

Effect of *Trichinella spiralis* intervention on *Citrobacter rodentium*-induced experimental colitis in mice

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Abstract

Materials and Methods: In the present study, 36 male BALB/c mice aged 6-8 weeks were randomly divided into four groups: the control group (PBS), the *T. spiralis* infection group (TS), the *C. rodentium*-induced colon inflammation model group (CR) and the *T. spiralis* pre-infected and *C. rodentium* induced colitis group (TS+CR). The mice were sacrificed 7 and 14 days after the establishment of the model. The changes in various colitis indicators were used to investigate the effect of *T. spiralis* infection on the *C. rodentium*-induced mouse CD model. **Results:** The results showed that the weight, DAI score, and macroscopic and microscopic colon damage in the TS+CR group was significantly decreased compared with that observed in the CR group. Flow cytometry results revealed that *T. spiralis* infection could reduce the expression of IFN- γ , IL-17 and IL-12 and increase that of IL-4 and IL-10 in colitis mice. Compared with that observed in the control group, the number of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in the spleens and mesenteric lymph nodes (MLNs) of mice in the TS group was increased ($P<0.01$). Compared with that observed in the TS group mice, the expression of CD4+CD25+Foxp3+Tregs in the spleens and MLNs of the TS+CR group mice was decreased ($P<0.05$). ELISA results revealed that compared with the control group, the TS group produced a strong IgG1 response ($P<0.001$) and low level of IgG2a response ($P>0.05$), while increased expression of IgG2a in serum was detected in the CR group mice ($P<0.05$).

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List of abbreviations

IBD: inflammatory bowel disease

T. spiralis : *Trichinella spiralis*

C. rodentium : *Citrobacter rodentium*

Tregs: regulatory T cells

MLNs: mesenteric lymph nodes

UC: ulcerative colitis

CD: Crohn's disease

EPEC: *Escherichia coli*

EHEC: enterohaemorrhagic *E. coli*

TGF- β : Transforming growth factor- β

IL: interleukin

ELISA: Enzyme-linked immunosorbent assay

SPF: specific pathogen free

DAI: disease activity index

TNBS: 2,4,6-Trinitrobenzene sulfonic acid

DNBS: dinitrobenzenesulfonate

Summary

Objectives: Epidemiological surveys have shown that the incidence of inflammatory bowel disease (IBD) is relatively high in developed countries but relatively low in countries with poor sanitation and dense populations. Good hygiene and low parasitic infections increase the susceptibility of individuals to IBD. The goal of the present study was to assess the immune characteristics of hosts infected with *Trichinella spiralis* (*T. spiralis*) and the relationship between intestinal worms and IBD. The ability of *T. spiralis* infection to ameliorate the effects of *Citrobacter rodentium* (*C. rodentium*) in a mouse IBD animal model was assessed, and the possible immunological mechanism associated with this prevention and treatment of IBD was evaluated.

Materials and Methods: In the present study, 36 male BALB/c mice aged 6-8 weeks were randomly divided into four groups: the control group (PBS), the *T. spiralis* infection group (TS), the *C. rodentium*-induced colon inflammation model group (CR) and the *T. spiralis* pre-infected and *C. rodentium* induced colitis group (TS+CR). The mice were sacrificed 7 and 14 days after the establishment of the model. The changes in various colitis indicators were used to investigate the effect of *T. spiralis* infection on the *C. rodentium*-induced mouse CD model.

Results: The results showed that the weight, DAI score, and macroscopic and microscopic colon damage in the TS+CR group was significantly decreased compared with that observed in the CR group. Flow cytometry results revealed that *T. spiralis* infection could reduce the expression of IFN- γ , IL-17 and IL-12 and increase that of IL-4 and IL-10 in colitis mice. Compared with that observed in the control group, the number of CD4⁺CD25⁺Foxp3⁺regulatory T cells (Tregs) in the spleens and mesenteric lymph nodes (MLNs) of mice in the TS group was increased ($P < 0.01$). Compared with that observed in the TS group mice, the expression of CD4⁺CD25⁺Foxp3⁺Tregs in the spleens and MLNs of the TS+CR group mice was decreased ($P < 0.05$). ELISA results revealed that compared with the control group, the TS group produced a strong

IgG1 response ($P < 0.001$) and low level of IgG2a response ($P > 0.05$), while increased expression of IgG2a in serum was detected in the CR group mice ($P < 0.05$).

