

# L-Ergothioneine Exhibits Protective Effects against Dextran Sulfate Sodium-Induced Colitis in Mice

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Cite This: *ACS Omega* 2022, 7, 21554–21565



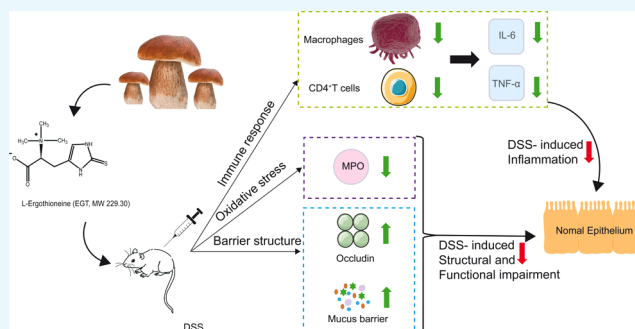
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**ABSTRACT:** **Background:** Ulcerative colitis (UC) is a chronic disease of the intestinal tract in which excessive activation of inflammatory response is correlated. L-Ergothioneine (EGT) widely existing in mushrooms has various physiological activities. In this study, the protective effects of EGT on dextran sulfate sodium (DSS)-induced colitis mice were investigated. **Results:** It was observed that EGT administration, especially at the high dose level, prevented the body weight loss, the colon shortening, and the increase in disease activity index and spleen index caused by DSS. Moreover, EGT supplementation attenuated DSS-induced gut barrier damage by enhancing the expression of tight-junction protein and recovering the loss of gut mucus layer. Furthermore, EGT considerably decreased the colonic myeloperoxidase (MPO) activity induced by DSS, but no significant differences were observed in the concentrations of IL-6 and TNF- $\alpha$  in colon tissues. Additionally, EGT downregulated the populations of CD4<sup>+</sup> T cells and macrophages, indicating that EGT stabilized the immune response caused by DSS. **Conclusion:** Together these results suggest that EGT can alleviate DSS-induced colitis and provide important insights concerning the potential anticolitis activity of such food products.



## 1. INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestinal tract, which is usually classified into Crohn's disease (CD) and ulcerative colitis (UC).<sup>1</sup> CD affects any region of the intestine and displays histologically submucosal thickening, transmural inflammation, fissuring ulceration, and granulomas, while UC usually affects the colon and may involve the rectum and its inflammation is typically limited to the mucosa and submucosa with cryptitis and crypt abscesses.<sup>2,3</sup> The worldwide incidence and prevalence of UC are rising, especially in newly industrialized countries.<sup>4</sup>

Although the etiology remains largely unknown, studies have shown that the complex interactions among genetic predisposition, inflammatory mediators, intestinal barrier defects, mucosal immune dysregulation, microbes, and environmental factors are closely related to the pathogenesis of UC.<sup>5</sup> At present, the current effective treatment strategies for UC patients are surgery and medical therapy, which usually induce a series of complex side effects. In recent years, food-derived functional ingredients as the adjuvant treatment for UC have been gradually investigated and it is shown that dietary polysaccharides,<sup>6,7</sup> conjugated linoleic acid,<sup>8</sup> phenolic compounds,<sup>9</sup> and peptides<sup>10,11</sup> can relieve colitis by inhibiting inflammatory reactions.

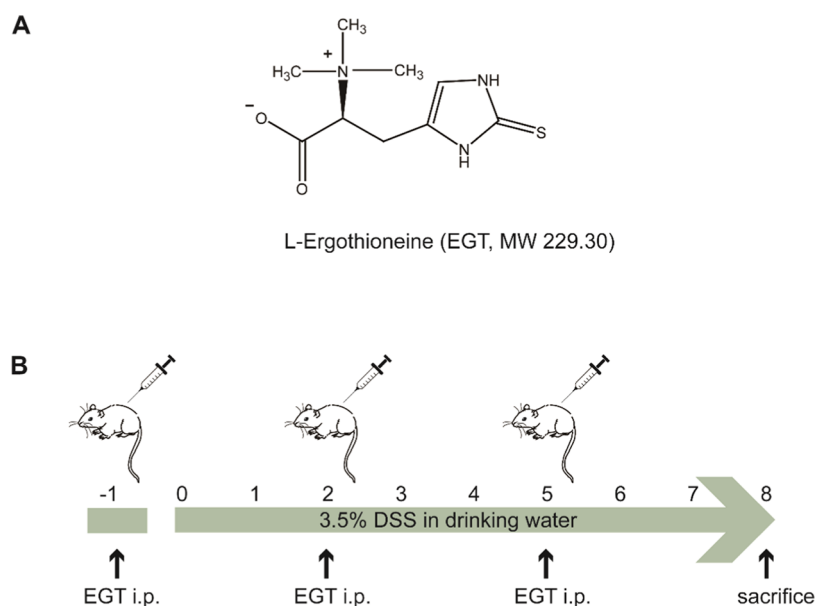
It is worth mentioning that L-Ergothioneine (EGT) (Figure 1A), a 2-thio-imidazole amino acid first discovered in 1909 in Ergot fungus, is widely distributed in fungi and a few other microbes.<sup>12</sup> Now, it has important implications in the treatment of diseases because of its powerful antioxidant activity. At physiological pH, EGT is stable since it mainly exists in the form of thione, making it resistant to autoxidation.<sup>13</sup> EGT can scavenge reactive oxygen and nitrogen species, thereby reducing oxidative stress and acting as an anti-inflammatory agent. Repine and Elkins<sup>14</sup> observed that EGT can reduce acute lung injuries and inflammation in cytokine-insufflated rats by inhibiting oxidative stress, TNF- $\alpha$ -induced NF- $\kappa$ B activation, and IL-8 release in alveolar epithelial cells. Moreover, Asahi et al.<sup>15</sup> found that EGT can inhibit inflammation-related DNA halogenation. Again, it was proved that EGT could facilitate adjuvant vaccine immunotherapy by modulating the tumor microenvironment.<sup>16</sup> Recently, Samuel et al.<sup>17</sup> have reported that EGT mitigates

**Received:** March 6, 2022

**Accepted:** June 2, 2022

**Published:** June 14, 2022





**Figure 1.** L-Ergothioneine and experimental design. (A) Structural formula and molecular weight of L-Ergothioneine (EGT). (B) Experimental schedule; 32 female mice were randomly divided into four groups: control group (control), DSS-induced model mice group (DSS), DSS-induced model mice group treated with 4  $\mu\text{g}$  EGT (EGT-4  $\mu\text{g}$ , 0.2 mg/kg body weight), and DSS-induced model mice group treated with 40  $\mu\text{g}$  EGT (EGT-40  $\mu\text{g}$ , 2 mg/kg body weight). The control group was fed with normal drinking water, and the other three groups were treated with 3.5% DSS for 7 days, together with three intraperitoneal injections of PBS for the DSS group or EGT for the EGT groups on days 0, 2, and 5. DSS: dextran sulfate sodium; EGT: L-Ergothioneine; i.p.: intraperitoneal injections; PBS: phosphate-buffered saline.

telomere shortening under oxidative stress conditions. Additionally, the EGT concentration in the plasma of patients with CD was remarkably lower than that in the healthy group,<sup>18,19</sup> indicating the potential role of EGT. Specifically, the latest research showed that EGT from *Pleurotus ostreatus* can significantly alleviate ulcerative colitis induced by DSS in rat model.<sup>20</sup> Nevertheless, despite the great promise of EGT, little information regarding its anti-inflammatory effects and the detailed mechanisms accounting for these effects are available.

Against this background, we investigate whether EGT administration could improve the clinical indicators and histological alterations in colitis mice induced by dextran sodium sulfate (DSS) by evaluating the suppressing disease severity, organ damages, oxidative stress, inflammatory responses, regulating immune response, and protecting gut barrier.

