Cinnamtannin D1 ameliorates DSS-induced colitis by preventing Th17/Treg imbalance through activation of the AMPK/mTOR pathway

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**Abstract**

**Background:** Inflammatory bowel disease (IBD) is a chronic idiopathic gastrointestinal disease, including ulcerative colitis (UC) and Crohn’s disease (CD), which is typically characterized by chronicity and relapse. Cinnamtannin D1 (CTD1), extracted from *Cinnamomum tamala*, has been found to exert good immunosuppressive activity. However, the role of CTD1 in IBD is unclear.

**Methods:** The colitis mice model was established by dextran sulfate sodium (DSS) treatment. Protein levels (p-STAT3/STAT3, ROR-\(\gamma\)t, p-STAT5/STAT5, FOXP3, p-AMPK/AMPK, and p-mTOR/mTOR) were examined using Western blotting analysis. Changes in histopathology were detected through hematoxylin and eosin staining. The proportion of T helper 17 (Th17) cells and regulatory T (Treg) cells was measured by flow cytometry analysis.

**Results:** CTD1 improved body weight and colon length, and alleviated inflammation and histological damage in DSS-induced colitis mice model. DSS treatment also demonstrated a negative effect on Th17-Treg cells balance whereas CTD1 restored the balance of Th17-Treg cells in DSS-induced colitis mice model. Regulatory factors (such as STAT3, ROR-\(\gamma\)t, STAT5, and FOXP3) that closely related to the balance of Th17-Treg cells were regulated by CTD1. In addition, AMPK phosphorylation was increased and mTOR phosphorylation was inhibited by CTD1 in DSS-induced colitis mice model.

**Conclusion:** These findings established that CTD1 improved DSS-induced colitis by suppressing Th17-Treg cells balance by activating the AMPK/mTOR pathway. This study provided a new strategy for developing novel treatments for patients with IBD.

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Introduction

Inflammatory bowel disease (IBD) is a group of inflammatory disorders characterized by relapsing and remitting gastrointestinal (GI) tract mucosal inflammation, and is divided into ulcerative colitis (UC) and Crohn’s disease (CD).1 The incidence of IBD is increasing globally, accompanied by about 0.5% of the total population of developed countries, including Europe and the United States.2 An abnormal activation of the mucosal immune response is a major risk factor for developing IBD.1 Chronic intestinal inflammation contributes to GI cancer in patients with IBD.3 Therefore, IBD interventions can play a role in mitigating progression of disease and preventing the occurrence of GI cancer. Currently, various protocols have been applied for treating IBD, such as immunomodulators, and thiopurine agents; however, the therapeutic outcomes require further improvement.

*Cinnamomum tamala,* an endemic species, has been used as a food spice and herbal medicine in China for thousands of years. It is widely distributed in Taiwan in central mountains at a height of 800–2000 m.5-7 A previous study has established that extracts of *Cinnamomum tamala* played a hypoglycemic role in type 2 diabetic Lep db/Lep db mice model.8 Moreover, the species was found to have a spasmylytic potential in mice,9 suggesting that it can be involved in ameliorating the disease. Subsequently, Cinnamonnin D1 (CTD1), a dietary polyphenol compound extracted from *Cinnamomum tamala,* was found to exhibit good immunosuppressive activity. CTD1 could suppress concanavalin A (ConA)-induced lymphocyte proliferation and inhibit 2,4-dinitrofluorobenzene-induced delayed-type hypersensitivity response through down-regulation of excessive T-cell activation.10 CTD1 demonstrated anti-apoptotic effect on palmitic acid-stressed B-cells by suppressing oxidative stress.11 However, the role of CTD1 in IBD is unclear.

Effectors CD4+ T helper (Th) cells and regulatory CD4+ regulatory T (Treg) cells play a fundamental role in IBD, and participate in regulating pro-/anti-inflammatory cytokine production in the gut.12 The present study investigated the mechanism underlying the effects of CTD1 on Th cells and Treg cells in mice with dextran sulfate sodium (DSS)-induced colitis.

Materials and Methods

Animals

Male C57BL/6 mice (6-7-week old) were purchased from Better Biotechnology Co. Ltd. (Nanjing, China) and divided in the following five groups (n = 6/group): (A) control; (B) DSS; (C) DSS + CTD1 (25 mg/kg); (D) DSS + CTD1 (50 mg/kg); and (E) DSS + CTD1 (100 mg/kg). The DSS-induced mouse model was established as described previously.13 Briefly, the colitis was induced by treating with 3% DSS for 7 days, and mice were administrated with CTD1 (25, 50, or 100 mg/kg). Mice in the control group were given distilled water. The animals were observed for body weight every 2 days, and the colon length was examined. The experiment protocols were approved by the Guide for the Care and Use of Laboratory Animals and the Second Affiliated Hospital of Chongqing Medical University (Approval No. (2020) 558).