Conclusion: The results of the present study demonstrated that *T. spiralis* -infected mice induced balanced Th2 immune responses that balanced Th1 immune responses stimulated by *C. rodentium* to ameliorate intestinal inflammation.

Key words: *Trichinella spiralis* ; *Citrobacter rodentium* ; inflammatory bowel disease

Introduction

IBD is a chronic intestinal disorder that primarily manifests as a specific immune response of the mucosal immune system towards intestinal pathogenic microorganisms. Human IBD can be divided into two types: ulcerative colitis (UC) and Crohn's disease (CD) [1]. UC typically involves Th2- and Th17-type inflammation, and the superficial portion of the colon is prone to ulcers. CD is primarily based on Th1-type inflammation, and the lesions are typically transmural and may spread throughout the intestine [2]. Compared to developing countries, the incidence of inflammatory bowel disease in developed Western countries is relatively high. This phenomenon can be explained by the "hygiene hypothesis", which describes how improved sanitary conditions reduce the chances individuals will come into contact with pathogenic organisms, increasing the incidence of autoimmune and inflammatory diseases [3-5]. Antiparasitic drugs and good hygiene practices in developed countries have effectively controlled parasitic infections, but with the elimination of parasitic infections, the incidence of immune diseases, including inflammatory bowel disease, has also increased [6].

As a non-invasive organism, *C. rodentium* uses attaching and effacing (A/E) lesion formation as its major tissue-targeting mechanism, resulting in infection. This approach is consistent with the mechanisms observed in other enteropathogenic pathogens, such as *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC). As a natural mouse pathogen, *C. rodentium* provides an excellent *in vivo* model to study A/E lesion-forming pathogens, including EPEC and EHEC, because both EHEC and EPEC are poorly pathogenic in mice. However, infection with *C. rodentium* has also been investigated as a model for studying IBD, because the colonic pathology of the mouse shares several similarities with clinical IBD [7]. In addition, this model provides potential mechanisms for increasing the understanding of how host immunity that plays a role in limiting the infection of the mucosal epithelium of the colon and clearing pathogens [8].

Parasitic infection can induce an immune response in the host, causing polarization towards a Th2-type response, which is characterized by the expression of the Th2-type cytokines IL-4, IL-5 and IL-13 [9]. Parasites downregulate T and B cell responses with respect to Tregs, anti-inflammatory cytokines IL-10 and transforming growth factor TGF- β and regulates the Th1- and Th2-type immune responses [10]. Parasites can establish a parasitic lifestyle by regulating the host immune response, which simultaneously protects the host from excessive inflammation that causes damage to tissues and organs [11]. In recent years, experimental and clinical studies have shown that parasitic infections can protect the host from inflammatory bowel disease. For example, the intestinal parasites *T. spiralis*, Swine whipworm (*Trichuris muris*) and *Hymenolepis diminuta* can provide protection to IBD in mice [12, 13]. Similarly, UC and CD symptoms in IBD patients infected with *T. muris* are alleviated [14]. The mechanism of intestinal parasite treatment of IBD is not yet clear, and many aspects of this process remain to be elucidated. In the present study, we constructed an experimental mouse enteritis model induced by *C. rodentium*, studied the immune system effects of *T. spiralis* treatment on inflammatory bowel disease in mice, and elucidated the effects of *T. spiralis* intervention in IBD at the molecular and cellular levels to identify the potential immunological mechanisms involved to provide a theoretical basis for clinical treatment of human IBD.

Materials and methods

Parasite isolate, storage and recovery of *C. rodentium*, animals, culture medium and growth conditions

The *T. spiralis* isolate ISS534 used in the present study was obtained from domestic pigs in Henan Province, China, and maintained in Kunming mice in the Jilin Provincial Engineering Research Center of Animal

Probiotics. *C. rodentium* ATCC 51459 was provided by Dr Haining Shi (Harvard Medical School, Boston, MA, USA) and cultured in Luria-Bertani medium at 37°C with shaking. Male BALB/c mice (specific pathogen free, SPF) aged 6-8 weeks were purchased from Beijing Hua fu kang Biotechnology. The study protocol was approved by the Jilin Agriculture University Veterinary Research Ethics Committee, and all procedures were performed in strict accordance with the guidelines of the Chinese National Institute of Health Guide for the Care and Use of Laboratory Animals. Care was taken to minimize the number of animals used and to reduce their suffering. Before the experiment, the mice were allowed to eat and drink freely for at least 4 days to adapt to the feeding environment. Throughout the experiment, the mice were allowed to eat and drink freely, and the bedding materials were changed regularly to ensure an excellent feeding environment.