## 2. MATERIALS AND METHODS

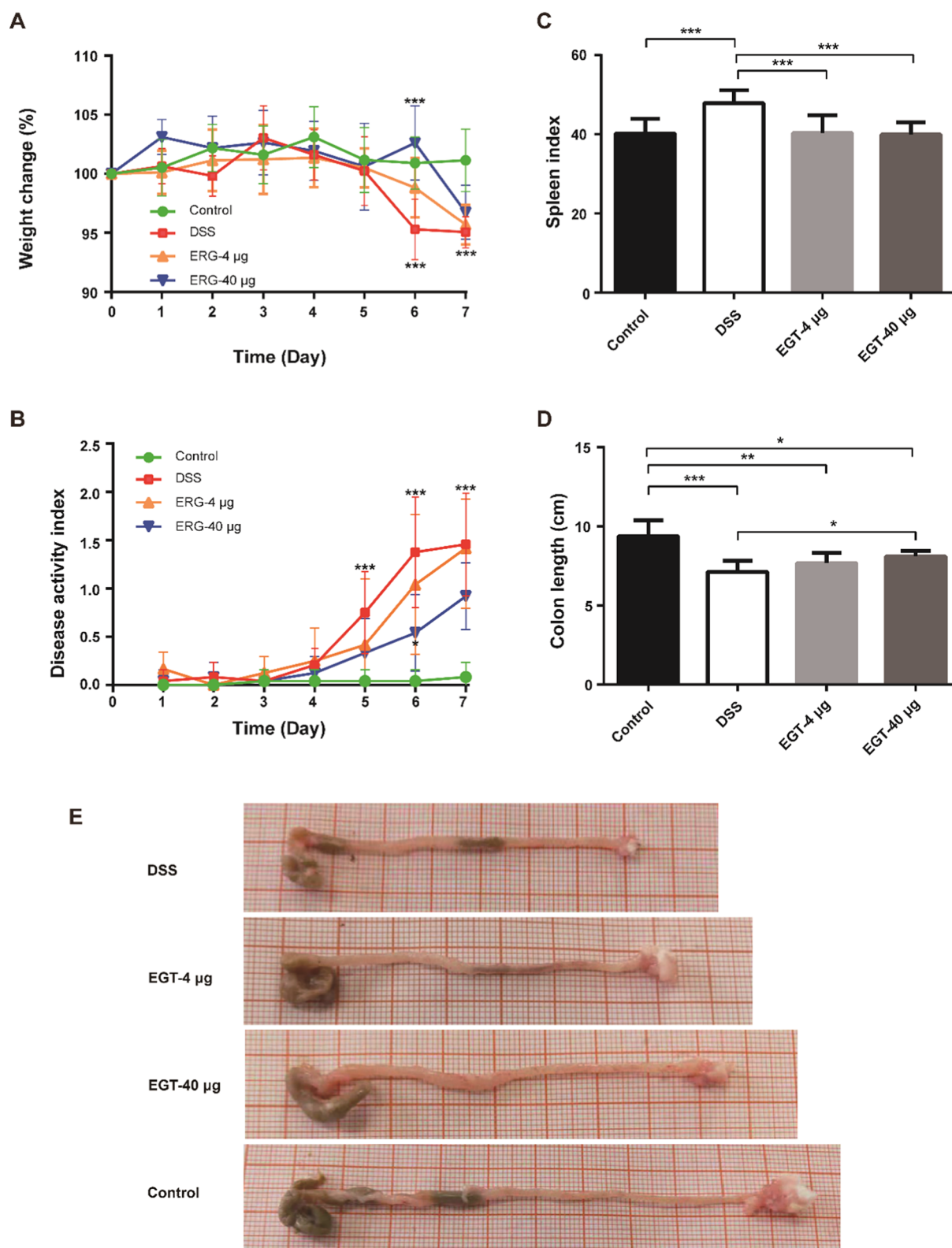
**2.1. Chemicals.** Dextran sodium sulfate (DSS, molecular weight 36 000–50 000) was purchased from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). L-Ergothioneine (EGT,  $\geq 98\%$ ) was bought from Shanghai Aladdin Biochemical Technology Co., Ltd. Fecal occult blood assay kit and myeloperoxidase (MPO) assay kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mouse IL-6, TNF- $\alpha$  ELISA kits, and Goat anti-rabbit IgG were purchased from Boster Biological Technology Co. Ltd. (Wuhan, China). The bicinchoninic acid (BCA) protein assay kit was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). GADPH antibody was obtained from Abcam (Cambridge, MA). The occludin antibody was purchased from Proteintech (Wuhan, China). Fixable Viability Stain 780 from BD Pharmingen is useful for discrimination of viable from nonviable cells. Antibodies for CD45, CD3, CD4, CD11b, and CD16/CD32 (Fc Blocker) were bought from BD

Pharmingen. Antibody for F4/80 was from Biologend. RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco. Penicillin, streptomycin, and glutamine were from Solarbio Life Technologies. Collagenase VIII and DNase I were from Sigma-Aldrich. Other chemicals were of analytical grade.

**2.2. Animals and Experimental Design.** A total of 32 female 6- to 8-week-old BALB/c mice weighing  $20 \pm 2$  g were obtained from Liaoning Changsheng Biotechnology Co., Ltd. All animals were bred under specific pathogen-free conditions with a temperature of  $22.5 \pm 2$  °C, relative humidity of  $40 \pm 10\%$ , 12 h/12 h light/dark cycle in the Animal Center of Shenyang Medical College. The animal experiment was performed according to the Guidelines for Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Ethical Committee of Shenyang Medical College (SYYXY2020091501).

EGT was prepared as a stock solution using autoclaved distilled water, then further diluted to the desired concentration with phosphate buffer saline (PBS) before injection. Then i.p. administration was performed because it is preferred over the oral route to avoid the gastrointestinal tract and potential degradation/modification.<sup>21</sup> The EGT dosage was based on the results of previous researches<sup>22,23</sup> and preliminary experiments with some modifications, including the low-dose group (0.2 mg/kg body weight) and the high-dose group (2 mg/kg body weight).

After acclimatizing for 1 week, the 32 mice were randomly divided into four groups ( $n = 8$  per group), namely, control group (control), DSS-induced model mice group (DSS), DSS-induced model mice group treated with 4  $\mu\text{g}$  EGT (EGT-4  $\mu\text{g}$ , 0.2 mg/kg body weight), and DSS-induced model mice group treated with 40  $\mu\text{g}$  EGT (EGT-40  $\mu\text{g}$ , 2 mg/kg body weight). All of the mice were given a free diet during the experiment. The control group was given normal drinking water, and the



**Figure 2.** Symptoms of DSS-induced colitis mice. (A) Body weight. The body weight of mice in the DSS group decreased compared with the control group from day 6 to day 7 ( $P < 0.005$ ). The administration of EGT-40  $\mu\text{g}$  significantly slowed down the loss of body weight on day 6 ( $P < 0.005$ ). (B) Disease activity index. The DAI levels significantly increased compared to the DSS group with the control group from day 5 to day 7 ( $P < 0.005$ ). EGT-40  $\mu\text{g}$  treatment significantly suppressed the DAI increase on day 6 ( $P < 0.05$ ). (C) Spleen index. EGT administration significantly suppressed the increase in spleen index caused by DSS ( $P < 0.005$ ). (D) Colon length. The colon length in the DSS group was significantly shorter than that in the control group ( $P < 0.005$ ) and the EGT-40  $\mu\text{g}$  group ( $P < 0.05$ ). (E) Macroscopic pictures of colons. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ . All data in (A–D) are presented as mean  $\pm$  standard deviation (SD).

other three groups were treated with 3.5% (w/v) DSS dissolved in drinking water DSS for 7 days, together with three intraperitoneal injections of PBS for the control group and DSS group or EGT for the two EGT groups on the day before DSS induction, day 2, and day 5 (Figure 1B). On day 8, the mice were sacrificed by cervical dislocation.

