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Novel immunomodulatory properties of cirsilineol through selective inhibition of IFN-γ signaling in a murine model of inflammatory bowel disease☆

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ABSTRACT

Regulation of signal transducer and activator of transcription (STAT) 1 signaling is being explored as a new approach to the treatment of inflammatory bowel diseases. However, few chemicals have been reported to inhibit IFN-γ/STAT1 signaling for Crohn's disease therapy. In the present study, we found that cirsilineol, a small natural compound isolated from *Artemisia vestita*, significantly ameliorated trinitro-benzene sulfonic acid (TNBS)-induced T-cell-mediated experimental colitis in mice, which was closely associated with reduced autoreactive T-cell proliferation and activation. Moreover, the regulatory action of pro-inflammatory and anti-inflammatory cytokine by cirsilineol treatment was found to decrease the activity of effector Th1 cells but increase the activity of regulatory T cells as characterized by down-regulation of IFN-γ and corresponding up-regulation of IL-10 and TGF-β. The therapeutic effect of cirsilineol was attributable to a novel regulatory mechanism with selective inhibiting IFN-γ signaling in colonic lamina propria $CD4^+$ T cells, which was mediated through down-regulating STAT1 activation and T-bet expression. Furthermore, cirsilineol was found to down-regulate the activation of JAK2, a critical kinase for IFN-γ/STAT1 signaling, and abrogate the expression of T-bet, resulting in markedly decreased proliferation and activation of T cells in vitro. Importantly, the inhibition of IFN-γ/STAT1 signaling by cirsilineol was reversible in the presence of high level of IFN-γ. These results strongly suggest that cirsilineol might be potentially useful for treating T-cell-mediated human inflammatory bowel diseases.

1. Introduction

Both excessive autoimmune T-cell responses and dysfunction of the homeostasis of immune system play a critical role in the pathogenesis of human autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease and lupus nephritis [1–4]. There is increasing evidence that these autoimmune diseases are characteristically associated with the over-production and activity of pro-inflammatory cytokines by autoreactive T cells [5–7]. Pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-17 play important roles in inflammatory bowel disease especially Crohn's disease, while anti-inflammatory cytokines, such as IL-4, IL-10 and TGF-β modulate self-inflicted injury by suppressing and counteracting the effect of pro-inflammatory cytokines [6,8–12]. To the cytokines-involved signaling, the family proteins named as signal transducers and activators of transcription (STAT) are intracellular effector molecules [13], which play an important role in the development of the immune system such as T-cell polarization as well as in the regulation of T-cell survival and function. For example, IFN-γ/STAT1/T-bet pathway has been identified to be critical for the development of chronic intestinal inflammation, in which tissue damage was significantly attenuated in STAT1-null mice compared with that in wild-type control mice in the dextran sulfate sodium-induced colon inflammation [14], and T-bet-deficient T cells failed to induce colitis in adoptive transfer experiments in SCID mice [15]. Specific targeting of this pathway may be a promising novel approach for the treatment of patients with Crohn's disease and other autoimmune diseases mediated by Th1 lymphocytes.

Artemisia vestita is a traditional Chinese and Tibetan medicinal plant which has been widely used in China for treating various inflammatory diseases such as rheumatoid arthritis [16]. Previously, we demonstrated that the aqueous extract from aerial parts of *A. vestita* reduced contact sensitivity in mice through down-regulating the activation, adhesion and metalloproteinase production of T lymphocytes [17]. We also found that the ethanol

☆ This work was done in Nanjing University.

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extract from *A. vestita* exerted anti-sepsis action through down-regulating the MAPK and NF-κB pathways [18]. To explore the chemical basis of the herbal extract against the inflammations, a special attention was paid to active ingredients contained in *A. vestita*. Under the activity-guided isolation, a series of active ingredients with anti-inflammatory and immunosuppressive activities from *A. vestita* were separated and identified [19]. Among them, cirsilineol is a special natural small compound with potent immunosuppressive and anti-tumor properties [19,20]. The purpose of this study, thus, was to report a novel strategy for Crohn's disease therapy involving selective inhibition of IFN-γ/STAT1 signaling by means of this small molecule.

2. Materials and methods

2.1. Mice

Specific pathogen-free, 8–10-week-old female C57BL/6, BALB/c and DO11.10 transgenic mice (BALB/c background) were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, the United States) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Extraction and isolation

The aerial parts of *A. vestita* used in this study were purchased from Tibet pharmaceutical factory of Tibet university (Lhasa, China) and identified as *A. vestita* Wall. by Dr. Ciren Dunzhu (Tibet Tibetan Medicine College, Lhasa, China). The dried aerial part of the *A. vestita* (3 kg) was extracted with 75% ethanol, and then was applied on a macroporous adsorption resin HP-20 (250–850 μm; Mitsubishi Chemical, Japan) and eluted following the procedure: water, 30% ethanol, 60% ethanol and 90% ethanol to yield four fractions and evaporate them to dryness under reduced pressure to afford AV1–AV4 (AV1: 30.5 g, AV2: 60.6 g, AV3: 90.6 g, AV4: 33.1 g). Then AV3 was subjected on a silica gel column eluted with CH_2Cl_2–MeOH (100: 0–30:70) step gradient to give 11 fractions (AV3-1 to AV3-11). AV3-2 was rechromatographed over silica gel (100–200 mesh; Qingdao Oceanic Chemical Plant, China) eluted with CH_2Cl_2–MeOH (49:1) to yield J-03 (cirsilineol, purity 98% by HPLC). In the in vitro study, cirsilineol was dissolved at a concentration of 0.01 mol/L in 100% DMSO as a stock solution, stored at −20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% DMSO throughout the study (all the control groups are composed of 0.1% DMSO).

2.3. HPLC analysis and structural elucidation

HPLC analysis was applied on a Waters 600 series HPLC system consisting of a Waters 600 pump, a 2487 UV detector, an online degasser and a LC Work Station equipped with Empower™ software. J-03 was applied to YMC-pack Pro C18 column (5 μm, 150 mm × 4.6 mm, YMC Co., Ltd., Japan) and detected at 275 nm. Column temperature was set up at 25 °C and the flow rate was 1 mL/min. The gradient elution program (methanol:water) was 0 min, 40:60; 10 min, 55:45; 25 min, 65:35; and 26 min, 40:60, respectively. NMR and ES-MS were used for structure elucidation. The ^1H and ^{13}C NMR measurements were carried out in Bruker DPX-300 spectrometer operating at 300 and 100 MHz, respectively. ES-MS experiments were recorded on ABI Mariner ESI-TOF mass spectrometer.