Chemicals

The molecular weight of CTD1 (purity >95%), extracted from the bark of *C. tamala,* was 864 Da, and was provided by the Second Affiliated Hospital of Chongqing Medical University.

Assessment of disease activity index (DAI)

The DAI assessment was done as described previously.14 Briefly, body weight of mice was determined, and diarrhea and rectal bleeding were visually inspected to determine DAI. The DAI score was assessed as follows: weight loss, 0–4 (score 0, no loss; score 1, 1–5% loss; score 2, 6–10% loss; score 3, 11-20% loss; and score 4, >20% weight loss); stool bleeding, 0–4 (score 0, no blood; score 2, presence; score 4, gross blood); and stool consistency, 0–4 (score 0, normal; score 2, loose stool; score 4, diarrhea).

Enzyme-linked immunosorbent serological assay (ELISA)

Colon tissues of mice were collected and homogenized for 1 min in a buffer containing protease inhibitors. The supernatant was obtained after centrifuging at 10,000 g for 15 min. Levels of tumor necrosis factor-α (TNF-α), Interleukin (IL)-18, and IL-6 in supernatants were measured using corresponding ELISA kits (NJJC, Nanjing, China).

Myeloperoxidase (MPO) activity

Colon tissues from more or less the same site were weighed and homogenized using ice-cold phosphate-buffered saline (PBS) solution. MPO activity analysis was carried out according to manufacturer’s instructions. The O-dianisidine method was used to detect MPO activity, reported as units per gram of wet tissue.

Histological analysis

Samples of colon tissue were obtained and fixed in 4% paraformaldehyde. The samples were embedded in paraffin and sliced in 4-μm thickness. Subsequently, hematoxylin and eosin (HE) was used for staining of sections. The images were captured using a light microscope with a magnification of 200×. A previously established scoring system was used for evaluating histopathological changes15: 0-5, inflammatory infiltration; 0-4, crypt injury; 0-3, ulcer; 0, presence of edema; and 1, absence of edema. Histological scoring was carried out by two investigators blinded to investigations.

Flow cytometry analysis

Mesenteric lymph nodes (MLNs) were harvested from mice and a single cell suspension (1 × 10⁶ cells/mL) was prepared in straining buffer. Cells were incubated with CD4-FITC (BD
Biosciences, New York, USA) for 30 min for Th17 cells. After fixation and permeabilization, cells were subsequently covered with IL-17A-APC antibody for 1 h. For Treg cells, cells were covered with CD4-FITC and CD25-PE for 30 min followed by fixation and permeabilization and exposed to Foxp3-APC antibody for 1 h. Finally, cells were measured using flow cytometry and analyzed by the FlowJo 10 software (TreeStar, Ashland, OR, USA).

**Western blotting analysis**

Cells from mesenteric lymph nodes were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Takara, Dalian, China), and total protein was extracted. Equal amount of protein (30-40 µg) was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skimmed milk for 40 min, membranes were incubated overnight at 4°C with primary antibodies. Then membranes were covered with secondary antibodies for 1 h at room temperature. Bands were observed using enhanced electrochemiluminescence (ECL) substrate (Beyotime, Shanghai, China). Primary antibodies included phospho-signal transducer and activator of transcription 3 (p-STAT3; 1:1000), STAT3 (1:1000), retinoid-related orphan receptor gamma t (ROR-γt; 1:1000), p-STAT5, STAT5, forkhead box P3 (FOXP3; 1:1000), phospho-AMP-activated protein kinase (p-AMPK; 1:2000), AMPK (1:3000), phospho-mammalian target of rapamycin (p-mTOR; 1:1000), mTOR (1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000) (all obtained from Abcam, Cambridge, MA, USA).

**Statistical analysis**

GraphPad Prism software 5.0 was used for statistical analysis. Data were expressed as mean ± standard deviation (SD). Multiple comparison testing was conducted with one-way Analysis of Variance (ANOVA); P < 0.05 was considered statistically significant.