**2.3. Assessment of Colitis.** The severity of DSS-induced colitis was evaluated according to a previous study with some modifications.<sup>24</sup> In detail, the body weight of the mice before DSS administration was considered 100% and was monitored daily. Moreover, body weight loss, fecal traits, and hematochezia were evaluated daily and used for calculating the colitis disease activity index (DAI) according to the grading rules and calculating formula as follows

$$\text{DAI} = \frac{1}{3}(\text{body weight loss score} + \text{fecal traits score} + \text{hematochezia score})$$

On the 8th day, the spleen and colon were collected. The colon length was measured, and then a 1 cm colon proximal to the anus was cut and fixed in 4% paraformaldehyde for pathological evaluation. The remaining colon was washed with PBS and stored at  $-80\text{ }^{\circ}\text{C}$  for evaluation of other indicators. Meanwhile, the spleen weight was measured immediately and used for calculating organ indexes according to the following formula

$$\text{spleen index} = [\text{spleen weight (mg)}/\text{body weight (g)}] \times 10$$

**2.4. Histological Injury and Hematoxylin and Eosin (H&E) Staining.** To observe pathological changes, colon tissues cut from the same part of each mouse were selected and fixed in 4% paraformaldehyde, followed by paraffin embedding, slicing, and staining according to the previous method.<sup>25</sup> Based on the cell infiltration of inflammatory cells and epithelial damage, the sections were evaluated and graded under biological microscopes and the images were taken using a digital camera (Nikon, Japan).<sup>26</sup>

**2.5. Alcian Blue and Periodic Acid-Schiff (AB-PAS) Staining.** The adherent mucus layer present in colon tissues was visualized by co-staining paraffin tissue sections, which were obtained the same as the above, with AB and PAS according to the manufacturer's instructions. After dewaxing of tissue sections and staining with AB and PAS, the distribution of mucus was visualized as the proteoglycan with different acidity and alkalinity in the colon tissues can present with different colors: acid mucus is in blue, and neutral mucus is in red.

**2.6. Western Blot Analysis.** The total proteins were extracted after colon tissues were homogenized in ice-cold RIPA lysis buffer and centrifuged, followed by quantifying with the bicinchoninic acid (BCA) method and resolving in SDS-PAGE gels. The proteins in gels were further transferred on PVDF membranes, blocked with skimmed milk, and incubated with primary antibodies (anti-occludin or anti-GAPDH antibodies) and corresponding secondary antibodies successively. Finally, an ECL chemical solution was applied to visualize the proteins, and images were captured by a chemiluminescence imaging system. The optical density was analyzed by ImageJ software.

**2.7. Measurements of MPO, IL-6, and TNF- $\alpha$  in Colon Tissues.** MPO activity, and IL-6 and TNF- $\alpha$  levels in colon

tissues were determined using the corresponding kits according to the manufacturer's instructions. Briefly, the excised colon was homogenized in tissue buffer by a high-throughput sample homogenization processing system 5 times at 50 Hz for 10 s with 30 s intervals. Subsequently, MPO activity in the homogenates was measured following the instructions of MPO kit. Regarding IL-6 and TNF- $\alpha$  levels detection, the homogenates were further centrifuged at 10 000 rpm at  $4\text{ }^{\circ}\text{C}$  for 20 min and repeated the centrifugation for 10 min. Before the ELISA testing, the total protein levels in supernatants collected from homogenates were quantified by the BCA method using the BCA assay kit.

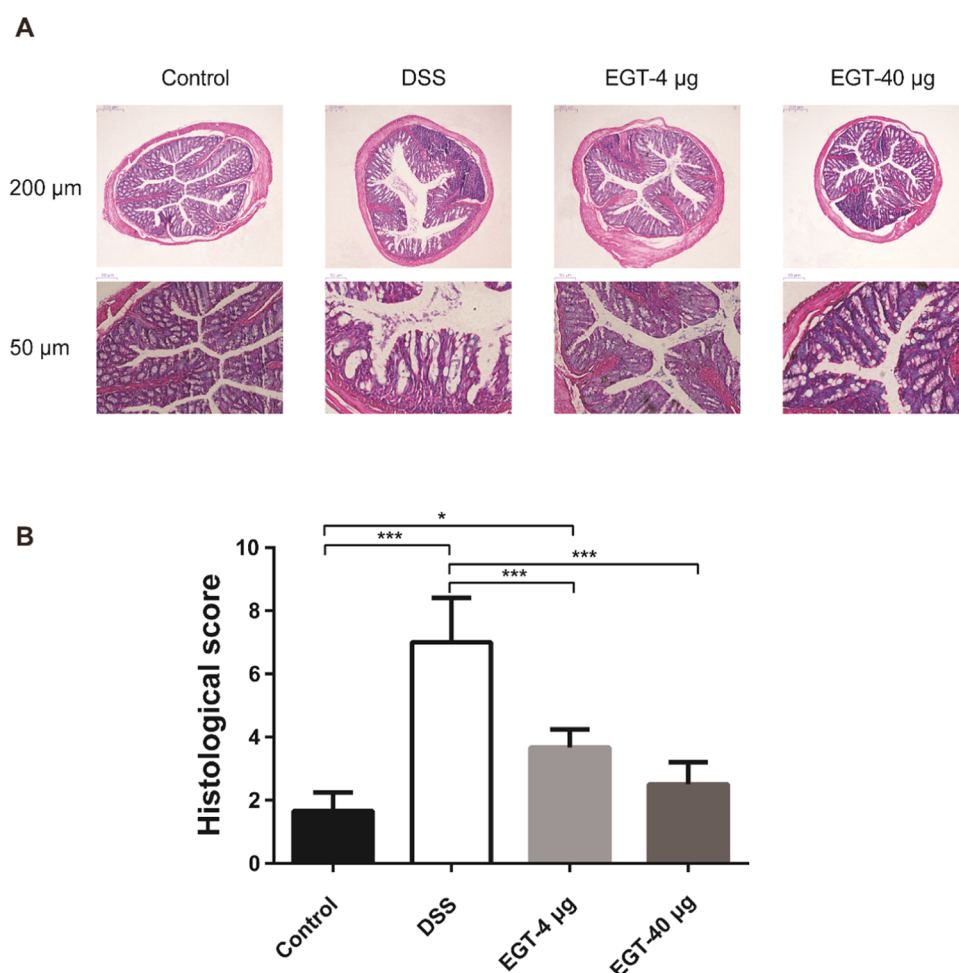
**2.8. Measurement of CD4<sup>+</sup> and Macrophages Populations in Colonic Tissue by Flow Cytometry.** Single-cell suspensions from colon lamina propria were prepared as previously described.<sup>27</sup> Briefly, the colons removed from mesentery and stool were cut into small fragments, and then treated with EDTA in 15 mL of HBSS under rotation for  $3 \times 15$  min (replacing buffer each time) at  $37\text{ }^{\circ}\text{C}$  to separate the epithelium from the lamina propria fraction. Subsequently, the colon pieces were transferred into 15 mL of RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 2 mM L-glutamine, 1.5 mg/mL collagenase VIII, and 40  $\mu\text{g}/\text{mL}$  DNase I and placed into the shaker at  $37\text{ }^{\circ}\text{C}$  for 15 min. After that, cell suspensions were filtered through a 70  $\mu\text{m}$  nylon cell strainer. The spin and resuspension of the pellet were repeated once more and ready for further analysis. The single cells obtained were incubated with Fc blocker at  $4\text{ }^{\circ}\text{C}$  for 15 min and then incubated with the Fixable Viability Stain 780 and antibodies (CD45, CD3, CD4, CD11b) on the ice away from the dark for 30 min for cell surface marker staining. Finally, the cell populations were detected by BD LSRFortessa. The data were analyzed by FlowJo V10 software.