2.4. Drugs and reagents

Mouse $CD4^+$ T cells from splenocytes or lamina propria mononuclear cells were purified using magnetic beads (Miltenyi Biotec, Auburn, CA) with more than 95% purity. The cells were incubated in RPMI 1640 medium supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Concanavalin A (Con A), trinitro-benzene-sulfonic acid (TNBS), 3-(4, 5-dimethyl-2-thiazyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), carboxy-fluorescein diacetate succinimidyl ester (CFSE), mitomycin C and dexamethasone (Dex) were purchased from Sigma Chemical Co. (St. Louis, MO). $OVA_{323–339}$ peptide was purchased from Genscript (Nanjing, China). Purified anti-mouse CD3 (145-2C11), purified anti-mouse CD28 (37.51), anti-STAT1 (pY701) Alexa Fluor-488 conjugate and anti-CD4 APC conjugate were purchased from BD PharMingen (San Diego, CA). Annexin V-FITC/PI kit was purchased from BD Biosciences (San Jose, CA). ELISA kits for interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), IL-4, IL-5, IL-10, IL-17 and transforming growth factor-β (TGF-β) were purchased from R&D Systems (Minneapolis, MN). Recombinant murine IFN-γ was purchased from Peprotech (Rocky Hill, NJ). PE-Cy5-anti-mouse CD4 mAb were purchased from eBioscience (San Diego, CA). FITC-anti-mouse CD69 mAb and FITC-anti-mouse CD25 were purchased from Biolegend (San Diego, CA). Anti-JAK2, anti-STAT1 and anti-phospho-STAT1 were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal anti-IFN-γ receptor subunit α antibody, protein A/G, anti-T-bet and anti-α Tubulin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Light Shift Chemiluminescent EMSA kit was purchased from Pierce (Rockford, IL). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.5. Single mixed lymphocyte reaction

Single mixed lymphocyte reaction was determined as previously described [17]. Briefly, splenocytes (4×10^5) from C57BL/6 mice, which had been treated with mitomycin C (500 μg/mL) for 1 h, were co-cultured with splenocytes (4×10^5) from BALB/c mice in the presence or absence of the various concentrations of cirsilineol at 37 °C in 5% CO_2 for 72 h. The proliferation of the lymphocytes from BALB/c mice was measured by MTT method. The OD_{540} was determined by an ELISA reader (Sunrise, Tecan, Austria).

2.6. Cell proliferation

Splenocytes were cultured in 96-well plates at a density of 4×10^5 cells/well in RPMI 1640 medium (0.2 mL) and stimulated with 5 μg/mL of Con A for 72 h at 37 °C in 5% CO_2/air. Then cell growth was evaluated with modified MTT assay. Cell proliferation was also assayed by incorporation of [methyl-^3H] thymidine (ICN Pharmaceuticals, Costa Mesa, CA) at 0.5 μCi/well during the last 8 h of incubation, and the uptake was measured as counts per minute (c.p.m.). In some cases, cell proliferation was also determined by CFSE assay as we previously reported [21].

2.7. TNBS-induced colitis in mice

Colitis was induced by intrarectal injection of TNBS as previously described [22]. Briefly, BALB/c mice were fasted for 24 h with free access to drinking water. They were anesthetized by sodium pentobarbital (50 mg/kg, i.p.). Next, 100 μL of a 10 mg TNBS in 2 mL of 50% ethanol solution was injected intrarectally through a flexible catheter of 3.5 cm length. To ensure distribution of the agent within the entire colon and cecum, mice were held in a vertical position for 30 s. Negative control mice were administered

by 50% ethanol solution using the same technique. In the drug treatment group, cirsilineol (3, 10, and 30 mg/kg) and dexamethasone (1 mg/kg) were injected intraperitoneally daily from day 1 post immunization onwards. The positive control animals with TNBS-induced colitis were given intraperitoneally the same solvent (1% methylcellulose in normal saline) instead of the drugs. In all protocol studies, mice were monitored for the appearance of diarrhea, loss of body weight, and overall mortality for eleven days after TNBS administration.

2.8. Macroscopic inflammation scores

Eleven days after TNBS administration, mice were sacrificed, and the colon was removed and carefully opened to score colonic damage macroscopically. Four parameters were taken into account: presence of adhesions, degree of colonic ulcerations, wall thickness, and degree of mucosal edema. Each parameter was given a score from 0 (normal) to 3 (severe) as previously described in the literature [22]. The total score ranged from a minimum of 0 to a maximum of 12. Grading was performed in a blinded fashion.

2.9. Microscopic inflammation scores

Three days after TNBS administration, mice were sacrificed and the colon was removed and fixed in 10% buffered formalin phosphate, embedded in paraffin, and cross sections of 5 μm were stained with hematoxylin and eosin. To grade inflammation we adapted the histological damage score as previously described in the literature [22]. Inflammatory infiltrate, 0–3; number of gut wall layers infiltrated, 0–3; loss of mucosal architecture, 0–3; and edema, 0 or 1. The total score ranged from a minimum of 0 to a maximum of 10. Grading was performed in a blinded fashion.

2.10. Preparation of lamina propria mononuclear cells isolated from colonic tissue

Colon lamina propria mononuclear cells (LPMC) were isolated as previously described [22]. Briefly, colon tissue was opened longitudinally, cut into 5-mm pieces, and incubated in 0.5 mM EDTA in calcium- and magnesium-free Hank's balanced salt solution for 20 min at 37 °C. This was repeated after thorough washing. Tissue was then incubated for 20 min at 37 °C in 20 mL RPMI 1640 containing 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, 5 μg/mL gentamycin, and 100 μg/mL streptomycin as well as 1 mg/mL type IV collagenase (Sigma). At the end of the incubation the tissue was subjected to further mechanical disruption using a 1-mL syringe. Then the LPMC were washed once and layered onto a 30: 70% gradient Percoll column (Sigma). Cells were spun at 2200 × *g* for 20 min to obtain the leukocyte-enriched population (LPMC) at the 30–70% interface. $CD4^+$ T cells from colon LPMC were subsequently isolated by magnetic beads as described by the manufacturer (Miltenyi Biotec, Auburn, CA).

