Colitis can be Improved by (-)-Epigallocatechin Gallate through Targeting Notch in DSS-induced UC mice

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Abstract

Abstract Background: Ulcerative colitis (UC) is a chronic and non-specific inflammatory bowel disease. Previous research shows that Notch plays a role in the pathogenesis of UC and (-)-Epigallocatechin Gallate(EGCG) could attenuate colitis. However, the mechanism of EGCG to improve colitis remains unclear. Methods:The human epithelial colorectal adenocarcinoma Caco-2 cells were intervened with EGCG (10ug/ml or 30ug/ml) with or without Lipopolysaccharide. A mouse model of UC was induced by 3% dextran sulfate sodium and EGCG treatment was administered to mice at a dose of 10 and 20 mg/kg. The stool consistency, rectal bleeding and weight were recorded daily. The disease activity index (DAI) of mice was calculated, and the pathological injury scores were assessed through hematoxylin and eosin staining. Immunohistochemical analyses were performed for iNOS, F4/80, Notch1 and hes1. Inflammatory cytokines were detected using ELISA kits .Western blot assays were performed for TNF-α, IL-1β, , Notch1, Cleaved-Notch1, Notch2,, Hes-1, COX2, iNOS from colon tissues and Caco-2 cells. Results: In this study, we found that the cytokine secretion and inflammation protein expression were reduced with EGCG treatment in LPS induced Caco-2 cells. And the levels of Notch1, Cleaved-Notch1, and Hes-1 expression were decreased by EGCG administration in the cell. Moreover, we found the pro-inflammation cytokine secretion and the macrophages accumulation were reduced by oral EGCG in DSS-induced mice colon which indicates EGCG ameliorates colitis in vivo. And we also found the phenotype of macrophages could alter to M1 was inhibited by oral EGCG in vivo. In addition, we demonstrate that EGCG could attenuate the levels of Notch1, Cleaved-Notch1, and Hes-1 expression in the colon. Conclusion: This study demonstrates that colitis can be improved by EGCG through targeting Notch in DSS-induced UC mice. Key words: EGCG; Notch; colitis; inflammation

Introduction

Ulcerative colitis (UC) is a chronic and non-specific inflammatory bowel disease (IBD), which is characterized by complicated and relapsing inflammation caused enormous multidimensional burdens on patients and health care systems[1]. Over 1 million residents in the USA and 2.5 million in Europe are estimated to have IBD and it also has emerged in newly industrialized countries in Asia, South America, and the Middle East and has evolved into a global disease with rising prevalence in every continent[2]. In China, the age-standardized rate of prevalence and incidence of IBD was increased between 1990 and 2017[1]. It can impede career aspirations, instill social stigma and impair quality of life in patients[1,3]. Some drug-like anti-inflammatory, immunosuppressive, 5-aminosalicylic acid or corticosteroids, have been used in clinical UC patients. But these clinical medicines are no cure for relapsing and there are certain side effects[3,4]. Hence, it is necessary to find some new and safer treatment drugs, such as natural products.

The pathogenesis of UC includes environmental factors, genetic predisposition, dysregulated immune responses, mucous barrier and so on[5]. However, the pathogenesis of UC is still unclear. The Notch signaling pathway is a major regulator of cell-fate determination during development and cellular differentiation[6]. Some studies have been reported that the Notch signaling pathway plays a role in the pathogenesis of UC[7,8].

And Notch could ensure integrity and homeostasis of the intestinal epithelium[7]. It also plays a role in regulating inflammatory respond[6]. Thus, Notch maybe as a potential target to treatment UC.

The (-)-Epigallocatechin-3-gallate (EGCG) is a kind of polyphenol that is abundant in tea[9]. Previous study has been reported that EGCG could attenuate colitis which induced by dextran sulfate sodium (DSS)[10]. However, the mechanism of EGCG to improve colitis remains unclear. In our previous studies, we have demonstration that Notch is a receptor of EGCG[11,12]. Thus, we hypothesized that EGCG may attenuate colitis in UC mice by targeting Notch1 to inhibit inflammation.

To prove this hypothesis, the human epithelial colorectal adenocarcinoma Caco-2 cell line[13] and DSS-induced UC model of C57 mice[4] were used to study the effects of EGCG on Notch1 to attenuate colitis. The results showed that the activation of Notch1 was attenuated by treatment with EGCG in vitro and in vivo. Furthermore, the inflammation response and clinical symptoms of colitis in UC mice can be also improved by EGCG. These findings suggest that EGCG can attenuate colitis by targeting Notch1. Therefore, the identification effect of EGCG on Notch1 provides a new idea for the treatment of UC and EGCG as a potential drug for the treatment of IBD.