**2.9. Statistical Analysis.** Quantitative data were expressed as mean  $\pm$  standard deviation (SD). The differences among multiple groups were analyzed by one-way analysis of variance (ANOVA), the least significant difference (LSD) multiple-range test, or the Dunnett multiple-range test.  $P$ -value  $<0.05$  was considered statistically significant. Cytoscape 3.6.0 was used for the construction of network diagrams to understand the correlation between biochemical indexes. Statistical analyses were performed with GraphPad Prism 9 and SPSS 22.0 software.

### 3. RESULTS

**3.1. EGT Improved the Colitis Symptoms.** The daily change of the body weight upon the addition of DSS was presented in Figure 2A. There were no significant differences in the initial body weights of each group from day 1 to day 5. However, the body weight of mice in the DSS treatment group declined compared with the control group from day 6 to day 7 ( $P < 0.005$ ). The administration of EGT-40  $\mu\text{g}$  significantly slowed down the decrease of body weight on day 6 ( $P < 0.005$ ), while EGT-4  $\mu\text{g}$  showed little impact on the body weight loss induced by DSS. These results suggested the preventive potentials of EGT at a high dosage.

Simultaneously, DAI was monitored daily throughout the whole experimental period. It can be seen from the data in Figure 2B that differences were displayed since the 5th day after taking drinking water containing 3.5% DSS. With respect to the DSS group, the DAI levels were significantly increased compared with the control group from day 5 to day 7 ( $P <$



**Figure 3.** Effects of EGT on histopathological changes in DSS-induced mice colon. (A) H&E staining images of each group. EGT supplementation relieved the ulceration or damage caused by DSS. (B) Histological score of each group. EGT-4  $\mu\text{g}$  and EGT-40  $\mu\text{g}$  EGT treatments significantly inhibited the increase in histological score induced by DSS ( $P < 0.005$ ). Data are presented as mean  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

0.005), while the other two colitis groups showed a decreasing trend in DAI after EGT dealing. The results suggested that EGT treatment reduced the levels of DAI, especially EGT-40  $\mu\text{g}$  treatment significantly suppressed the DAI increase on day 6 ( $P < 0.05$ ).

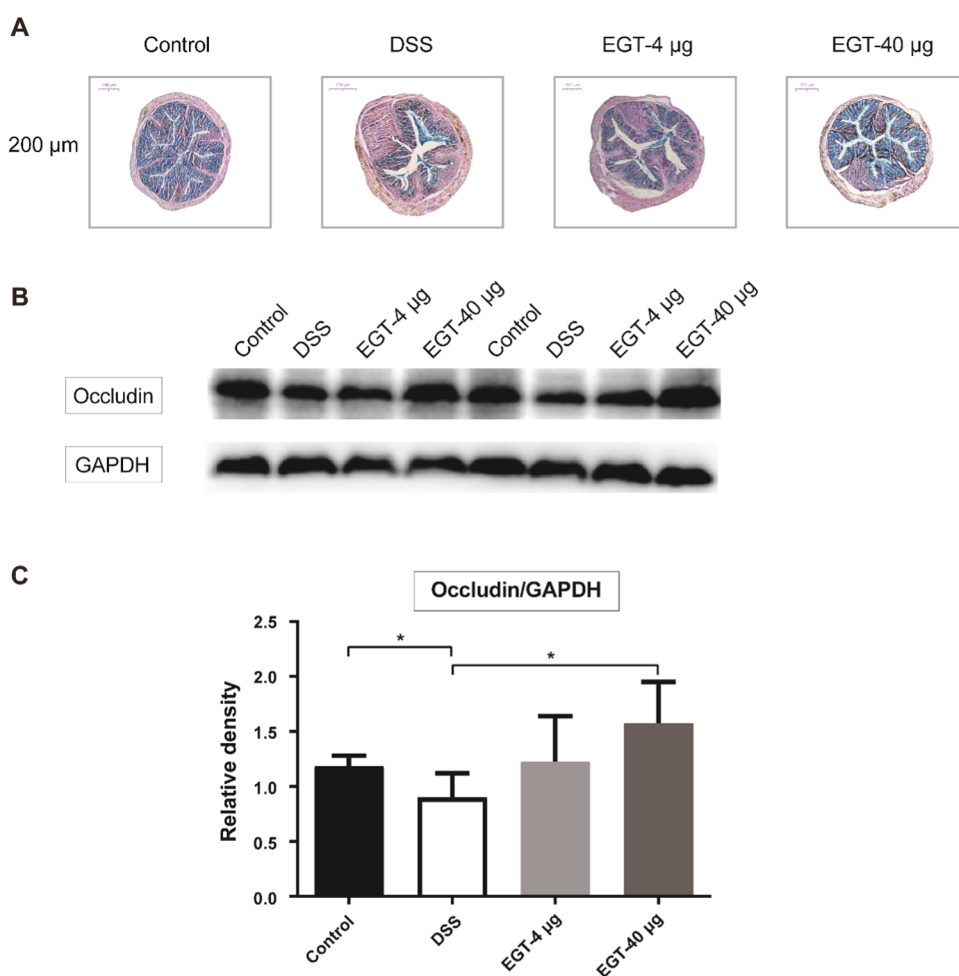
The increase in spleen index was positively associated with the degree of inflammation. As can be seen from Figure 2C, all of the mice in the DSS group exhibited an increase in the spleen index compared with the control group ( $P < 0.005$ ). It was noted that the average spleen index of mice in the EGT-4  $\mu\text{g}$  group and EGT-40  $\mu\text{g}$  group significantly declined compared with that in the DSS group ( $P < 0.005$ ), while no significant difference between the EGT-4  $\mu\text{g}$  group and EGT-40  $\mu\text{g}$  group was evident. Taken together, these results suggest that EGT administration significantly decreased the inflammatory response caused by DSS.

The DSS-induced colitis also has an impact on the colon length, so we further compared the colon length among the four groups. As shown in Figure 2D,E, the colon length in the DSS group was the shortest ( $7.13 \pm 0.70$  cm), followed by the EGT-4  $\mu\text{g}$  group ( $7.69 \pm 0.65$  cm), EGT-40  $\mu\text{g}$  group ( $8.10 \pm 0.35$  cm), and control group ( $9.38 \pm 1.01$  cm). Particularly, the colon length in the DSS group was significantly shorter than that in the control group ( $P < 0.005$ ) and the high-dose EGT-treated group ( $P < 0.05$ ). Collectively, these results implied

that administration of EGT alleviated DSS-induced colitis symptoms.

**3.2. EGT Recovered the Colonic Histological Alterations Caused by DSS.** The colon histological alterations were evaluated to assess the protection of EGT against colonic damage. As can be seen from Figure 3A, the colon tissues in the control group had an intact intestinal mucosa and crypt structure and were enriched for goblet cells. In contrast, the mice in the DSS group showed intestinal mucosal and submucosal erosion, irregular crypts, severe infiltration of inflammatory cells, and loss of goblet cells. What was highlighted in Figure 3A was that the ulceration or damage in colons from mice treated with EGT was relieved or prevented. In other words, the colon morphology in 4 and 40  $\mu\text{g}$  EGT-treated mice was similar to that in control mice, especially the latter.