2.11. Cytokine assay

Cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-17, IL-10, TGF-β) were determined using ELISA kits from R&D systems (Minneapolis, MN).

2.12. Intracellular staining and flow cytometric analysis

Freshly isolated colon lamina propria $CD4^+$ T cells were fixed with Fix Buffer I (BD PharMingen, San Diego, CA) and permeablized with Perm Buffer III (BD PharMingen) for 30 min on ice. After washing the cells twice with BD Pharmingen Stain Buffer, they were stained with anti-STAT1 (pY701) Alexa Fluor®-488 conjugate (BD PharMingen) and anti-CD4 APC conjugate (BD PharMingen). Then they were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

2.13. Western blot

Proteins were extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a horse radish peroxidase-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.14. Determination of STAT1 DNA binding by electrophoretic mobility shift assay (EMSA)

Nuclear extracts of the cells were subjected to EMSA assay. The double-stranded oligonucleotide probe containing a gamma-activated sequence (GAS) (5′ biotin-AGCCTGATTTCCCCGAAATGACGGC-3′; and 5′ biotin-GCCGTCATTTCGGGGAAATCAGGCT-3′) corresponding to the STAT1 consensus DNA-binding site, was obtained from Invitrogen (Carlsbad, CA). Reactions for nuclear protein–DNA binding were performed using the Light Shift Chemiluminescent EMSA kit. Briefly, five micrograms of nuclear protein was incubated in a 20 μL reaction volume at room temperature for 20 min and then loaded onto a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate-ethylenediaminetetraacetic acid buffer at 4 °C. Then, binding reactions were transferred to nylon membrane. After cross-link transferred DNA to membrane, the bands were detected by chemiluminesecence. Specificity of DNA–protein complex was confirmed by competition with a 100-fold excess of unlabeled GAS binding sequences (Invitrogen, Carlsbad, CA).

2.15. Statistical analysis

Data are presented as mean ± SEM. Analyses of the nonparametric data (body weights, macroscopic and microscopic inflammation score) were performed by Kruskall–Wallis tests followed by Mann–Whitney *U* post hoc with Bonferroni correction when appropriate. The parametric data (proliferation assay and ELISA results) were analyzed by one-way ANOVA with Student–Newman–Keuls (SNK) post hoc analysis. $P < 0.05$ were considered to be significant.

3. Results

3.1. Identification of cirsilineol

J-03 was subjected to HPLC analysis and structure determination. The purity of J-03 was confirmed to be 98% by HPLC (Fig. 1A). The structure of J-03 was identified as cirsilineol (Fig. 1A) by MS and NMR spectral analyses and compared with the reported data [23]. The chemical name of cirsilineol is 5-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6,7-dimethoxychromen-4-one. ES-MS: $[M+H]^+$ 345.1534. ^1H NMR (DMSO-*d*6, 500 Hz) δ (ppm): 3.73 (3H, s, C6–OCH_3), 3.90 (3H, s, C7–OCH_3), 3.94 (3H, s, C3′–OCH_3), 6.93 (1H, s, C3-H), 6.96 (1H, s, C8–H), 6.98 (1H, d, C5′–H), 7.60 (1H, d, C2′–H), 7.63 (1H, dd, C6′–H), 10.02 (1H, s, C4′–H), 12.95 (1H, s, C5–H).