Materials and Methods

Animal experimental design

Twenty-four 7-to 8-week-old male C57BL/6 mice were purchased from CAVENS LAB ANIMAL Co., (Changzhou, China). After two weeks of adaptive feeding, they were randomly divided into four groups: Normal group (6 mice), DSS group (6 mice), DSS+LE group (6 mice), DSS+HE group (6 mice). Except for the normal group treatment with drinking water, the remaining groups received 3% DSS in drinking water for 8 days. From the first day of adding DSS to the drinking water, EGCG treatment was administered to mice at a dose of 10 and 20 mg/kg per day by gavage for 1 week. During modelling and drug treatment, the stool consistency, rectal bleeding and weight were recorded daily. The disease activity index (DAI), a score used to assess the severity of colitis, was calculated as previously described[14]. All animal experiments were undertaken according to the institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the Yunnan Agricultural University.

At the end of the experiment, all mice were killed with sodium pentobarbital and received colon tissue,whose length was measured, and then portions of colon were stored at -80 for the western blot and enzyme-linked immunosorbent assay (ELISA).Portions of colons were fixed in formalin for histopathological and immunohistochemical analysis.

Culture and treatment of Caco-2 cells

The human epithelial colorectal adenocarcinoma Caco-2 cells were purchased from KCB (Kunming, China). and were cultured in DMEM comlete medium comprising 10% fetal bovine serum, and 1% penicillin–streptomycin at 37 in a humidified 5% CO2 atmosphere. The Caco-2 cells were starved for 12h and intervened with EGCG (10ug/ml or 30ug/ml) with or without Lipopolysaccharide (LPS, 25ug/ml) for 24 h. The supernatant from each treatment was used to determine the levels of TNF-α protein released by the cells using a ELISA kit. Cells were collected for Western blot analysis.

Western blot analysis

Total proteins were extracted from colon tissues and Caco-2 cells using RIPA buffer supplemented with 1% PMSF (protease inhibitors), and protein concentrations were determined by the bicinchoninic acid (BCA) method. Equal amounts of protein (45μg) samples and 5× reduction loading buffer were mixed in the tubules and boiled at 95 for 10min. Total protein samples were separated by 10% SDS-PAGE and were transferred onto the 0.2um PVDF membrane. The membranes were blocked using 5% skimmed milk for 1h and were incubated for 16h at 4degC with TNF-α(sc-133192),IL-1β(sc-32294),F4/80(sc-52664,Santa Cruz, USA), Notch1(SJ205, Hangzhou HuaAn Biotechnology, China), Cleaved-Notch1(Val1744), Notch2(D76A6, Cell Signaling Technology , MA, USA), Hes-1 (ab-71559), COX2 (ab179800), iNOS (ab15323,abcam, MA,

USA). And then were incubated with anti-rabbit IgG (# HAF008), anti-mouse IgG (# HAF007) and anti-Rat IgG (# HAF005) secondary antibodies for 1h at room temperature. The membrane target proteins was detected by the enhanced chemiluminescence western blot detection system (Millipore, Billerica, MA, USA).

Histopathological and Immunohistochemistry analysis

Formalin-fixed mouse colon tissues were embedded in paraffin wax and cut into 3μM thick sections for histopathological and immunohistochemical examination. For histopathology, sections were dewaxed and hydrated, stained with hematoxylin (Sigma, USA) and eosin (Solarbio), and photographed with a Leica microscope to evaluate the morphology. Immunohistochemical analyses were performed for iNOS, F4/80, Notch1 and hes1. Colon sections were dewaxed and hydrated, repaired with microwave in PH 6, 0.1M sodium citrate buffer, washed with PBS for three times, immunohistochemical pen drew circles on the sections, and endogenous catalase inhibitors were dropped in the circles for 20min, and the sections were sealed with the serum working liquid in the ABC kit for 20min. Sections were incubated overnight with anti-iNOS, anti-F4/80, anti-Notch1 and anti-Hes1 antibodies at 4. Biotin-labeled secondary antibody was incubated at 37 for 30 minutes, peroxidase (DAB) was stained for 5 minutes, the positive area was stained brown, and finally stained with hematoxylin, dehydrated, transparent and sealed. Images were captured using a Leica microscope.

Enzyme-linked immunosorbent assay (ELISA) analysis

Part of the frozen colon tissue was obtained and placed in the lysate with a tissue crusher to break the homogenate. The lysates were centrifuged at 8000g for 10 min at 4 , and the supernatants were transferred for ELISA. Human TNF-α, Mouse TNF-α, IL-1β, MCP-1 and IL-6 concentrations were determined using the ELISA MAX Deluxe set (BioLegend, USA) according to the manufacturer's instructions.

Statistical analysis

One-way ANOVA followed by Tukey's test was used to analyse the data expressed as means ± SEM for comparing differences among each group. Statistical evaluations was analyzed via SPSS 22.0 (IBM, Armonk, NY, USA) and Prism 5 (GraphPad Software, La Jolla, CA, USA). Level of statistical significance was set to $p < 0.05$.