The histological scores in four groups were shown in Figure 3B. The colon injury score in the colitis mice group was significantly higher than that in the other groups ( $P < 0.005$ ). Although the histological score in the low-dose EGT-treated group showed a significant increase compared with the control group, treatments with 4 and 40  $\mu\text{g}$  EGT still significantly protected the colon tissues against damage induced by DSS. Taken together, these results implied that EGT administration



**Figure 4.** Effects of EGT on mucosubstance and occludin expression in the colon tissues of DSS-induced colitis mice. (A) Images of Alcian Blue and Periodic Acid-Schiff (AB-PAS) Staining. EGT intervention, particularly EGT-40  $\mu\text{g}$  prevented the loss of mucous layer and goblet cells induced by DSS. (B) Western blot of occludin. (C) Occludin density analysis of each group. EGT-40  $\mu\text{g}$  administration significantly improved the decrease in occludin expression caused by DSS ( $P < 0.05$ ). Quantitative data are presented as mean  $\pm$  SD. \* $P < 0.05$ .

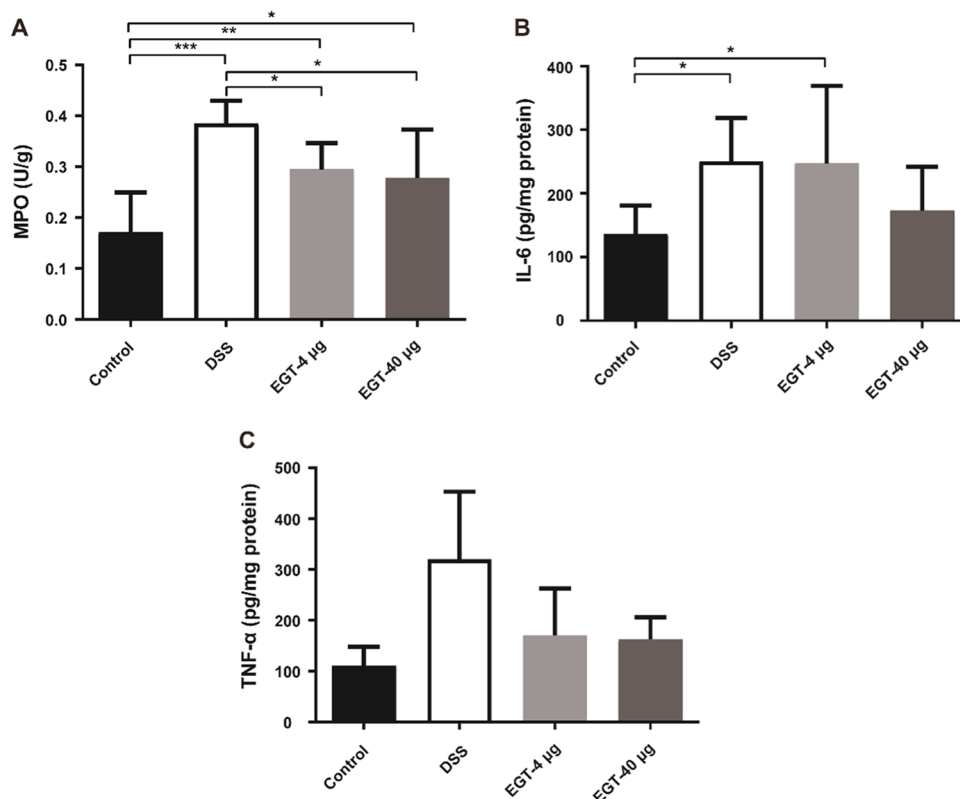
can alleviate the histopathological injury of the colon caused by DSS.

**3.3. EGT Protected the Intestinal Barrier.** Goblet cells in the colon can secrete a large number of mucous particles, i.e., mucin, into the intestinal lumen, which form a dense mucous layer protecting the host against the development of colitis. To evaluate the protective effect of EGT on the mucous layer and goblet cells, the AB-PAS staining was conducted. What is striking about Figure 4A is that colonic tissues in the control group showed a normal thickness of the inner mucous layer and a normal density of goblet cells, while the colon tissues displayed the inner mucous layer with less organized and the goblet cell loss in the DSS group. However, more goblet cells and a relative increase in the thickness of mucus layer were observed after EGT intervention, particularly EGT-40  $\mu\text{g}$ , compared with that in the DSS group.

As can be seen in Figure 4B,C, the expression of occludin was significantly decreased during DSS administration, compared with the control group ( $P < 0.05$ ). Compared with the DSS group, the levels of occludin in the EGT-4  $\mu\text{g}$  group showed a downward trend, but with no significant difference. However, as for the mice treated with EGT-40  $\mu\text{g}$  during DSS administration, occludin expression was significantly improved ( $P < 0.05$ ), even beyond the levels in the

control group. Together these results demonstrated that EGT prevented the gut barrier damage in DSS-induced colitis mice.

**3.4. EGT Regulated MPO Activity and Production of Inflammatory Cytokines.** To investigate the effect of EGT on the activity of inflammatory enzyme and cytokine production in the colon, MPO activities, IL-6, and TNF- $\alpha$  in colon tissues were determined. As can be seen from Figure 5A, the results suggest that MPO activity is associated with DSS addition. Compared with the control group, DSS group displayed a higher MPO activity ( $P < 0.005$ ), as well as EGT-4  $\mu\text{g}$  group ( $P < 0.01$ ) and EGT-40  $\mu\text{g}$  group ( $P < 0.05$ ). Moreover, the MPO activity of mice in the EGT-4  $\mu\text{g}$  group and the EGT-40  $\mu\text{g}$  group both presented significant differences compared with that of the DSS group ( $P < 0.05$ ). Additionally, the MPO activity of the EGT-40  $\mu\text{g}$  group was lower than that of the EGT-4  $\mu\text{g}$  group, although no significant differences were observed. Together these results suggest that EGT treatment significantly prevented the DSS-induced activation of colonic MPO activity. Concerning IL-6, the concentrations of IL-6 in colons of the DSS group and EGT-4  $\mu\text{g}$  group were significantly higher than those in the control group ( $P < 0.05$ , Figure 5B). The DSS group and EGT-4  $\mu\text{g}$  group had similar levels of IL-6 suggesting EGT-4  $\mu\text{g}$  supplementation EGT cannot inhibit the increase in IL-6



**Figure 5.** Effects of EGT on MPO activity, IL-6, and TNF- $\alpha$  production in colonic tissues of DSS-induced colitis mice. (A) MPO activity. EGT-4  $\mu$ g and EGT-40  $\mu$ g treatments significantly prevented the DSS-induced activation of colonic MPO activity ( $P < 0.05$ ). (B) IL-6 production. EGT-4  $\mu$ g and EGT-40  $\mu$ g administrations did not significantly inhibit the increase in IL-6 induced by DSS. (C) TNF- $\alpha$  production. Compared with the DSS group, TNF- $\alpha$  levels in the EGT-4  $\mu$ g group and EGT-40  $\mu$ g group showed a declining trend, but no significant differences were observed. Data are presented as mean  $\pm$  SD,  $n = 6$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

concentration caused by DSS. Compared with the DSS group, the IL-6 level of the EGT-40  $\mu$ g group showed a declining trend, but no significant difference was observed. Regarding TNF- $\alpha$ , the concentrations of colonic TNF- $\alpha$  in the control group, EGT-4  $\mu$ g group, and EGT-40  $\mu$ g group decreased by 2.89-, 1.88-, and 1.97-fold, respectively, compared with the DSS group (Figure 5C), however, no significant differences were observed for all four experimental groups. To sum up, these results implied that administration of EGT only partially alleviated DSS-induced inflammation response.