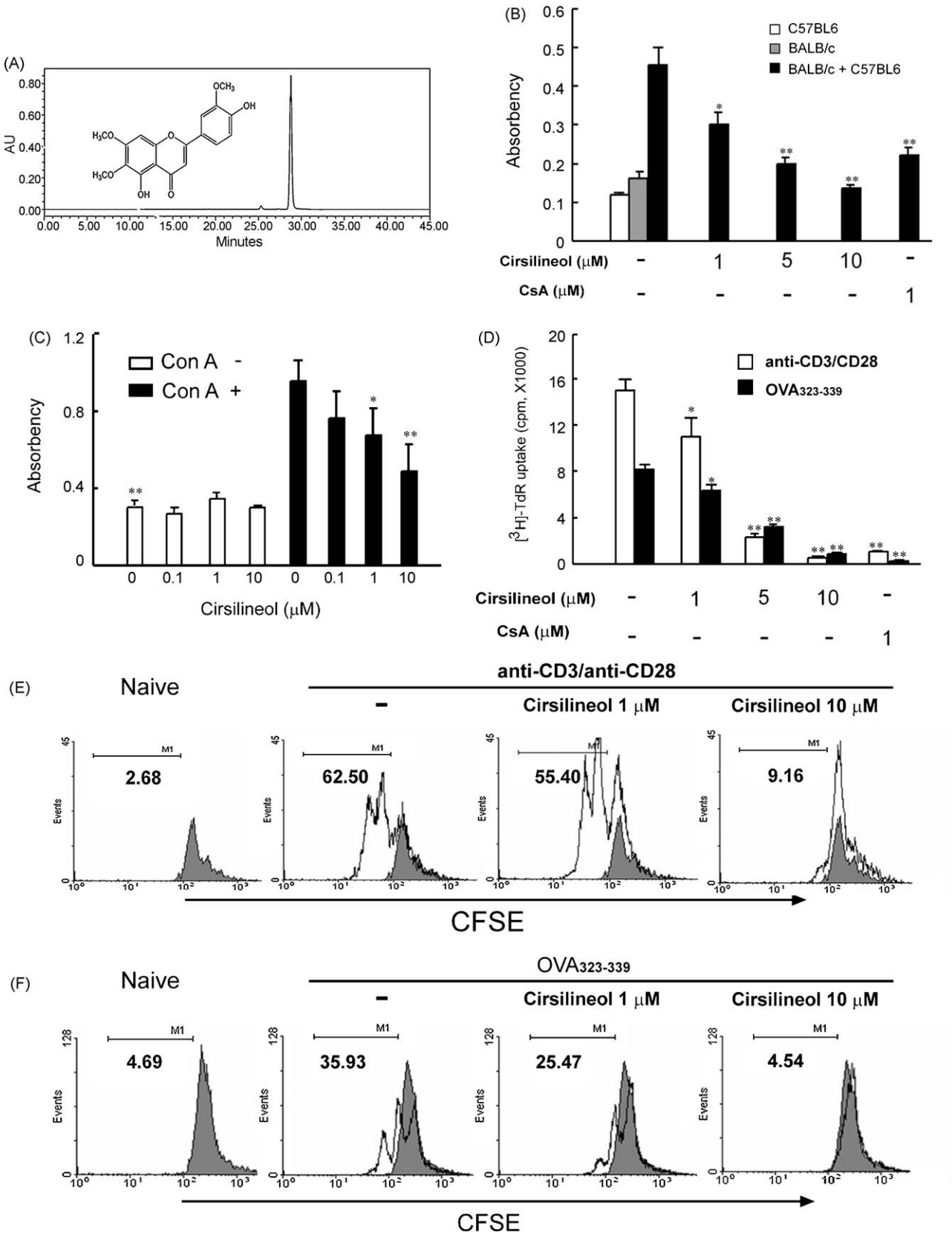

Fig. 1. Effects of cirsilineol on T-cell proliferation induced by multiple stimulators in vitro. (A) The chemical structure of cirsilineol and HPLC analysis of cirsilineol isolated from *Artemisia vestita* Wall. The purity of cirsilineol was confirmed to be more than 98%. (B) Splenocytes (4×10^5/well) from C57BL/6 mice, which had been treated with mitomycin C (500 μg/mL) for 1 h, were co-cultured with splenocytes (4×10^5/well) from BALB/c mice in the presence or absence of the various concentrations of cirsilineol at 37 °C in 5% CO_2 for 72 h. The proliferation was measured by MTT assay. $^*P < 0.05$, $^{**}P < 0.01$ vs control group. (C) BALB/c splenocytes (4×10^5/well) were stimulated with Con A (5 μg/mL) for 96 h, at increasing concentrations of cirsilineol in a 96-well plate in triplicate. For cell viability measurement, the resting splenocytes were cultured with cirsilineol at increasing concentrations for 48 h in triplicate. Cell number was counted by using MTT assay. $^*P < 0.05$, $^{**}P < 0.01$ vs Con A alone group. (D) Mouse $CD4^+$ T cells (2×10^5/well) purified from splenocytes of BALB/c mice were stimulated by anti-CD3 (10 μg/mL) plus anti-CD28 (1 μg/mL) antibodies for 96 h, or mouse $CD4^+$ T cells

3.2. Cirsilineol inhibited mixed lymphocyte reaction and T-cell proliferation in a dose-dependent manner

As shown in Fig. 1B, cirsilineol significantly inhibited single mixed lymphocyte reaction in a concentration-dependent manner. Furthermore, cirsilineol remarkably inhibited T-cell proliferation induced by Con A in a concentration-dependent manner by MTT assay (Fig. 1C). It is important to note that cirsilineol at the concentrations mentioned above did not affect T lymphocyte's viability by MTT uptake assay (Fig. 1A) and annexin V-propidium iodide assay (Supplemental Fig. 1). These results also indicated that the inhibition of cirsilineol on T-cell proliferation, at concentrations which are up to 10 μM, is not due to a cytotoxic activity. Moreover, cirsilineol also significantly inhibited anti-CD3/anti-CD28-stimulated [^{3}H]-thymidine incorporation of mouse CD4$^+$ T cells in a concentration-dependent manner, with more than 85% inhibition rate at 5 μM and almost completely at 10 μM (Fig. 1D). The similar result was also seen in CFSE assay (Fig. 1E).

3.3. Cirsilineol suppressed $OVA_{323-339}$-specific CD4$^+$ T-cell proliferation

DO11.10 transgenic mice, with BALB/c background, were used to examine the effect of cirsilineol on the response of CD4$^+$ T cells, specific for the peptides $OVA_{323-339}$, because more than 90% of the TCR in the T cells of the mice can recognize the $OVA_{323-339}$ peptide. CD4$^+$ T cells from DO11.10 transgenic mice were stimulated by $OVA_{323-339}$-loaded splenocytes from BALB/c mice in the presence and absence of cirsilineol. The results were similar to those of Con A or anti-CD3/anti-CD28-induced mouse T-cell proliferation experiment. Cirsilineol remarkably inhibited $OVA_{323-339}$-specic CD4$^+$ T-cell proliferation in a concentration-dependent manner (Fig. 1D and F).

3.4. Cirsilineol significantly ameliorated TNBS-induced Th1-mediated colitis through inhibiting IFN-γ/STAT1/T-bet signaling in CD4$^+$ T cells

To determine if cirsilineol affects T cells in vivo in the same manner as it does in vitro, we used a TNBS-induced T-cell-mediated colitis in mice and treated with cirsilineol. In the pre-experiment, we found that the drug concentration in the serum of mice with intraperitoneally administered 30 mg/kg of cirsilineol daily for three days reached a peak at about 8 μM by a high performance liquid chromatography assay, which just fell within the range of the drug concentrations (1–10 μM) in vitro. So 3, 10 and 30 mg/kg of cirsilineol were selected for in vivo experiments. As illustrated in Fig. 2A and B, when intraperitoneally administered daily from day 1 post immunization onwards, cirsilineol showed a significant improved effect on the body weights and survival rate of mice as compared with vehicle control (PBS). The ameliorative effect became overt at the time of disease onset (days 5 and 6) and persisted over the course of colitis. In addition, the macroscopic damage such as edema, inflammation and colon length was clearly less pronounced after treatment with cirsilineol (Fig. 3B and D). The observed macroscopic effect of cirsilineol was consistent with microscopic pathological score, including markedly reduced inflammatory infiltration, restoration of the destructive mucosal architecture and remission of edema from cirsilineol-treated mice as compared with those from vehicle control by histological analysis (Fig. 3A and C). The positive drug dexamethasone also significantly attenuated the intestinal inflammation (Fig. 3).