Results

EGCG attenuates LPS induced inflammation by target Notch in Caco-2 cell

Previous studies have been reported that the inflammation could be induced by LPS in Caco-2 cell[13]. But the effect of EGCG in LPS induced Caco-2 cells was still unclear. To evaluate the anti-inflammatory effects of EGCG in LPS induced Caco-2 cells, we perform the experiment as described in methods. The level of TNF-α secretion was increased by LPS treated compared with the normal group (figure 1A). The expression of COX2, which is a kind of inflammation protein, was also added by LPS treatment (figure1B and 1C). The effect of EGCG on inflammation has been reported in some studies, and it also plays an anti-inflammation role in LPS induced Caco-2 cells. The level of TNF-α secretion and COX2 expression were significantly decreased by EGCG add compared with LPS group (figure1A, 1B and 1C). Notch was a novel drug target of EGCG[11]. To evaluate the mechanism of EGCG anti-inflammation in the intestinal cell, we detected the protein of Notch signaling pathway. The results showed that EGCG could significance decrease the Notch1 expression compared with LPS treatment (figure1D, 1E). Similarly, the expression of Notch2 also decreased by EGCG treatment (figure1D, 1F). The LPS treatment could induce Notch1 activation (figure1D, 1G) and promote the Notch signaling transduction through increased expression of HES-1(figure1D, 1H). But, the expression of Notch1 and Notch2 were decreased by EGCG intervention. The activation of Notch1 (figure1D, 1G) and the downstream signal protein HES-1(figure1D, 1H) were reduced through EGCG treatment. These results reveal a potential mechanism of EGCG anti-inflammation to attenuate colitis by target to Notch pathway.

EGCG ameliorates colitis in DSS-induced UC mice

The typical features in the DSS-induced mice model of UC have sustained body weight loss, diarrhea, and rectal bleeding[16]. The colitis was induced in mice by administration of 3% DSS for 8days as described in methods. We found that the bodyweight of mice in DSS group continued to decrease significantly from day6 (figure2A) and EGCG administration could dramatically improve body weight loss in mice by treat with high dose (figure1A, $p<0.05$). The colon shortening was restored by EGCG therapy compared with DSS treatment group (figure2B and 2C). The spleen weight was generally measured as an indirect marker of inflammation in all experiment groups. Different concentrations EGCG could significantly reversed the DSS-mediated increase in the spleen index ($P<0.05$, Figure 2D). Furthermore, the DAI score, which is used as indicator to assessing the severity of colitis, increased distinctly after DSS treatment and it was markedly lower in the HE group (figure2E and 2F). In addition, we also observed epithelial crypt damage, mucosa edema, disappearance of intestinal crypts and goblet cells, and inflammatory cell infiltration by histopathological analysis using H&E staining to assessing severity of colonic ulceration and inflammation during experiment groups. The DSS group was sharp contrast to the normal group (figure 2G) and EGCG could exhibited obvious protection of mucosa damage and less histological inflammation (figure 2G), which showed a lower histopathological score (figure 2H). Thus, these data confirm that EGCG exerts therapeutic effects on DSS-induced colitis.

EGCG reduced the level of inflammatory cytokines in colon tissues of DSS-treated mice

The increased inflammation cytokines, such as TNF-α, IL-1β, IL-6, MCP-1, plays a critical role in DSS-induced ulcerative colitis[4]. To investigate the effect of EGCG on inflammation-associated molecules, ELISA assay was performed to confirm the effects of EGCG on the protein levels of TNF-α, IL-1β, IL-6 and MCP-1. Compared with the Normal group, these cytokines in colonic tissue were upregulated markedly in colonic tissues of mice with oral administration of DSS (figure3A, 3B, 3C and 3D). While secretion levels of these inflammatory cytokines were significantly suppressed in mice treated with EGCG (figure3A, 3B, 3C and 3D). Furthermore, we evaluated the expression levels of TNF-α and IL-1β using western blot assays. The levels of TNF-α and IL-1β was significantly reduced by EGCG treat compare with those of the DSS treatment group (figure 3E, 3F and 3G). Taken together, these data indicated that EGCG could inhibit the overwhelming inflammatory response in colonic tissue of mice with DSS-induced colitis.

Macrophage phenotype could change by administration of EGCG in colitis mice

In our previous study, the macrophage phenotype could be changed with EGCG treat[17]. To further understand the potential mechanism by which EGCG therapy mitigate intestinal inflammation in this study, we analyzed the macrophages accumulation and the polarization of M1 macrophage by IHC and western blot analyses. The results showed that the levels of F4/80, which is marker of macrophages, were increased by DSS treat compare with normal group. And EGCG treatment could attenuate the macrophages accumulated (figure 4A). More importantly, the nitric oxide synthase (iNOS, M1 specific mark) were down-regulated with EGCG treat (figure 4A). The protein of iNOS expression was also decreased by EGCG treatment compared with mice in the DSS group (figure 4B and 4C). These results show that EGCG resolve inflammation of the colons partly because of reduced macrophages, especially the type of M1 macrophages.