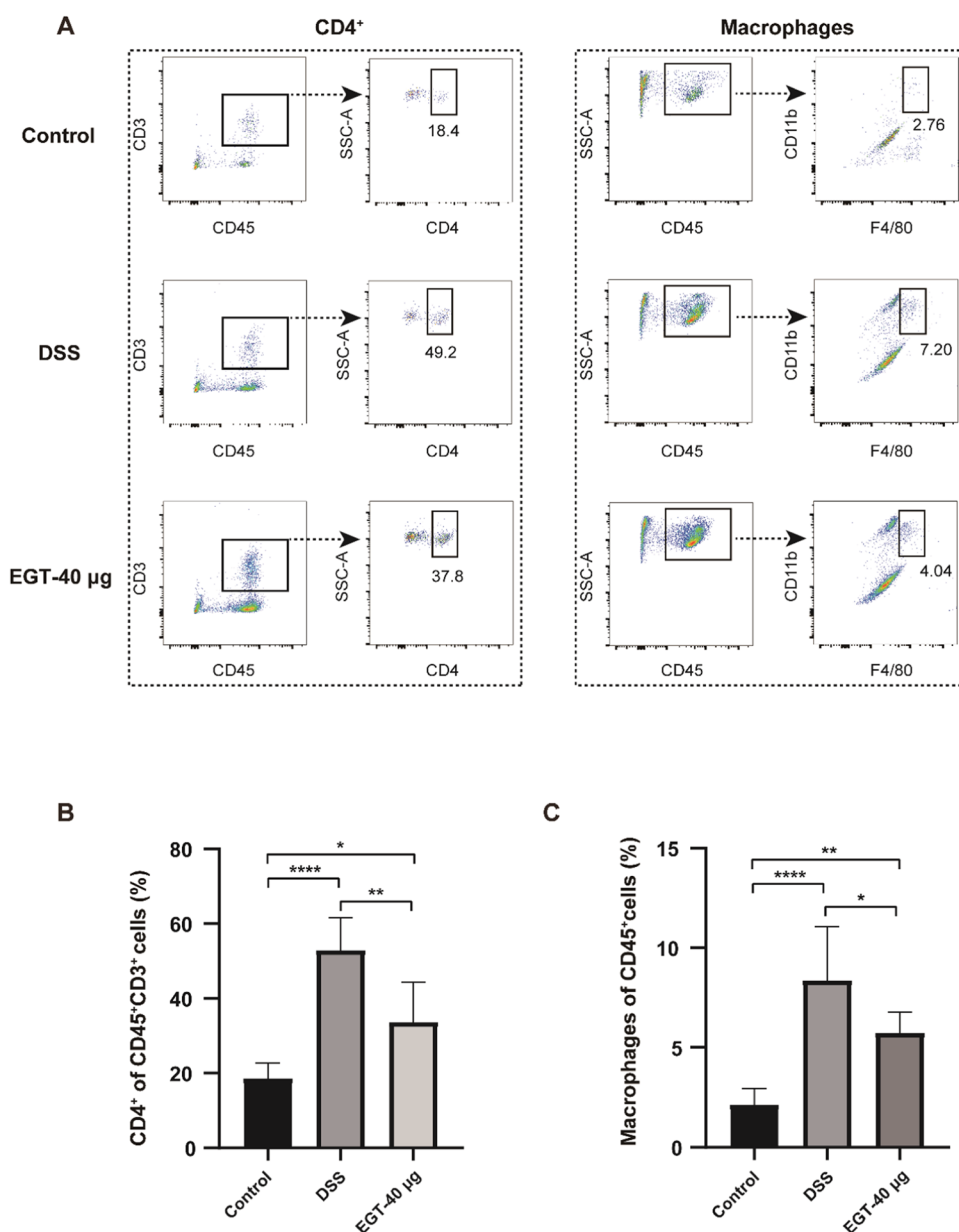
**3.5. EGT Downregulated Populations of CD4<sup>+</sup> T Cells and Macrophages.** To determine if the percentage of some immune cells was influenced by EGT, we compared CD4<sup>+</sup> T cells and macrophages subsets percentages in the colonic lamina propria of the control group, DSS group, and EGT-40  $\mu$ g group by flow cytometry. As can be seen from Figure 6A, both CD4<sup>+</sup> T cells and macrophages population were increased in colons of DSS-induced colitis mice. However, EGT-40  $\mu$ g treatment caused a significant decrease in CD4<sup>+</sup> T cells and a decline in macrophages cells. What stands out in Figure 6B is that all of the mice in the DSS group exhibited a higher percentage of CD4<sup>+</sup> T cells compared with that in the control group ( $P < 0.001$ ), whereas the administration of EGT-40  $\mu$ g significantly slowed down the increase of CD4<sup>+</sup> T cells ( $P < 0.01$ ). With respect to macrophages (Figure 6C), the percentage of the DSS group was significantly increased compared with that in the control group ( $P < 0.001$ ), while EGT-40  $\mu$ g treatment significantly suppressed the increase of macrophages ( $P < 0.05$ ). Taken together, these results

indicated that the EGT administration can downregulate the immune response caused by DSS.

### 3.6. Correlation between MPO Activity and Cytokine Release Regulated by EGT and Colitis Indexes in Mice.

To evaluate the effects of EGT on macroscopic and microscopic colitis indicators, weight change on day 6, spleen index, DAI on day 6, colon length, histological score, the expression of occludin, MPO activity, the concentrations of IL-6 and TNF- $\alpha$  were analyzed by Cytoscape among DSS group, EGT-4  $\mu$ g group, and EGT-40  $\mu$ g (Figure 7A). Two concentrations of EGT had varied effects on colitis indicators. When comparing 4  $\mu$ g EGT treatment with the control group, the spleen index and histological score ( $P < 0.005$ ) showed a higher degree of correlation than that of MPO ( $P < 0.05$ ). However, the expression of occludin, weight change, colon length, DAI, and the levels of TNF- $\alpha$  and IL-6 did not display a correlation with EGT treatment. Moreover, 40  $\mu$ g EGT treatment had an impact on weight change, histological score, and spleen index ( $P < 0.005$ ). In addition, 40  $\mu$ g EGT showed a high degree of significant relation with DAI-6, MPO activity, the expression of occluding, and colon length ( $P < 0.05$ ) but had no significant correlation with IL-6 and TNF- $\alpha$ .

To further investigate the effect of two concentrations of EGT on inflammatory markers of colitis, the interdependent quantitative relationships between the macroscopic and microscopic colitis indicators of the DSS group, EGT-4  $\mu$ g group, and EGT-40  $\mu$ g group were analyzed using unary linear regression. After correlations analysis between the macroscopic and microscopic colitis indicators, a total of four sets of



