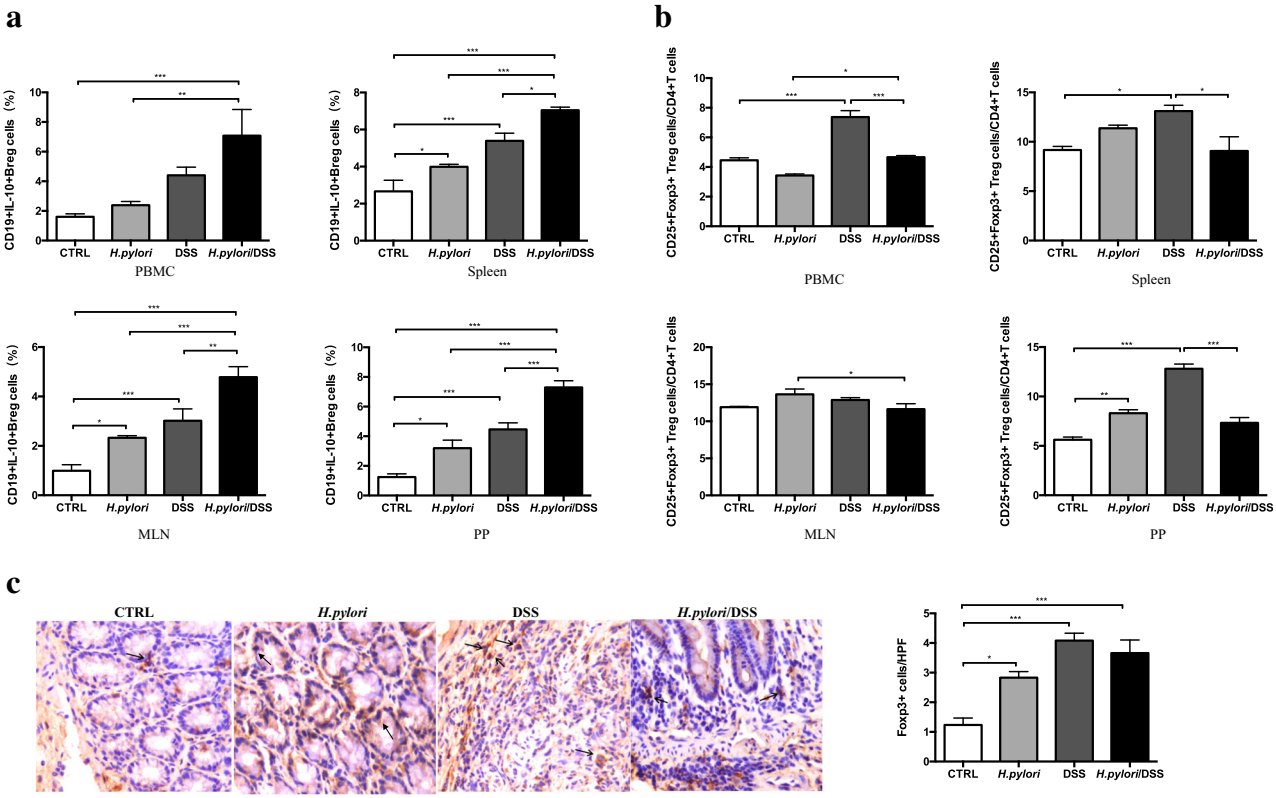

Fig. 4. CD19$^+$IL-10$^+$Breg cells and CD4$^+$CD25$^+$Foxp3$^+$Treg cells in chronic colitis mice. **a** The percentages of CD19$^+$IL-10$^+$Breg cells in PBMC, spleen, MLN, and PP were detected by flow cytometry. **b** CD4$^+$CD25$^+$Foxp3$^+$Treg cells in PBMC, spleen, MLN, and PP were detected by flow cytometry. **c** Foxp3 + Treg cells in colon were stained *via* immunohistochemistry.(*$P < 0.05$; **$P < 001$; ***$P < 0.005$; NS, no significant.)

cells may play a more important role compared with Treg cells in the process. The inverse association between *H. pylori* infection and IBD provides a potential strategy, and the Breg cells may be a promising target for IBD clinical treatment.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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