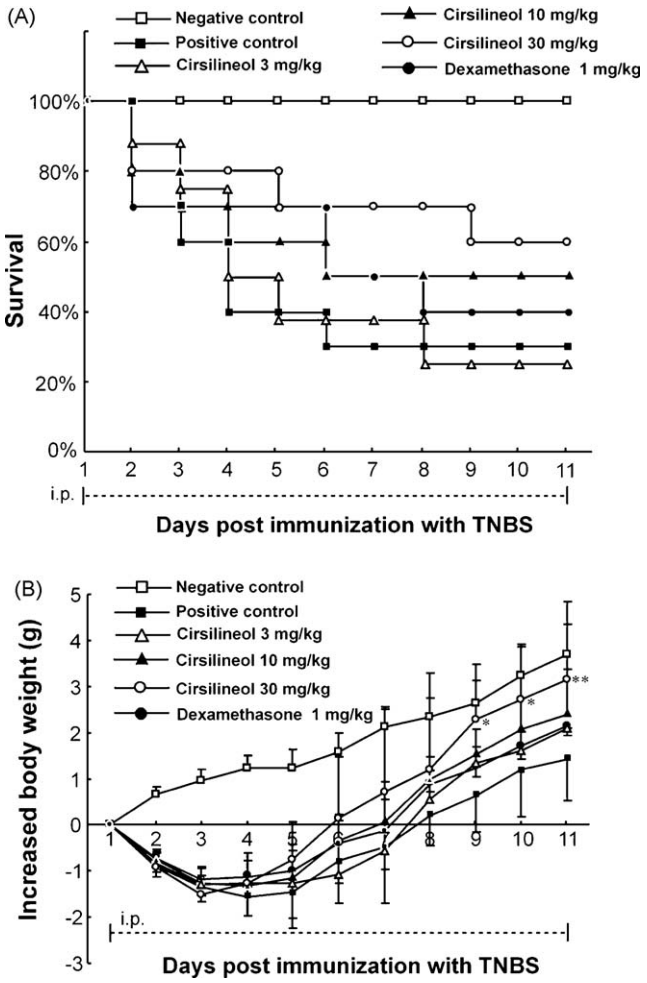

Fig. 2. Effects of cirsilineol on survival rate and body weights of mice with TNBS-induced colitis. (A) The survival rate of mice with TNBS-induced colitis. (B) Body weights of mice with TNBS-induced colitis. Data are presented as mean ± SEM. n = 8–10; $^*P < 0.05$, $^*P < 0.01$ vs corresponding positive control.

The significant ameliorative effect of cirsilineol in colitis prompted us to investigate in detail potential regulatory mechanisms of the compound as to how it affected lamina propria T-cell responses and to identify the possible signaling pathway through which cirsilineol might regulate inflammatory diseases. To this end, in the present study colonic LPMC were isolated from cirsilineol-treated and vehicle control mice and characterized for in vitro antigen-specific T-cell reactivity and cytokine profiles in response to in vitro challenge by the disease-eliciting TNBS antigen. The results revealed that cirsilineol altered T-cell responses in two ways. First, the activation and proliferation of TNBS-reactive T cells derived from cirsilineol-treated colitis mice was significantly inhibited compared with that of vehicle control mice (Fig. 4A and B). Second, colonic LPMC derived from cirsilineol-treated mice displayed unique cytokine profiles characterized by selective reduction of IFN-γ ($P < 0.01$; Fig. 5A and B). The other pro- or anti-inflammatory cytokines commonly associated with experimental colitis, including IL-17, IL-2, TNF-α, IL-4 and IL-5, were not markedly altered, whereas IL-10 and TGF-β were significantly up-regulated ($P < 0.05$; Fig. 5A). At the same time,

(2×10^5/well) purified from splenocytes of DO11.10 transgenic mice were stimulated with 1 μg/mL of $OVA_{323-339}$ and 4×10^5 BALB/c splenocytes per well for 96 h, in the presence/absence of various concentrations of cirsilineol and their proliferation was evaluated using [^{3}H]-thymidine uptake assay. $^*P < 0.05$, $^{**}P < 0.01$ vs corresponding control group. Results from columns were expressed as the mean ± SEM of three experiments. (E and F) Mouse CD4$^+$ T cells (2×10^5/well) from BALB/c or DO11.10 transgenic mice were labeled with 2.5 mM CFSE for 10 min, then they were stimulated with anti-CD3 (10 μg/mL) plus anti-CD28 (1 μg/mL) (E) or $OVA_{323-339}$ (1 μg/mL) plus 4×10^5 BALB/c splenocytes (F) for 96 h, respectively. The proliferation of CFSE-labeled CD4$^+$ T cells was evaluated using flow cytometry. Data are one of three independent experiments with similar results.