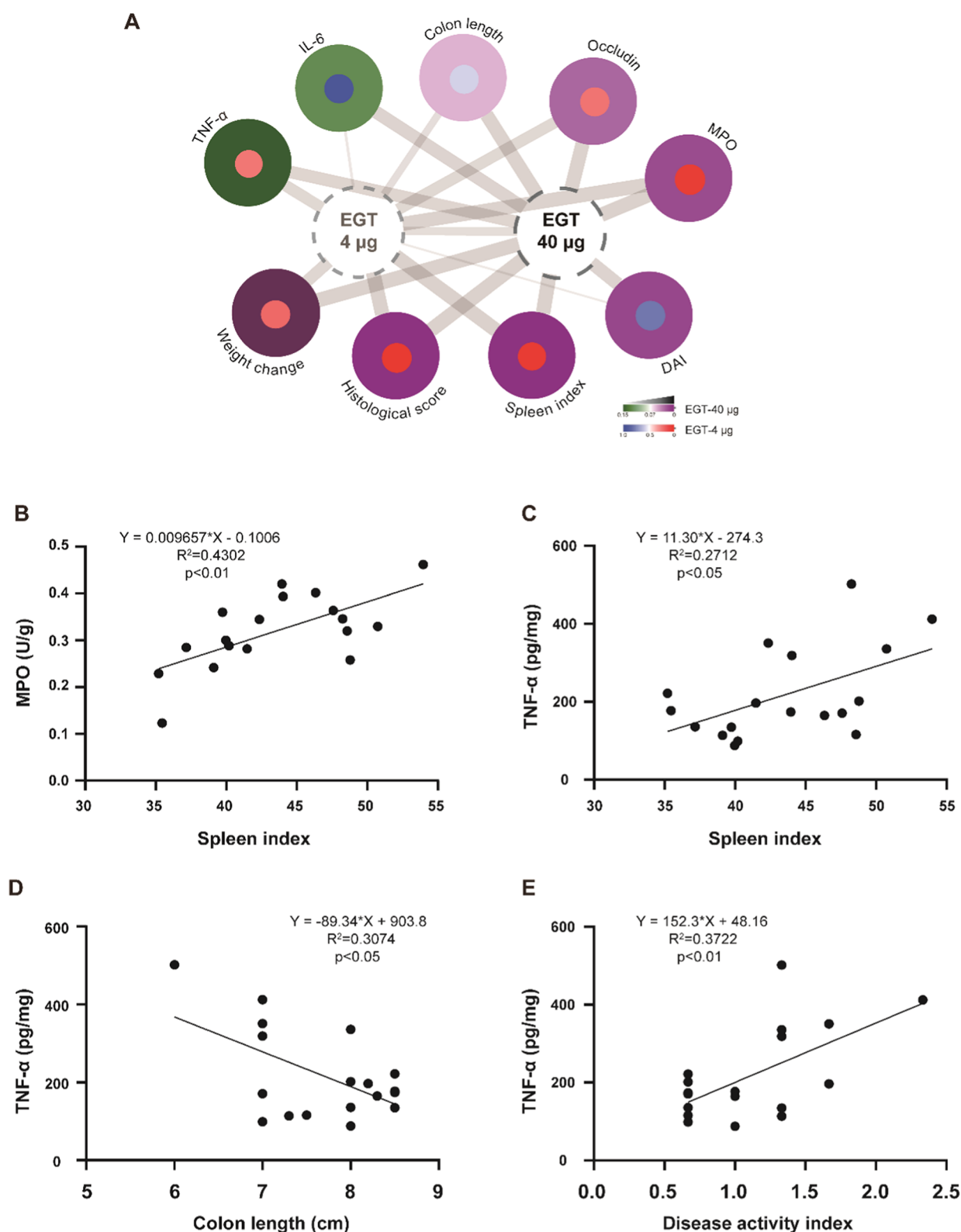
**Figure 6.** EGT downregulated CD4<sup>+</sup> T cell and macrophage populations in colonic tissues of DSS-induced colitis mice. (A) Representative biaxial plots depicting the gating strategy for CD4<sup>+</sup> T cell and macrophage subsets derived from colonic lamina propria analyzed by flow cytometry. (B) CD4<sup>+</sup> T cells populations of control, DSS, and EGT-40 μg groups. The administration of EGT-40 μg significantly slowed down the increase of CD4<sup>+</sup> T cells caused by DSS ( $P < 0.01$ ). (C) Macrophage populations of control, DSS, and EGT-40 μg groups. EGT-40 μg treatment significantly suppressed the increase of macrophages induced by DSS ( $P < 0.05$ ). CD4<sup>+</sup> and CD4<sup>+</sup>CD3<sup>+</sup> cells were set gate from viable cells. CD4<sup>+</sup> T cells were from CD45<sup>+</sup>CD3<sup>+</sup> cells and CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages were from CD45<sup>+</sup> cells. Quantitative data are presented as mean  $\pm$  SD,  $n = 6$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$ .

parameters were correlated. The spleen index displayed a significantly higher positive correlation with MPO activity (Figure 7B,  $P < 0.01$ ) than that with the levels of TNF- $\alpha$  in colon tissues (Figure 7C,  $P < 0.05$ ). Moreover, the levels of TNF- $\alpha$  in colon tissues showed a significantly negative correlation with colon length (Figure 7D,  $P < 0.05$ ) but a positive correlation with DAI (Figure 7E,  $P < 0.01$ ). Overall, MPO activity in colon tissues was significantly positively related to spleen index, colon length, and DAI, but TNF- $\alpha$  levels in colon tissues were negatively related to these indicators, revealing that the dose of EGT was correlated with the colitis alleviation.

#### 4. DISCUSSION

UC is characterized by mucosal inflammation of unknown etiology affecting the colon and rectum.<sup>28</sup> Nowadays, the global incidence of UC is increasing, while the etiology is complex and still unclear, hence investigating the pathogenesis mechanisms and exploring newly effective treatments become more important.<sup>29,30</sup> At present, conventional anti-inflammatory drugs for UC, including 5-aminosalicylic acid and anti-TNF- $\alpha$  antibodies, have limited therapeutic effects and often bring some side effects.<sup>3,31</sup> Although some active ingredients of herbs, such as atropine, hyoscyne, and hyoscyamine, in belladonna are used to treat IBD because of their anticholinergic properties, there are many side effects including





**Figure 7.** Correlation analysis among biochemical indexes in DSS-induced colitis mice. (A) Correlation analysis of different parameters by Cytoscape. Each node represents an index. The node center represents the correlation between 4  $\mu\text{g}$  EGT treatment and parameters and the periphery represent the correlation between 40  $\mu\text{g}$  EGT treatment and parameters. Blue colors indicate a weak correlation, red colors indicate a strong correlation for 4  $\mu\text{g}$  EGT treatment, green colors indicate a weak correlation, and purple colors indicate a strong correlation for 40  $\mu\text{g}$  EGT treatment. The line thickness indicates the degree of correlation. (B) Interdependent quantitative relationships between spleen index and colonic MPO. (C) Interdependent quantitative relationships between spleen index and colonic TNF- $\alpha$ . (D) Interdependent quantitative relationships between colon length and colonic TNF- $\alpha$ . (E) Interdependent quantitative relationships between disease activity index and colonic TNF- $\alpha$ . Data in (B–E) are presented as mean,  $n = 6$ .

dilated pupils, blurred vision, tachycardia, staggering, rash, flushing, urinary retention, constipation, and convulsions.<sup>32</sup> Food-derived natural bioactive ingredients have therefore been proposed to prohibit long-term inflammation, of which EGT as a derivative of the amino acid histidine mainly from edible fungi receives progressive attention because of its good absorption, long half-life, and safety.<sup>33,34</sup> However, systematic research on EGT function to attenuate ulcerative colitis is currently lacking. Recently, Pang et al.<sup>20</sup> treated DSS-induced colitis in rats by oral administration at a low EGT dose (20 mg/kg) and high EGT dose (40 mg/kg), respectively. The results indicate that EGT can significantly alleviate colon length shortening and colonic pathological damage, mediated by downregulating the expression of pro-inflammatory factors and inhibiting the TLR4/MyD88/NF- $\kappa$ B signaling pathway.<sup>20</sup> Compared with the research of Pang et al., although we use lower doses of EGT (0.2 and 2 mg/kg) to treat DSS-induced mice by i.p. administration, results still suggest that EGT can reduce inflammation, improve tight junctions and barrier function abnormalities and maintain intestinal homeostasis by regulating the mucosal immune response.