EGCG attenuate the expression levels of Notch in colitis mice

It have been confirmed that Notch is a new target of EGCG[17]. In order to further understand the mechanism of EGCG improved the colitis, the expression levels of protein which is belong to Notch signaling pathway were detect by WB in colon from colitis mice. The expression levels of Notch1 and Notch2 were increase in colitis mice colon compared with normal group (figure5A, 5B and 5C). And the phenomenon is alleviated by EGCG treatment (figure5A, 5B and 5C). The Notch1 was activated in DSS group and deactivated with EGCG (figure5A and 5D). The expression levels of Notch downstream target protein HES-1 were also decreased in EGCG group compared with DSS group. The same results were observed in the colon stained by IHC of Notch1 and HES-1 (figure 5F). These results indicated that EGCG attenuates colitis in mice through the Notch pathway.

Discussion

UC is one of the main types of IBD which is affect the bowel[5] and it could develop into colorectal cancer(CRC)[7]. EGCG is a major compound of tea[9] and it could attenuate colitis in UC mice[10]. However, the mechanism of EGCG in the treatment of colitis should be further investigated. A previous study reported that regulation Notch signaling pathway could enhance intestinal barrier function in colitis mice[4] and it could be a target of chronic inflammatory diseases, such as IBD[6]. In our previous studies, we reported that Notch is a receptor of EGCG[11,17]. In this study, we focused on the Notch signaling pathway which plays a role in the development of UC and aberrant activation of Notch1 was attributed to the severity of CRC[18]. Our results found that the inflammation state and Notch expression were altered by EGCG treatment in Caco-2 cell which induced by LPS. Furthermore, we also identified the mechanism is present in UC mice, which is induced by DSS in C57/BL6 mice. These results suggested that EGCG could play a role to against colitis by regulation Notch signaling pathway in UC mice.

Previous studies have been reported that Notch could regulation inflammation[19,20] and modulates macrophages polarization[21]. Some studies also propose that targeting the Notch signaling pathway could be a potential therapeutic strategy to improve chronic inflammatory diseases[6,22,23]. A new research reported that the transcription levels of Notch1 and Hes-1 genes were significantly elevated in UC patients[22] which reminder us to reflect on important of Notch in UC. And recent studies have revealed the unexpected importance of epithelial cells in the pathophysiology of IBD[24]. So, in this study, we take human epithelial colorectal adenocarcinoma Caco-2 cell line as inflammation model[25] to assess the inflammatory responds with LPS treatment. Our research found that the inflammation responds was increased by LPS treatment in Caco-2 cell (figure 1A and 1B) or enhance intestinal immune response in DSS induced UC mice colon (figure3 and figure 4). And the levels of Notch1 and cleaved-Notch1 expression were also increase by LPS treatment in Caco-2 cell (figure 1D) or DSS induced UC mice colon. These results was similar to the previous study report[22,26,27]. And our finding also showed that Notch could be a target to therapy hyper-inflammatory respond and UC. Moreover, this research also proved that Notch activate could contribute to M1 macrophages accumulation. These founds suggest that activate Notch play a role to induced UC as other studies report[7,8,22].

The effect of EGCG on perfect IBD has been reported in previous studies[28,29]. And our previous studies have been demonstration that Notch was a target of EGCG[11,30] to attenuate inflammation responds or other chronic disease[17]. So, we think the Notch maybe was a target of EGCG to attenuate UC. In this study, we found that EGCG could decreased the cytokine level which induced by LPS in Caco-2 cell (figure 1A and 1B). The levels of Notch expression were increased and Notch was activation by LPS stimulation in cell, but, the phenomenon was disappeared by administration EGCG in vitro (figure 1D). Moreover, the inflammation state of colon and macrophages accumulation were also decreased by oral EGCG (figure 3 and figure 4). To further understand the mechanism of EGCG on attenuate colitis, we detected Notch signaling protein and found that the level of Notch expression, Notch activation, and downstream protein were also significance decreased by administration EGCG (figure 5). Our results revealed the effect of EGCG on UC and expound the mechanism.

In conclusion, our study provides direct evidence that EGCG reduces inflammatory response by targeting Notch and reveals the mechanism by which EGCG alleviates colitis in UC mice (Figure 6). EGCG suppressing inflammation may be a promising treatment option for chronic inflammatory diseases in patients with IBD.