**Results**

**CTD1 alleviated inflammation and histological damage in DSS-induced colitis mice model**

The effect of CTD1 on DSS-induced inflammation and damage in mice was measured. ELISA identified that TNF-α, IL-1β, and IL-6 levels were elevated by DSS. However, DSS-induced TNF-α, IL-1β, and IL-6 levels were suppressed by CTD1 treatment in a dose-dependent manner (Figure 2A). Moreover, DSS treatment up-regulated MPO whereas CTD1 down-regulated DSS-induced MPO (Figure 2B). HE staining assay revealed that DSS enhanced histological score whereas CTD1 reduced DSS-induced histological score (Figure 2C). These results suggested that CTD1 alleviated DSS-induced inflammation and histological damage in mice.

**CTD1 restored Th17/Treg balance in DSS-induced colitis mice model**

The subsequent experiments evaluated whether CTD1 can affect Th17/Treg balance in DSS-induced colitis mice model. Cells from mesenteric lymph nodes were collected and subjected to flow cytometry analysis. The results revealed that Th17 (CD4+IL17+) cells in mesenteric lymph nodes were increased in the DSS group (0.48%) compared to the control group (2.88%) whereas CTD1 inhibited DSS-induced Th17 (CD4+IL17+) cells in a dose-dependent manner compared to the DSS group (Figure 3). Furthermore, CD4+CD25+FOXP3+ T cells in mesenteric lymph nodes were reduced in the DSS group (1.16%) compared to the control group (4.47%). However, CTD1 alleviated results in the DSS group (Figure 3). Along with the data of gating strategies...
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Figure 2  CTD1 alleviated inflammation and histological damage in DSS-induced colitis in mice. (A) TNF-α, IL-1β, and IL-6 levels were detected using ELISA. (B) Myeloperoxidase was measured using an MPO assay kit. (C) Histological change was examined using hematoxylin and eosin staining, and score calculated. Scale bar: 200 μm; n = 6. ***P < 0.001; ###P < 0.001. *vs. control. #vs. DSS.

Figure 3  CTD1 restored Th17-Treg cells balance in DSS-induced colitis mice model. Th17 (CD4+IL17+) cells and CD4+CD25+FOXP3+ T cells from mesenteric lymph nodes were subjected to flow cytometry analysis; n = 6. ***P < 0.001; ###P < 0.001. *vs. control. #vs. DSS.
(Supplemental Figures S1), these findings implied that CTD1 restores DSS-induced Th17/Treg balance in mice.

**CTD1 regulated Th17/Treg balance-associated transcription factors**

The expression levels of transcription factors required for Th17/Treg balance were examined in DSS-induced mesenteric lymph nodes of mice. As shown in Figure 4, Western blotting analysis demonstrated that p-STAT3/STAT3 (2.59-fold) and ROR-γt (17.75-fold) protein levels were enhanced by DSS treatment compared to the control group whereas these protein levels were reduced by CTD1 in a dose-dependent manner. Moreover, DSS treatment suppressed p-STAT5/STAT5 (5.51-fold) and FOXP3 (6.22-fold) protein levels compared to the control group, but CTD1 increased these protein levels. These results indicated that CTD1 regulated the expression levels of Th17/Treg balance-associated transcription factors.

**CTD1 restored Th17/Treg balance by AMPK pathway**

The present study explored the involvement of CTD1 in Th17/Treg balance factors. Western blotting assay verified that DSS treatment decreased the p-AMPK/AMPK protein level (7.7-fold) compared to the control group, but CTD1 increased the DSS-reduced p-AMPK/AMPK protein levels in a dose-dependent manner. However, the p-mTOR/mTOR protein level (21.29-fold) was elevated by DSS compared to the control group, but the DSS-induced p-mTOR/mTOR protein level was suppressed by CTD1 (Figure 5A). Moreover, flow cytometry analysis proved that CTD1 inhibited DSS-induced Th17 (CD4+IL17+) cells but increased the reduced levels of Th17 and Treg cells.
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CD4+CD25+FOXP3+ T cells caused by DSS. However, compound C, an AMPK inhibitor, reversed the effects of CTD1 on Th17 (CD4+IL17+) cells and CD4+CD25+FOXP3+ T cells (Figure 5B). Along with the data of gating strategies these data implied that CTD1 restores Th17/Treg balance by AMPK pathway.