Experimental grouping

The experimental mice were randomly divided into four groups, with more than nine mice in each group: the control group (PBS), the *T. spiralis* -infected group (TS), the *C. rodentium* -induced colitis model group (CR), and the *T. spiralis* -pre-infected *C. rodentium* -induced colitis group (TS+CR). At the beginning of the experiment, each mouse in the TS and TS+CR groups was orally infected with 400 individual *T. spiralis* muscle larvae. After 21 days of infection, the control and TS groups were administered PBS, and the CR and TS+CR groups were administered 2×10^9 CFU/mL *C. rodentium*. Subsequently, the faeces of mice in the CR and TS+CR groups were collected every day from days 1 to 14 post *C. rodentium* infection, the colony count of faeces was determined, and the number of *C. rodentium* per gram of faeces (CFU/g) was calculated. The mice were sacrificed under euthanasia on days 7 and 14, and the weights of the mice in the four groups was determined daily.

Collection and oral administration of *T. spiralis* larvae in mouse muscles

T. spiralis were maintained in our laboratory through serial infection of rats. The parasites were isolated from the infected mice through dissection, were their muscle tissues were harvested and bones were removed. The muscles were treated with an artificial digestion solution (1% pepsin and 1% concentrated hydrochloric acid) for 4 h at 37°C. Subsequently, the mixture was filtered using an 80-mesh filter and rinsed thrice. The larvae were subsequently harvested in accordance with the modified Bellman's method. Then, the vitality of the larvae was assessed, and they were enumerated under a microscope. The mice in the TS and TS+CR groups were orally infected with 400 individual larvae.

C. rodentium-induced colitis

Jiang et al. (2016) established the *C. rodentium* -induced colitis model approach [15] that was used the present study. The mice in the CR and TS+CR groups were rapidly administered 2×10^9 CFU/mL *C. rodentium* 24 h after fasting, while the mice in the control and TS groups were administered the same amount of PBS. The colitis mouse model elicited a Th1-type immune response, which was similar to that observed in human CD. Inflammation was assessed based on bellowed parameters, including clinical disease activity and the macroscopic and microscopic inflammation scores in the colon tissue. The grading was performed by three investigators blinded for the treatment of the mice.

T. spiralis infection

After the mice were sacrificed, their diaphragms were examined under a microscope to assess the success of *T. spiralis* infection, and the success rates of infection and parasitic density were calculated.

Disease activity index evaluation

The mice were observed daily in terms of the changes in their mental status, activity, hair gloss, appetite, and defecation (presence of blood, stool pattern, defecation frequency). Each group of mice was scored for the disease activity index (DAI) according to international standards: $DAI = (\text{weight loss score} + \text{stool score} + \text{blood stool score})/3$. To detect faecal occult blood, we used the benzidine method.

Macroscopic and microscopic assessment of colon injury

After the mice were sacrificed by cervical dislocation, the abdominal wall was opened, and the intestine was exposed. Then, the entire segment of the colon from the rectum to the cecocolic junction was removed, opened and rinsed thoroughly with normal saline, after which the isolated colon was examined for macroscopic damage. Scores were assessed by using the following damage scoring system [16]: 0: no damage; 1: localized hyperaemia without ulcers; 2: linear ulcers with no significant inflammation; 3: linear ulcer with inflammation at one site; 4: ulcer and inflammation at two or more locations; and 5: two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending more than 1 cm along the colon. Then, colon specimens were fixed in 10% paraformaldehyde for hour(s), after which paraffin sections were generated and haematoxylin and eosin (H&E) staining was performed. The pathological sections of the colon were observed under an optical microscope (Leica, GER). Histological damage was assessed using the criteria of Wallace and Keenan [17]: 0: intact tissue construction with no apparent damage; 1: damage limited to surface epithelium; 2: localized ulcer confined to mucosa; 3: focal, transmural inflammation and ulceration; 4: extensive transmural ulceration and inflammation adjacent to normal mucosa; and 5: extensive transmural ulceration and inflammation involving entire section. All data were obtained from three separate experiments, and the scores were assigned by three observers without knowing the state of the mice (Table 1).

Detection of IgG1 and IgG2a in serum by ELISA

Serum samples were collected 14 days after *C. rodentium* infection. The changes in IgG1 and IgG2a levels in serum were assessed using an ELISA kit (Bioss, CHN) following the manufacturer's instructions for cytokine detection. Cytokine levels were expressed per milligram of total protein. All data were obtained from three separate experiments.