Author contributions

Q.Q.Z., J. S. and X. J. W. designed the study. X.Y.Y., J.F.L., Y.Z.W, M.M.L. and K.C. performed the experiments. X.Y.Y. and Q.Q.Z.wrote the manuscript. X. J. W. supervised the overall study. J. S. is the guarantor of this work; as such, Q.Q.Z.had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the analysis.

Ethics guidelines

Reporting of animal data in this study followed the recommendations set out in the ARRIVE guidelines.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Conflicts of interest

The authors have no conflict of interest to disclose.

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Figure Legends

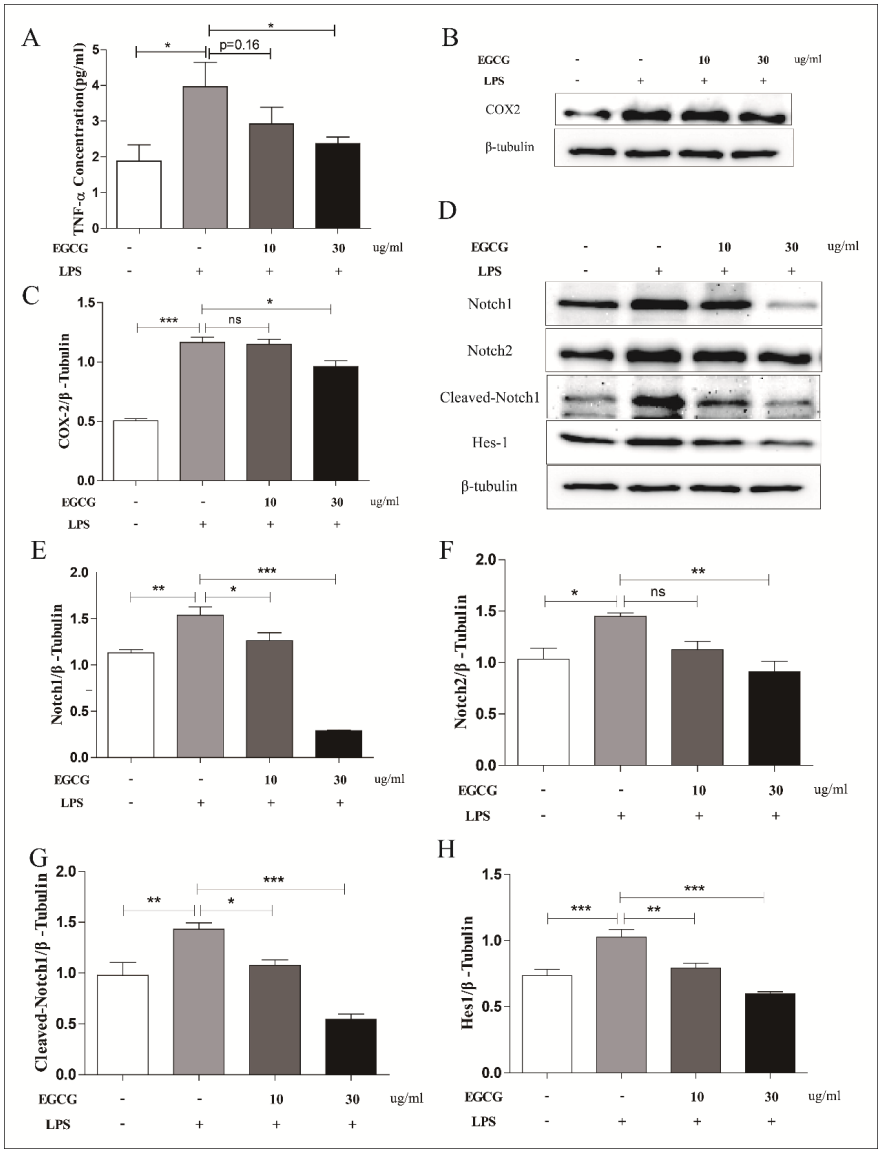

Figure 1 EGCG could attenuate inflammatory responses by targeting the Notch pathway in Caco-2 cells. The supernatant and samples were obtained from the Caco-2 cell line after LPS stimulation with or without EGCG treatment for 24 h. To measure the pro-inflammatory marker TNF-α, the supernatant was treated as described earlier using ELISA. 60 μg of each sample was applied to each well for detection of inflammation protein and Notch signaling pathway protein. (**A**) TNF-α was detected to demonstrate whether EGCG attenuates inflammatory response in Caco-2 cells. (**B, C**) The protein expression of COX2 was measured by western blotting, and the ODs were determined. (**D**) The protein levels of Notch1,

Notch2, Cleaved-Notch1, and Hes-1 were detected by western blotting, and the ODs were calculated to demonstrate the effect of EGCG on the Notch signaling pathway in the Caco-2 cell. Data are pooled from two experiments. Data are presented as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA). EGCG, (-)-epigallocatechin gallate; LPS, Lipopolysaccharide.