Discussion

The present study was performed in the colitis mice model established through DSS treatment. As summarized in Figure 6, CTD1 fostered DSS-mediated phosphorylation of AMPK and mTOR, and thus suppressed the DSS-induced levels of TNF-α, IL-1β, IL-6, and MPO. Subsequently, CTD1 was involved in restoring Th17/Treg balance. CTD1 decreased DSS-induced Th17 (CD4+IL17+) cells, and supressed the phosphorylation of STAT3 and reduced ROR-γt protein level. Conversely, CTD1 increased the reduced CD4+CD25+FOXP3+ T cells, phosphorylation of STAT5, and FOXP3 protein level caused by DSS.

Increasing evidences revealed that the enhancement of inflammatory cytokine levels in serum indicated a reasonable target for IBD therapy.16 The DSS colitis model presented the elevation of MCP-1, TNF-α, IL-6, IL-1β, and IFN-γ mRNA levels in the colon.16,17 Interestingly, the present study demonstrated that CTD1 improved DSS-induced inflammation, survival rate, and histological damage in mice, as evidenced by reduction in DSS-induced DAI score, down-regulation of DSS-induced TNF-α, IL-1β, and IL-6 levels, and reduction of DSS-induced histological score. Additionally, CTD1 was found to suppress inflammation-related and endoplasmic reticulum (ER) stress-related proteins in high levels of glucose and palmitic acid (PA)-stressed INS-1 cells,18 suggesting that CTD1 increased inflammation in DSS-induced colitis.

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Figure 5  CTD1 restored Th17-Treg cells balance by AMPK pathway. (A) Western blotting analysis was used to analyze p-AMPK, AMPK, p-mTOR, mTOR, and GAPDH protein levels. (B) Flow cytometry analysis was used for measuring Th17 (CD4+IL17+) cells and CD4+CD25+FOXP3+ T cells; n = 6. ***P < 0.001; **P < 0.01; ###P < 0.001. *vs. control or DSS. #vs. DSS or DSS+CTD1 (100 mg/kg).
Imbalance between Th17 and Treg cells was demonstrated in IBD. Correcting the imbalance between Th17 and Treg cells contributed to the prevention and treatment of IBD. Th17 cells could aggravate intestinal inflammatory response via pro-inflammatory cytokines. Treg cells demonstrated a fundamental role in maintaining immune tolerance and balance, and contributed to the pathogenesis of IBD in intestinal microenvironment. In the present study, CTD1 could decrease Th17 (CD4+IL17+) cells and increase CD4+CD25+FOXP3+ T cells in mesenteric lymph nodes, restoring DSS-induced Th17–Treg cells balance in mice model. These data implied that CTD1 could contribute to the regulation and maintenance of Th17–Treg cells balance. Previous studies have established that some regulatory factors, such as STAT3, ROR-γt, STAT5, and FOXP3, were involved in the regulation of Th17–Treg balance. Activated STAT3 contributed to the differentiation of Th17 cells via up-regulation of ROR-γt. Moreover, activated STAT5 was found to promote differentiation of Treg cells by up-regulating FOXP3. Importantly, mTOR signaling has been reported to increase STAT3 but inhibit STAT5. The present study demonstrated that these regulatory factors (STAT3, ROR-γt, STAT5, and FOXP3) were regulated by CTD1. Moreover, CTD1 increased p-AMPK/AMPK protein levels and down-regulated p-mTOR/mTOR protein levels in DSS-induced colitis mice model. Additionally, CTD1 suppressed DSS-induced Th17 (CD4+IL17+) cells but elevated the decreased CD4+CD25+FOXP3+ T cells caused by DSS. However, these effects were reversed by compound C. These results indicated that CTD1 played a role in Th17–Treg cells balance and regulated Th17–Treg cells balance through AMPK/mTOR pathway. However, the mechanism underlying the functional role of CTD1 in IBD needs to be further explored. Moreover, the effects of CTD1 on clinical samples are not evaluated, and require more experimental exploration in the near future.

**Conclusion**

In brief, the present research demonstrated that CTD1 could alleviate inflammatory response and histological damage in DSS-induced colitis mice model. Moreover, CTD1 had an important role in inhibiting imbalance between Th17 and Treg cells in DSS-induced colitis mice model through AMPK pathway. These data implied that CTD1 could provide a new therapeutic strategy for patients with IBD.

**Availability of Data and Materials**

All data generated or analyzed in this study are included in this published paper.

**Competing interests**

The authors stated that there were no conflicts of interest to disclose.
Author Contribution

Ying Xue Yang designed the study, and supervised data collection. Yuan Yuan analyzed and interpreted the data. Boning Xia prepared and reviewed draft of the manuscript for publication. All authors read and approved the final manuscript.

References

Supplementary

Figure S1  The gating strategies were provided in flow cytometry analysis.