Detection of CD4⁺CD25⁺Foxp3⁺Tregs and Th cells in spleens and MLNs by flow cytometry

Spleen and MLN lymphocyte single cell suspensions were prepared, and 1×10^6 cells were added to each tube. The cells were resuspended in PBS and incubated for 30 min in the dark with the following antibody combinations: FITC-rat-anti-mouse CD4, PE-rat-anti-mouse Foxp3 and APC-rat-anti-mouse CD25; FITC-rat-anti-mouse IL-17 and PE-Rat-anti-mouse IL-12; FITC-rat-anti-mouse IL-10 and PE-rat-anti-mouse IFN- γ or PE-rat-anti-mouse IL-4 (all antibodies were obtained from BD, USA). The cells were washed and resuspended again for flow cytometry analysis. All data were obtained from three separate experiments.

Statistical analysis

All results are presented as the means \pm standard error. Data were evaluated using one-way ANOVA with SPSS 13.0. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 7.

Results

Survival rate

The survival rate of mice in the CR group was only 65.78%, significantly lower than that observed for the TS+CR group (89.78%; $P < 0.05$), while the survival rate of mice in the control and TS groups was 100%.

Weight change

The day the *C. rodentium* model establishment was experimental day 0. The body weights of mice in each group were recorded daily from the beginning until the end of the experiment, and the results are presented in Fig. 1. The weights of mice in the CR group were significantly lower than those of the control group mice. The weights of mice in the CR group decreased by 6.62% on day 7 before gradually increasing by 0.76% on day 14. The weights of mice in the TS+CR group decreased relatively slowly compared to that observed in the CR group, decreasing by 2.22% on day seven to the lowest value observed before gradually increasing by 8% on day 14.

Changes in the amount of *C. rodentium* in faeces

From day 1 to 14 post *C. rodentium* infection, the excretion of *C. rodentium* in the faeces of the TS+CR group was lower than that observed in the CR group. From day 1 to 14 post *C. rodentium* infection, the *C. rodentium* excretion by the TS+CR group was significantly reduced, that observed in the CR group remained at a high level (Fig. 2).

DAI score

Twenty-four hours after the *C. rodentium* model was established, mice in the control colitis group manifested lethargy, sluggishness, appetite loss, rough hair, diarrhoea, bloody stools or faecal occult blood (+ ++), and weight loss. These symptoms were most evident 3 days after the start of modelling. All the symptoms of mice of the TS+CR group were less severe than those observed in the CR group and were significantly different on days 1 and 3 ($P < 0.05$). The conditions of the surviving mice in both groups started to gradually improve on day 4, and the mice were relieved on day 7. At this time, in contrast to the mice in the CR group, the mice in the TS+CR group were still recovering. The DAI scores of the three groups at different time points are shown in Fig. 3.

Colon pathological changes

To further evaluate the protective effects of *T. spiralis* in *C. rodentium* infection, on day 14 after the start of modelling, distal colon samples were collected and evaluated by H&E staining. As expected, no pathogenic changes were observed in either the control group or TS groups (Fig. 4AC), whereas the CR group exhibited typical pathological changes, including colonic epithelial cells proliferation, intestinal wall oedema, large amounts of inflammatory cell infiltration in the lamina propria, elongated crypts and the reduction or disappearance goblet cells (Fig. 4B). In the TS+CR group, the hyperplasia of colonic epithelial tissue disappeared, the lesions were limited to the mucosa, and the lamina propria of the mucosa was scattered with inflammatory cells ((Fig. 4D).

Changes in IgG1 and IgG2a levels in serum

After 14 days of *C. rodentium* infection, serum samples were collected, and ELISA was used to assess the changes in IgG1 and IgG2a levels in mouse serum. Compared with the control group, the TS group produced a strong IgG1 response ($P < 0.001$) and a low-level IgG2a response ($P > 0.05$), with an increased expression of IgG2a observed in serum from CR group mice ($P < 0.05$). This result showed that *C. rodentium* infection primarily induces a Th1-type immune response in mice, while *T. spiralis* infection tended to induce a Th2-type immune response. Compared with the CR group, the TS+CR group had increased IgG1 expression ($P < 0.01$) and decreased IgG2a expression ($P < 0.01$), while compared with the TS group, the TS+CR group exhibited increased IgG2a expression ($P < 0.01$). These results showed that the Th2 type immune response induced by *T. spiralis* inhibits the Th1 immune response caused by *C. rodentium* infection (Fig. 5).