In the study, the body weight loss, bloody stool, shorter colonic length, and higher DAI indicated that the 3.5% DSS-induced colitis mice model was successfully constructed. Moreover, EGT-40  $\mu$ g treatment ameliorated the response of body weight, colon length, and DAI induced by DSS, while EGT-4  $\mu$ g treatment did not bring out these effects. Interestingly, the body weight of the DSS group decreased, whereas EGT-40  $\mu$ g treatment significantly reduced this effect on day 6, but there was no significant difference in weight compared to that in the EGT-40  $\mu$ g administration group on day 7 (Figure 2A). Consistent with the trend of body weight, EGT-40  $\mu$ g treatments decreased DAI scores on day 6 but not on day 7 (Figure 2B). Furthermore, colon length was used to assess the protective effects of EGT against DSS-induced colitis in mice. Inflammation, congestion, and edema of the mouse colon brought about colon shortening in the DSS group, while EGT can partially ameliorate the health state of colitis mice in view of the recovered body weight, DAI, spleen index, and colon length. Overall, high-dose EGT exhibits a better effect on mice than low-dose EGT on relieving colitis, suggesting a dose-dependent manner in alleviating inflammation.

Previous studies revealed that dysfunctions in the mucosal barrier including mucus layer and barrier integrity are the primary pathological characteristic of colitis.<sup>35</sup> The mucus layer as the first line of intestinal barrier can protect the intestinal epithelium from the attack of pathogens and toxic metabolites; thus, decreases in mucins and goblet cells comprise key factors in UC development.<sup>36</sup> Our results demonstrate that EGT could reduce mucus destruction, loss of the goblet cells, and infiltration of inflammatory cells (Figures 3 and 4), thus attenuating the colitis-associated damages. Moreover, tight-junction proteins (TJs) prevent exogenous substances from invading the intestinal tissues, thereby suppressing inflammation and intestinal mucosal injury.<sup>37</sup> The present study suggests that EGT supplementation could restrain gut barrier damage by enhancing the expression of TJs protein occludin and maintaining the integrity of the colon barrier system.

MPO, a heme-containing lysosomal enzyme mainly secreted by activated neutrophils plays a key role in the host defense at the sites of inflammation by the production of halogenating molecules.<sup>15</sup> Moreover, a previous study has demonstrated that

the increase in MPO activity in colon tissues was generally accompanied by an increased inflammation level, and therefore as a biomarker of inflammation in mice with DSS-induced colitis.<sup>10</sup> Our research suggested both the EGT-4  $\mu$ g group and the EGT-40  $\mu$ g group significantly prevented the DSS-induced activation of MPO activity in colon tissues (Figure 5A), which confirmed the previous observation that EGT inhibited MPO activity *in vitro*.<sup>15</sup>

During the occurrence and development of colitis, the depletion of intestinal mucus, exhaustion of goblet cells, and the decreased expression of tight-junction proteins lead to an increase in intestinal permeability as well as damage to the intestinal barrier, which would make the gut antigen exposed to the immune cells and subsequently trigger inflammatory responses.<sup>38</sup> Upon activation, such immune cells secrete pro-inflammatory cytokines including IL-6 and TNF- $\alpha$ , leading to inflammation and remodeling of the morphology. Compared with the DSS group, the levels of IL-6 and TNF- $\alpha$  showed a declining trend in the EGT-40  $\mu$ g group, but no significant differences were observed. This may be due to the relatively large individual differences. Interestingly, although the levels of TNF- $\alpha$  are not significant among the four groups, they have a good linear relationship with colon length, spleen index, and DAI index (Figure 7C–E). These results indicate that the EGT can regulate the production of pro-inflammatory factors; however, the signaling pathway still needs to be explored.

Intestinal mucosal immunity consists of innate immunity and adaptive immunity, which is vital to resist pathogen invasion and prevent damage. Macrophages, dendritic cells, and cytokines are generally involved in innate immunity, while specific T and B lymphocytes play an important role in adaptive immunity.<sup>28</sup> CD4<sup>+</sup> T cells contribute to gut inflammation and accumulate in the mucosa of both UC and CD patients.<sup>39,40</sup> Consistent with the previous study regarding the increased number of CD4<sup>+</sup> T cells in the colons of colitis mice,<sup>39</sup> the current results indicate that EGT can downregulate the immune response caused by DSS in view of EGT-40  $\mu$ g treatment caused a significant decrease in CD4<sup>+</sup> T cells (Figure 6A,B). Moreover, a series of studies have shown that the macrophage subset is increased in IBD, which is important in the immunological and inflammatory responses.<sup>41,42</sup> Macrophages secrete many pro-inflammatory cytokines, such as IL-6 and TNF- $\alpha$ , and also release reactive metabolites of oxygen, nitrogen, and proteases that degrade the extracellular matrix.<sup>42</sup> In the study, macrophages cells were increased in colons of DSS-induced colitis mice, in agreement with Rugtveit's research;<sup>41</sup> however, EGT-40  $\mu$ g treatment caused a significant decrease in the percentage of macrophages (Figure 6A,C).

Although EGT can be found in a wide range of foods, it is only synthesized by certain fungi and bacteria, not by animals or higher plants.<sup>43</sup> Humans and other mammals can only obtain EGT through diet and accumulate it in their bodies by means of an intestinal transporter, OCTN1, while higher plants take EGT up from symbiotic nitrogen fixation systems and fungal production in the soil.<sup>44–46</sup> The mushrooms along with several other fungi are a major dietary source of EGT for humans, followed by beans, animal liver, garlic, etc.<sup>43</sup> Interestingly, EGT levels in different foods have large variations. For example, different species of mushrooms, *Boletus edulis*, *P. ostreatus*, *Agaricus bisporus*, and *Ramalina maitake*, have a relative content from 0.15 to 7.27 mg/g dry weight, among which the EGT content of *B. edulis* is the highest.<sup>47,48</sup> Additionally, a previous study showed cooking

procedures and storage conditions of mushrooms enhanced the accumulation of EGT in cultivated mushrooms.<sup>49</sup> In this respect, it is important to note the EGT amounts in foods and the effect of processing on the content of EGT in the future study.

In conclusion, EGT at a dose of 40  $\mu\text{g}$  significantly alleviated not all but DSS-induced abnormality in body weight, DAI, colon length, spleen index, histological score, tight-junction protein occludin, and MPO activity, which may indicate the underlying antioxidation mechanism of its protective functions against colitis. When comparing EGT groups with the DSS group, a declining trend in colonic IL-6 and TNF- $\alpha$  levels and a significant decrease in CD4<sup>+</sup> T cells and macrophages reveal the possibilities of specific beneficial potentials in immunomodulatory. Together these results elucidate the role of EGT in alleviating colitis, which may provide supportive data in our understanding of the mechanism in autoimmune inflammation and pursuing new therapeutic targets in UC. Nevertheless, despite the great promise of EGT as a safe and natural diet-derived antioxidant, further studies are required to explore the mechanisms of anti-inflammatory effects and to evaluate the general treatment of UC in practice.

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All authors have read and agreed to the published version of the manuscript.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

This research was supported by Shenyang Medical College Doctoral Research Initiation Fund Project (2019S071) and the Natural Science Foundation of Liaoning Province (2021-MS-352). The authors also thank Ying Zhang for her technical support in flow cytometry at the Institute of Health Sciences of China Medical University.

## ABBREVIATIONS

**AB-PAS**, Alcian Blue and Periodic Acid-Schiff; **CD**, Crohn's disease; **DAI**, disease activity index; **DSS**, dextran sodium sulfate; **ELISA**, enzyme-linked immunosorbent assay; **H&E**, hematoxylin and eosin; **IBD**, inflammatory bowel disease; **IL-6**, interleukin 6; **MPO**, myeloperoxidase; **PBS**, phosphate-buffered saline; **TJs**, tight-junction proteins; **TNF- $\alpha$** , tumor necrosis factor  $\alpha$ ; **UC**, ulcerative colitis

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