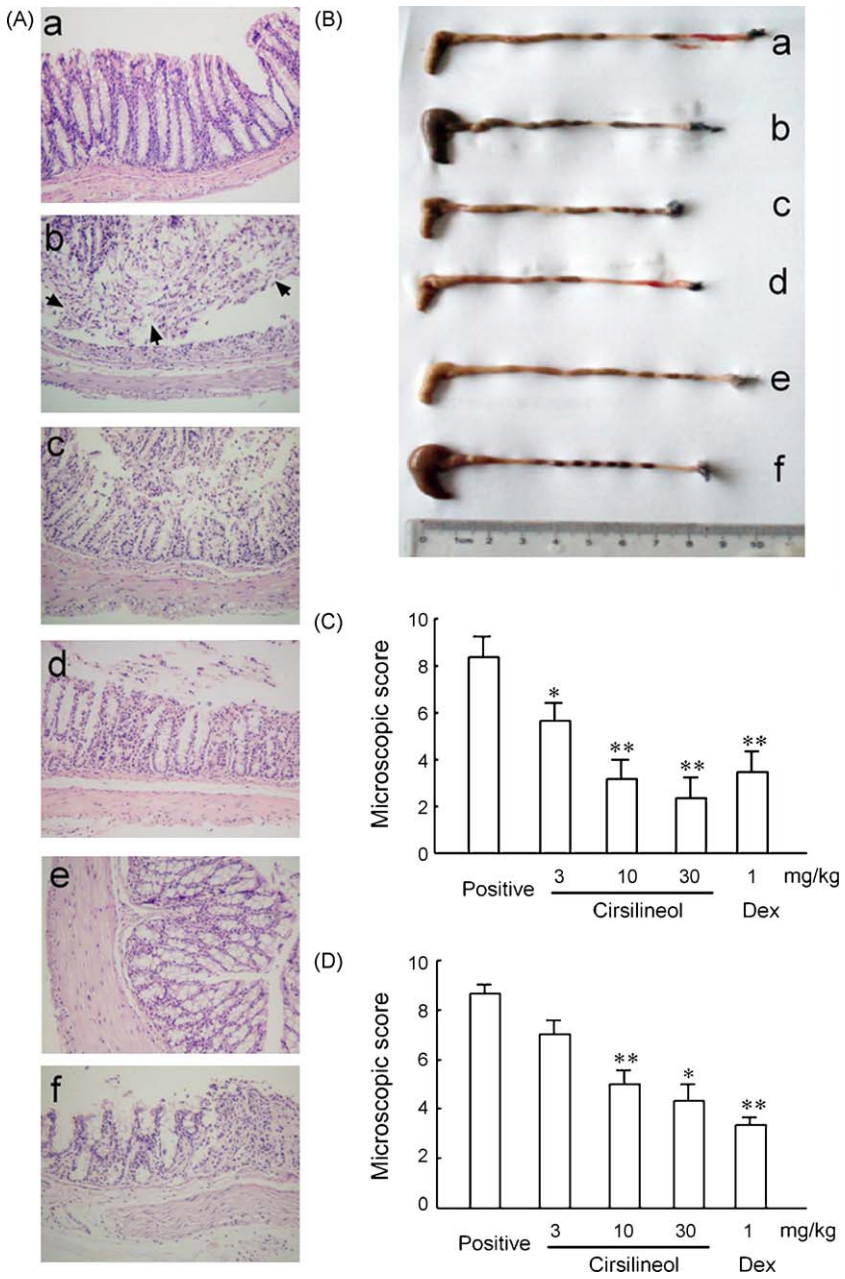

Fig. 3. Effects of cirsilineol on histological analysis, microscopic score and macroscopic score in TNBS-induced colitis mice. The colitis was induced by intrarectally injecting TNBS. Cirsilineol was given intraperitoneally once a day. Colons were taken out and observed the macroscopic change or examined the microscopic change as described in methods. Effect of cirsilineol on histopathological changes (A) and macroscopic appearance changes (B) of colons from TNBS-colitis mice. a, negative control; b, positive control; c, d and e, 3, 10 and 30 mg/kg cirsilineol, respectively; f, 1 mg/kg dexamethasone. Histopathological sections were stained by H&E, original amplification (×100). Black arrows indicate loss of mucosal architecture in the colon. Microscopic (C) and macroscopic (D) scores of colon from TNBS-colitis mice. Data are presented as mean ± SEM of experimental animals (n = 5–8). $^{*}P < 0.05$, $^{**}P < 0.01$ vs positive control.

the activation of STAT1/T-bet signaling in colonic LPMC from cirsilineol-treated mice was also notably down-regulated (Fig. 5C). Moreover, the significant reduction in STAT1 activation was also seen in colonic lamina propria $CD4^+$ T cells from cirsilineol-treated colitis mice, as compared with those of vehicle control mice by intracellular staining ($3.1 \pm 0.4\%$ from cirsilineol 30 mg/kg vs $17.9 \pm 2.1\%$ from positive control, $P < 0.01$) (Fig. 5D).

3.5. Cirsilineol inhibited STAT1 activation and T-bet expression in mouse $CD4^+$ T cells

To further analyze the mechanism of cirsilineol-mediated inhibition of IFN-γ signaling in mouse $CD4^+$ T cells, we examined the expressions of the phosphorylated STAT1 and T-bet by immunoblot analysis. Incubation of splenic $CD4^+$ T cells for 30 min with IFN-γ resulted in a marked enhancement of STAT1 tyrosine phosphorylation (Fig. 6A). Co-incubation of IFN-γ-treated $CD4^+$ T cells with cirsilineol (10 μM) completely inhibited the Tyr701 phosphorylation of STAT1, in line with the inhibition of DNA-binding activity of STAT1 treatment with cirsilineol (Fig. 6B). As a downstream molecule of STAT1, Th1-specific transcription factor T-bet was also suppressed by cirsilineol in a concentration-dependent manner (Fig. 6C).

3.6. Cirsilineol inhibited the activity of JAK2 in mouse $CD4^+$ T cells

JAK2 activation has been described to be an essential step for STAT1 activation. We further analyzed the mechanism of inhibition