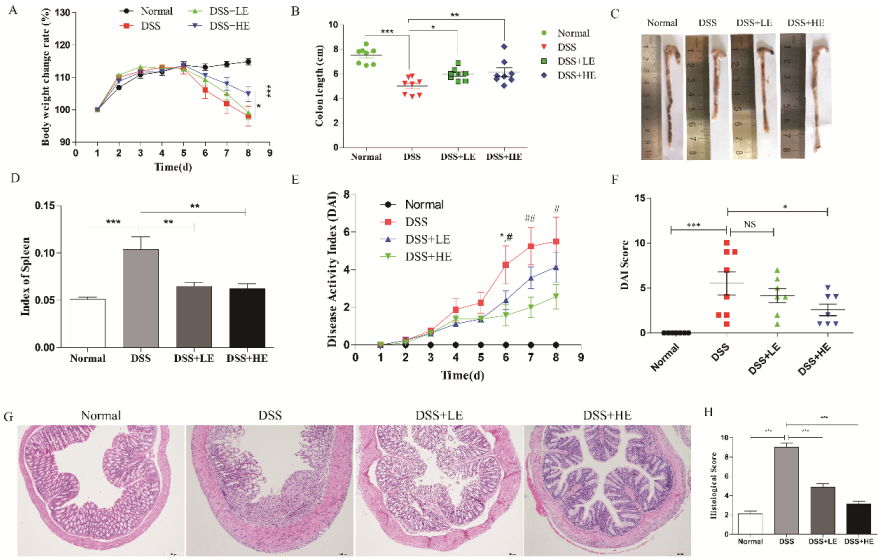

Figure 2 Colitis can be improved by the administration of EGCG in mice. Except for the normal group, mice in other groups were given DSS (3% w/v) for 8 days and the mice were treated with or without EGCG, respectively. EGCG promotes recovery of DSS-induced colitis in mice. (A) Body weight change rate (the change rate of body weight in each group of mice was compared with the body weight in the same group of mice on the 1st day). (B, C) Length of the colon (from the appendix to the anus) were detected on day8. (D)The index of the spleen; (E) Disease activity index; (F) The DAI score on day8 between groups; (G) Histopathological images showing paraffin-embedded sections stained with H&E in the colon; (H)Histopathologic scoring of the inflammation, depth of inflammation and crypt damage. Scale bars, 200 μm. Data are presented as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA). LE, Low dose of (-)-epigallocatechin gallate; HE, High dose of (-)-epigallocatechin gallate; DSS, Dextran sulfate sodium.