Changes in Th cells and CD4⁺CD25⁺Foxp3⁺Tregs in the spleens and MLNs of mice

The mice were sacrificed 7 and 14 days after the start of *C. rodentium* modelling. The spleens and MLNs of each group were removed, and the number of Th cells was assessed by flow cytometry. The results showed that 7 and 14 days after *C. rodentium* infection, the expression of IFN- γ and IL-12 in the spleens and MLNs of mice in the CR group increased ($P < 0.01$). Compared with that observed in the CR group, the expression of IFN- γ and IL-12 in the spleens and MLNs of mice the TS group was decreased ($P < 0.01$), and while that of IL-10 and IL-4 was increased ($P < 0.05$). These results showed that *C. rodentium* primarily induced the production of Th1 cytokines in mice, while *T. spiralis* infection primarily induced the production of Th2 cytokines. Compared with that observed in the CR group, the expression of IFN- γ and IL-12 in the spleens and MLN of mice in the TS+CR group was reduced ($P > 0.05$), showing that the Th2-type cytokine response caused by *T. spiralis* infection could inhibit the development of Th1-type inflammation induced by *C. rodentium*. Similarly, the expression of IL-10, IL-4 and IL-17 in the spleens and MLNs of mice in the TS+CR group was significantly lower than that observed in the TS group ($P < 0.05$). These results successfully showed that the Th1-type cytokine response caused by *C. rodentium* infection can inhibit the Th2-type immune response induced by *T. spiralis* (Fig. 6, 7).

The mice were sacrificed 14days after *C. rodentium* modelling. The spleen and MLN of each group were removed, and the expression of CD4⁺CD25⁺Foxp3⁺Tregs was detected by flow cytometry. The result showed that compared with that in the control group, the number of CD4⁺CD25⁺Foxp3⁺Tregs in the spleens and MLNs of mice in the TS group was increased ($P < 0.01$), showing that *T. spiralis* infection could induce CD4⁺CD25⁺Foxp3⁺Treg production in mice. Compared with that observed in the TS group mice, the number of CD4⁺CD25⁺Foxp3⁺ Tregs in the spleens and MLNs of mice in the TS+CR group mice was decreased ($P < 0.05$), showing that infection with *C. rodentium* could inhibit the expression of Tregs induced by *T. spiralis* (Fig. 6,7).

Discussion

2,4,6-Trinitrobenzene sulfonic acid (TNBS) and dinitrobenzenesulfonate (DNBS) mouse models are early-stage IBD animal models [18]. The damage caused in these models is highly concentrated, the course of the disease is acute, and the onset is short. Rat and mouse models of intestinal inflammation induced by the oral administration of dextran sulfate sodium (DSS) are also used. The DSS animal model is primarily an injury model, which is primarily used to study the influencing factors of promoting and inhibiting intestinal epithelial healing [19, 20]. In the present study, mice were administered *C. rodentium* to induce IBD animal models. *C. rodentium* is an adhesion and shedding pathogen of the family Enterobacteriaceae to which gerbils and mice are susceptible, whereas hamsters and rats are not. This model has been widely used to study the immunoprotective role of *E. coli* and the mechanism of the host immune response against intestinal pathogens. *C. rodentium* -induced animal model mice showed obvious inflammatory symptoms in the colon, and the pathological characteristics were consistent with the IBD animal model.

Compared with the TS group, the TS+CR group showed a higher survival rate, reduced intestinal damage, decreased DAI score, and significantly reduced symptoms of intestinal inflammation, indicating that *T. spiralis* infection could alleviate colitis caused by *C. rodentium*. This finding is consistent with that observed in other worm infection studies [21, 22].