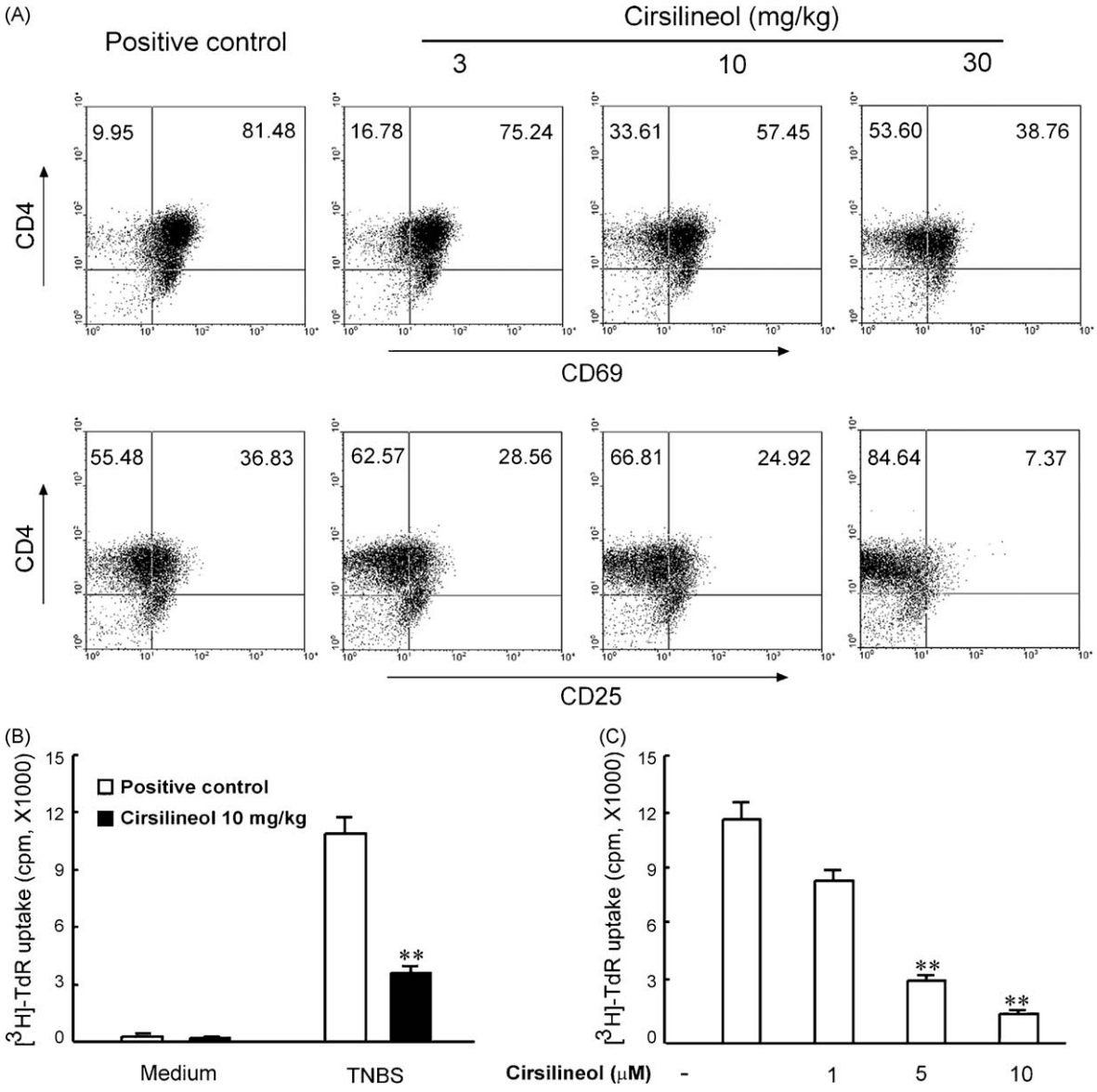

Fig. 4. Effects of cirsilineol on T-cell proliferation and activation in TNBS-induced colitis mice. (A) Colonic lamina propria $CD4^+$ T cells isolated 3 days postimmunization from colonic tissue of cirsilineol-treated mice or vehicle control mice were stained for CD69 and CD25 expression. Data are one of three independent experiments with similar results. (B) Lamina propria mononuclear cells (LPMC) from colonic tissue of cirsilineol-treated mice or vehicle control mice were isolated 3 days postimmunization and examined in vitro for proliferation in the presence (TNBS) or absence (medium) of TNBS. (C) LPMC from colonic tissue of vehicle control mice isolated 3 days postimmunization were cultured with the indicated concentrations of cirsilineol. Tritiated thymidine incorporation was measured at the end of the 72-h culture. Data are presented as mean cpm ± SEM of triplicates. $^{**}P < 0.01$ vs control.

of STAT1 activation by testing the effect of cirsilineol on JAK2 activity. The immunoblot analysis for JAK2 tyrosine phosphorylation (Fig. 6A) and immunoprecipitation analysis for tyrosine phosphorylation of the IFN-γ receptor subunit α (Fig. 6D) were performed. Both assays indicated that cirsilineol (10 μM) significantly inhibited IFN-γ-mediated JAK2 activation in mouse $CD4^+$ T cells.

3.7. High level of IFN-γ overcame the inhibitory effect of cirsilineol on JAK2/STAT1 signaling

To demonstrate whether the inhibition of STAT1 signaling by cirsilineol is reversible, we exposed splenic $CD4^+$ T cells to IFN-γ, a potent activator of STAT1, to examine whether increasing doses of IFN-γ are able to overcome the inhibitory effect of cirsilineol. As shown in Fig. 6E, IFN-γ (25 ng/mL) treatment enhanced the phosphorylation of JAK2 and STAT1 in splenic $CD4^+$ T cells. Consistently, T cells pretreated with cirsilineol for 3 h and then exposed to IFN-γ (25 ng/mL) showed significantly reduced phosphorylation of JAK2 and STAT1. Interestingly, high dose of IFN-γ (100 ng/mL) were able to overcome the inhibitory effect of cirsilineol on the activation of JAK2 and STAT1 (Fig. 6E).

4. Discussion

There has been growing interest to explore novel anti-inflammatory or immunomodulatory properties from natural herbal medicines [24–26]. Traditional Chinese medicine that has been practiced for thousands of years in clinic often provides a vast source of pharmaceutical material for the development of effective drugs and offers some unique advantages with low toxicity profiles [27,28]. The substantial obstacle, however, is that the detailed mechanisms of action of many of these natural active ingredients are largely unknown [29,30]. So finding effective anti-inflammatory and immunomodulatory natural compounds will not only