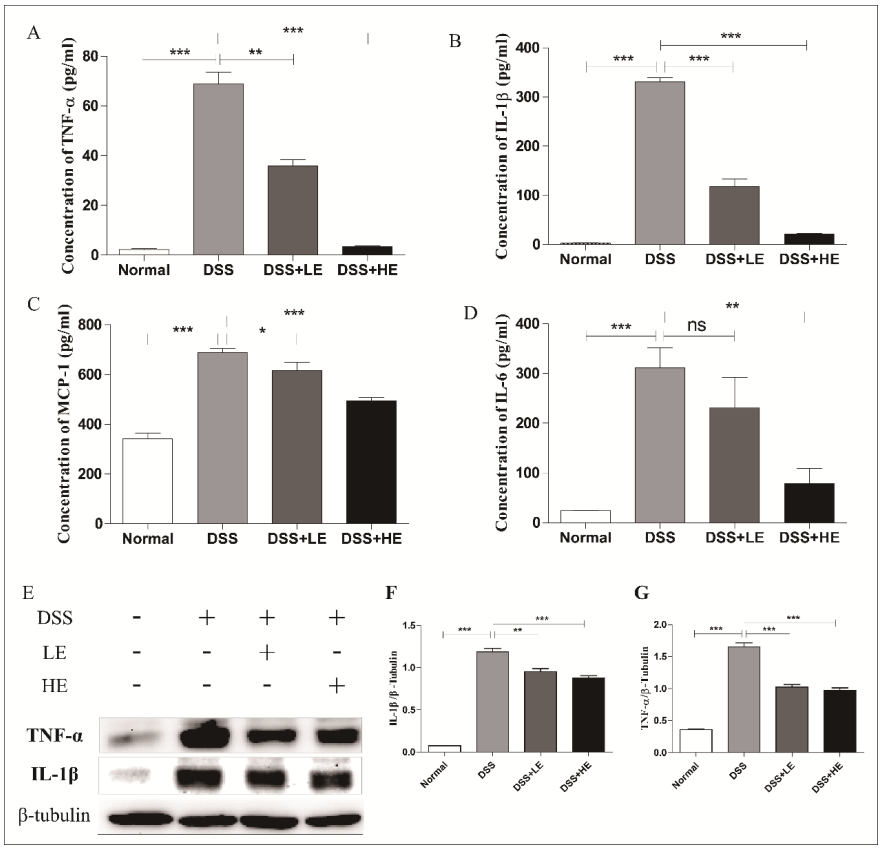

Figure 3 The level of cytokine secretion and protein expression can be inhibited by EGCG in colon of the UC mice. Colon in UC mice was treated with EGCG or placebo and harvested at day 8 post DSS induced. To measure the pro-inflammatory marker TNF-α (A), IL-1β (B), MCP-1 (C), and IL-6 (D), tissues were treated as described earlier using ELISA. The tissue was obtained from the colon of mice was homogenized in protein extraction solution and 60 μg for each well for detected. The protein expressions of TNF-α and IL-1β (E) were measured by western blotting. The value of OD (F, G) was detected by Image J (NIH). For all graphs, bars = mean ± SEM, n = 5. Statistical analysis was performed using one-way ANOVA. ***Mean value was significantly different for the EGCG and normal group at the same time point compared with the model group. LE, Low dose of (-)-epigallocatechin gallate; HE, High dose of (-)-epigallocatechin gallate; DSS, Dextran sulfate sodium.

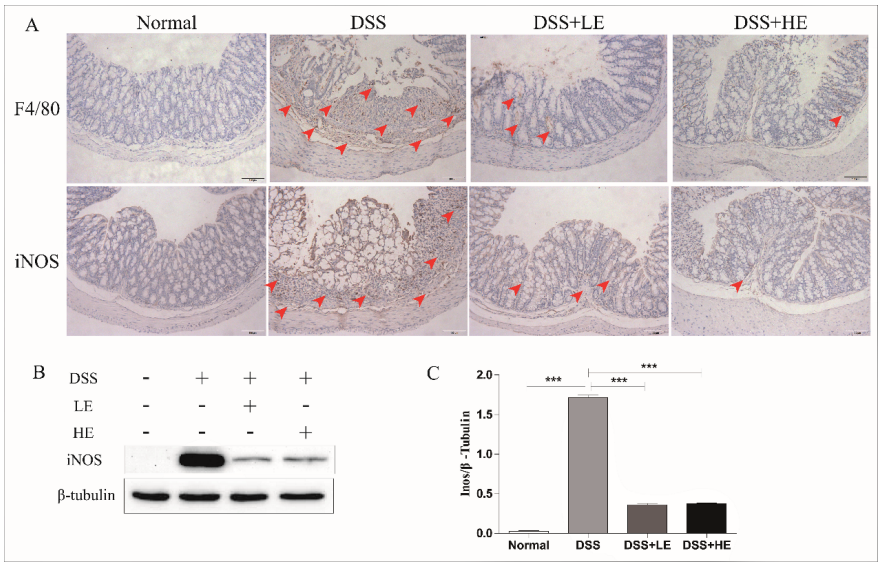

Figure 4 Macrophages were decreased by treat with EGCG in colon of the UC mice. Immunohistochemisty was performed on paraffin sections stained for F4/80 and

iNOS(A). The Brown area (red arrows) indicates positive staining. The tissue was obtained from the colon of mice was homogenized in protein extraction solution and 60 μg for each well for detected. The protein expressions of iNOS (B) were measured by western blotting. The value of OD (C) was detected by Image J (NIH). Data are pooled from two experiments (n=6 colon from six mice per group, repeated twice). Data are presented as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA, EGCG, and normal group vs. DSS group). Scale bars, 100 μm. LE, Low dose of (-)-epigallocatechin gallate; HE, High dose of (-)-epigallocatechin gallate; DSS, Dextran sulfate sodium.