To better understand how worms suppress the occurrence of immunomodulatory diseases, recent research has focused on the interaction between the Th2- and Th1-type immune responses [23]. Th1 cells primarily secrete IL-2, IFN- γ , IFN- α , and TNF- β , while Th2 cells primarily release IL-4, IL-5, IL-6 and IL-10 [24]. IL-12 plays an important role in Th1 cell-mediated immune response and inflammatory diseases [25]. IL-17 is a pro-inflammatory cytokine produced by T cells and is an early initiating factor of inflammatory responses induced by T cells [26]. In this study, IFN- γ and IL-4, IL-10 were selected as representatives of Th1- and Th2-type cytokines. The number of Th cells was assessed by flow cytometry, and the results showed that *T. spiralis* infection could reduce the expression of IFN- γ , IL-17 and IL-12 and increase that of IL-4 and IL-10 in colitis mice. These results showed that *T. spiralis* infection causes a colitis mucosal immune response to switch from a Th1-type response to a protective Th2-type response. ELISA results showed changes in the levels of IgG1 and IgG2a in mouse serum, indicating that the levels of Th2-type cytokines caused by *T. spiralis* infection have an inhibitory effect on *C. rodentium* -induced Th1 type inflammation. These reactions reduce the intensity of the Th1-type immune response, thereby alleviating intestinal inflammation, and these results are consistent with the expected experimental results. From another perspective, colitis was also shown to stimulate the switch from a Th2-type immune response induced by *T. spiralis* larvae infection into a Th1-type immune response, reduce the intensity of the Th2-type immune response, and possibly alleviate the pathological changes attributed to the parasite infection in the body. *T. spiralis* and *C. rodentium* interact together to maintain immune balance in mice.

Tregs have a wide range of immunosuppressive effects. By producing regulatory cytokines, these cells maintain the balance of the immune system and prevent the development of autoimmune diseases [27]. Foxp3 is a marker molecule for CD4⁺CD25⁺Tregs [28]. The results showed that compared with that observed in the control group, the number of CD4⁺CD25⁺Foxp3⁺Tregs in the spleens and MLNs of mice in the TS group was increased, showing that *T. spiralis* infection could induce CD4⁺CD25⁺Foxp3⁺Tregs production in mice. In contrast, compared with that observed in the TS group, the number of CD4⁺CD25⁺Foxp3⁺Tregs in the spleens and MLNs of mice in the TS+CR group were decreased, showing that *C. rodentium* infection can

affect Tregs in the immune response induced by *T. spiralis*. The results of the present study are consistent with those of a previous study of worm infections that showed increased levels of Foxp3⁺Tregs in mouse MLNs and intestinal mucosa lamina propria, inhibiting the mouse colon inflammatory response, as reported by Maloy et al [29].

In summary, the results of the present successfully showed that the effect of *T. spiralis* intervention on *C. rodentium* -induced colitis in mice is achieved by regulating the Th1/Th2 type immune response balance of the host.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Changes in the weights of the mice in each group. The data are presented as the means \pm SD of 3 mice per group.

Fig. 2 Changes in *C. rodentium* levels in faecal pellets. The data are presented as the means \pm SD of 3 mice per group.

Fig. 3 DAI assessment showing a lower value in the TS+CR group than that observed in the TS group. The data are presented as the means \pm SD of 3 mice per group.

Fig. 4 Histopathological analysis of the colons of mice. Colon samples were excised, fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with H&E for light microscopy assessments of epithelial damage (original magnification, 100 \times).

A, Mice were treated with PBS as control, the structure of the colon villus was integrated, and the gland was clearly visible.

B, In mice from the CR group, intestinal epithelial cells were necrotic and shedding, intestinal villi were shortened and fused, and the crypts were extended. In addition, a large number of lymphocytes, neutrophils, and eosinophils had infiltrated into the mucosa and submucosa.

C, In mice from the TS group, a high number of goblet cells were detected, but there was no obvious pathological damage.

D, Hyperplasia of colonic epithelial tissue in the TS+CR mice disappeared, the lesions were limited to the mucosa, and the lamina propria of the mucosa was scattered with inflammatory cells.

Fig. 5 Changes in the IgG1 and IgG2a contents of serum post *C. rodentium* infection. The data are presented as the means \pm SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 6 Demonstration of the gating strategy for the flow cytometry analysis of mouse CD4⁺CD25⁺Foxp3⁺Tregs and Th cells from spleens and MLNs 7 (A) and 14 days (B) after administration of *C. rodentium*. In this experiment single-cell suspensions were prepared from the spleens and MLNs of mice in each group and stained following surface and intracellular staining protocols. The data were collected with a FACSDiva flow cytometer and analysed. Lymphocytes were identified by their scatter properties (FSC-A x SSC-A plot).

Fig. 7 The number of Th cells in the spleens and MLNs of mice from the four groups at 7 and 14 days. Analysis of CD4⁺CD25⁺Foxp3⁺Tregs in the spleens and MLNs of mice from each group. The number of CD4⁺CD25⁺Foxp3⁺Tregs in mice from the TS+CR group increased compared with that observed in the CR group. The data are presented as the means \pm SD of 3 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Tables

Table 1. Disease activity index score parameters.

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