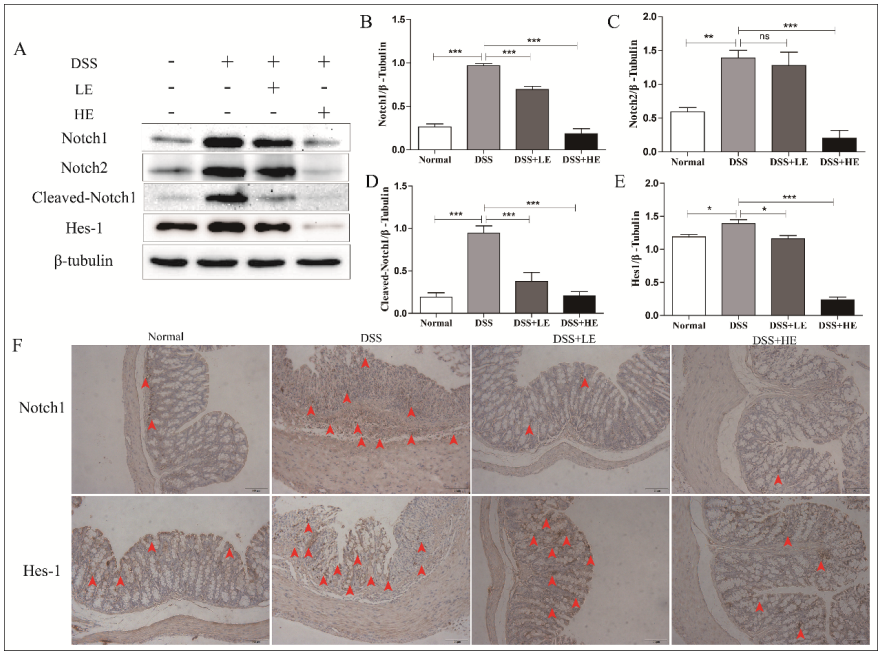

Figure 5 The Notch signaling pathway was weakened by treatment with EGCG in colon of the UC mice. The tissue obtained from the colon of mice was homogenized in a protein extraction solution; 60 μg of each sample was used for detection. (**A**) The expression levels of Notch1, Notch2, Cleaved-Notch1, and Hes-1 were measured by western blotting, and the ODs (B, C, D, E) were detected. (**F**) IHC was performed on paraffin-embedded sections stained for Notch1 and Hes-1: brown area (red arrows) indicates positive staining. Data are pooled from two experiments (n=6 colon from six mice per group, repeated twice). Data are presented as the means ± SEM. *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA, EGCG, and normal group vs. DSS group). Scale bars, 100 μm. LE, Low dose of (-)-epigallocatechin gallate; HE, High dose of (-)-epigallocatechin gallate; DSS, Dextran sulfate sodium.

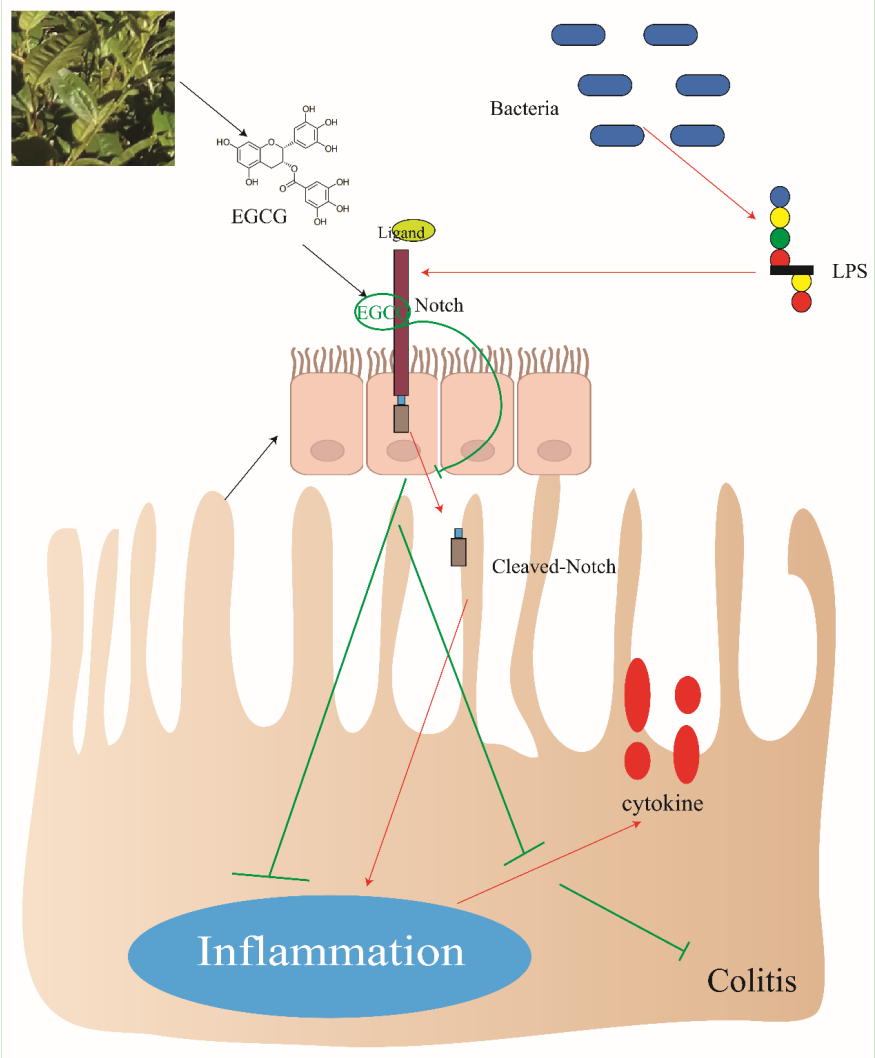

Figure 6 Colitis can be Improved by EGCG through Targeting Notch in mice