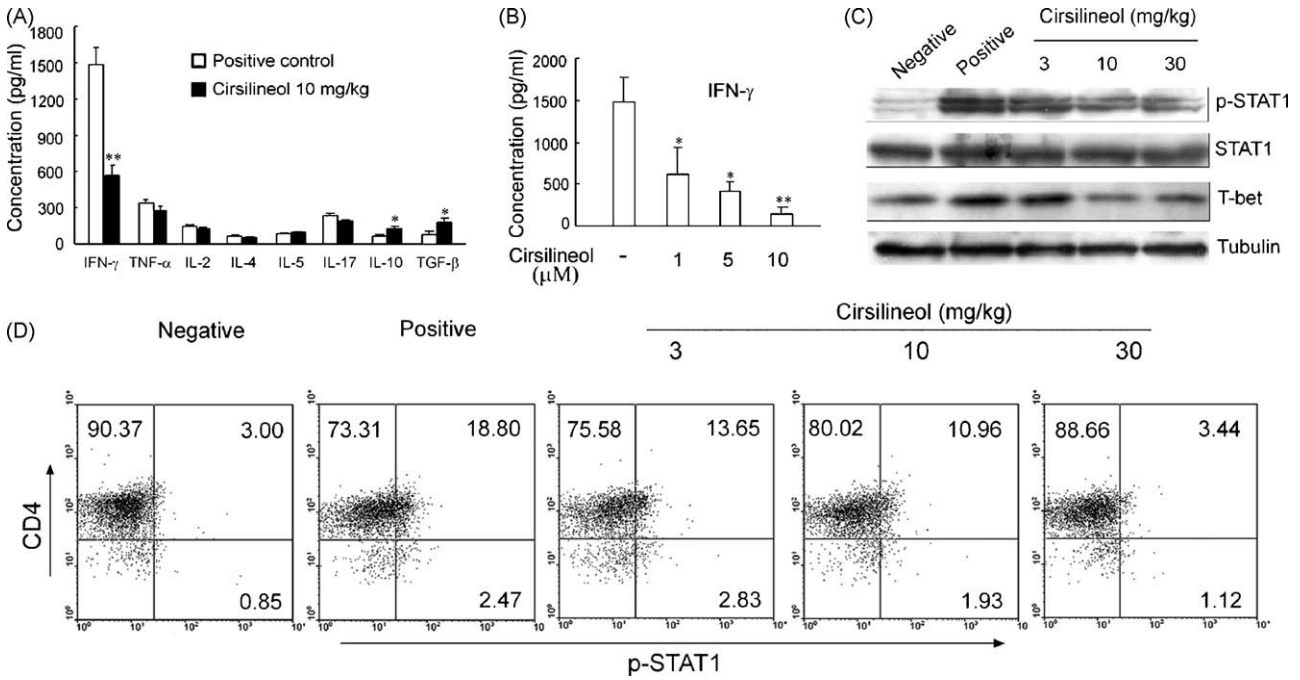

Fig. 5. Effects of cirsilineol on cytokine profiles and STAT1 activation in TNBS-induced colitis mice. (A) Lamina propria mononuclear cells (LPMC) from colonic tissue of cirsilineol-treated mice (solid bars) or vehicle control mice (open bars) isolated 3 days postimmunization were challenged with TNBS, and culture supernatants were collected at 48 h for cytokine measurement by ELISA. Data are presented as mean concentration (pg/mL ± SEM) of triplicate samples. $^{*}P < 0.05$, $^{**}P < 0.01$ vs corresponding positive control. (B) LPMC from colonic tissue of vehicle control mice isolated 3 days postimmunization were cultured with TNBS and various concentrations of cirsilineol for 48 h. Supernatants were collected for measurement of IFN-γ production by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control. (C) Proteins of LPMC from colonic tissue of cirsilineol-treated mice or vehicle control mice isolated 3 days postimmunization were extracted and subjected to Western blot. The blot shown here is one of three independent experiments. (D) Mouse colonic lamina propria $CD4^{+}$ T cells from cirsilineol-treated mice or vehicle control mice isolated 3 days postimmunization, were stained for intracellular pSTAT1. Data are one of the three independent experiments with similar results.

facilitate our understanding of regulation for the network of immune diseases, but also provide some useful small probes for investigating the interactions of critical signaling molecules involved in inflammation and autoimmune diseases. The present study indicates that cirsilineol is a novel anti-inflammatory and immunosuppressive agent acting through defined signaling mechanisms and provides important implications to evaluating the utility of natural compounds in treatment of autoimmune disease and inflammation.

Under the guidance of seeking a new immunosuppressant with high selectivity, our previous studies have identified some natural small compounds from Chinese herbs, and confirmed the selective immunosuppressive activities [31–33]. As a special natural small compound, cirsilineol, a natural flavone derived from *A. vestita*, showed a potent immunosuppressive feature on T-cell proliferation induced by various stimulators. It is important to note that cirsilineol did not affect T lymphocyte's viability, indicating that the immunosuppressive activity of cirsilineol observed here, at concentrations which are up to 10 μM, was not due to a cytotoxic activity, as evidenced by MTT uptake assay (Fig. 1A) and annexin V-propidium iodide staining assay (Supplemental Fig. 1) for cell death. In an attempt to confirm the immunosuppressive property of cirsilineol in vivo, we investigated the effect of this compound on TNBS-induced T-cell-mediated experimental colitis, a well-characterized murine model for Crohn's disease [34]. Acute experimental colitis was induced in BALB/c mice by TNBS administered intrarectally. It was manifested in weight loss, intestinal bleeding, and diarrhea, as well as by macroscopic and microscopic colon damage. Cirsilineol treatment led to striking amelioration of all of these manifestations, resulting in improved long-term survival of mice. Considering excessive T-cell responses always play a critical role in the pathogenesis of inflammatory bowel disease, we examined the effect of cirsilineol on the response of antigen-specific T cells in colitis mice. We found that cirsilineol treatment significantly inhibited the proliferation and activation of TNBS-reactive T cells in a dose-dependent manner. Further studies then elucidated the action of cirsilineol on cytokine production profile of lamina propria mononuclear cells from colonic tissue of mice.

A growing body of data implicates a dysfunctional mucosal immune response to commensal bacteria and imbalance between pro-inflammatory and anti-inflammatory cytokine responses are involved in the pathogenesis of inflammatory bowel disease, especially Crohn's disease [35,36]. As one of pro-inflammatory cytokines, IFN-γ has been shown to play a major role in the pathophysiology of several intestinal inflammatory diseases, including Crohn's disease and pouchitis [6,37]. Recently, the critical role of IL-17 in autoimmune inflammatory bowel diseases adds an additional dimension to this complex network involving a battery of pro-inflammatory cytokines and the activity of related cross-talking signaling pathways [38,39]. As to anti-inflammatory cytokines, IL-10 and TGF-β counteract self-inflicted injury by suppressing the effect of pro-inflammatory cytokines [40,41]. Interestingly, the observed inhibitory effect of cirsilineol on cytokine profile was remarkably specific for IFN-γ, a key mediator in T-cell-dependent inflammation. Moreover, this regulatory action of pro-inflammatory and anti-inflammatory cytokine by cirsilineol treatment was found to decrease the activity of effector Th1 cells but increase the activity of regulatory T cells as characterized by down-regulation of IFN-γ and corresponding up-regulation of IL-10 and TGF-β. However, the pro-inflammatory cytokine IL-17 recently associated with experimental colitis, as well as anti-inflammatory cytokines IL-4 and IL-5, were not markedly altered by cirsilineol. It is well known that the functional activity of regulatory T cells has been linked to the production of IL-10 and TGF-β, which is predominantly expressed in

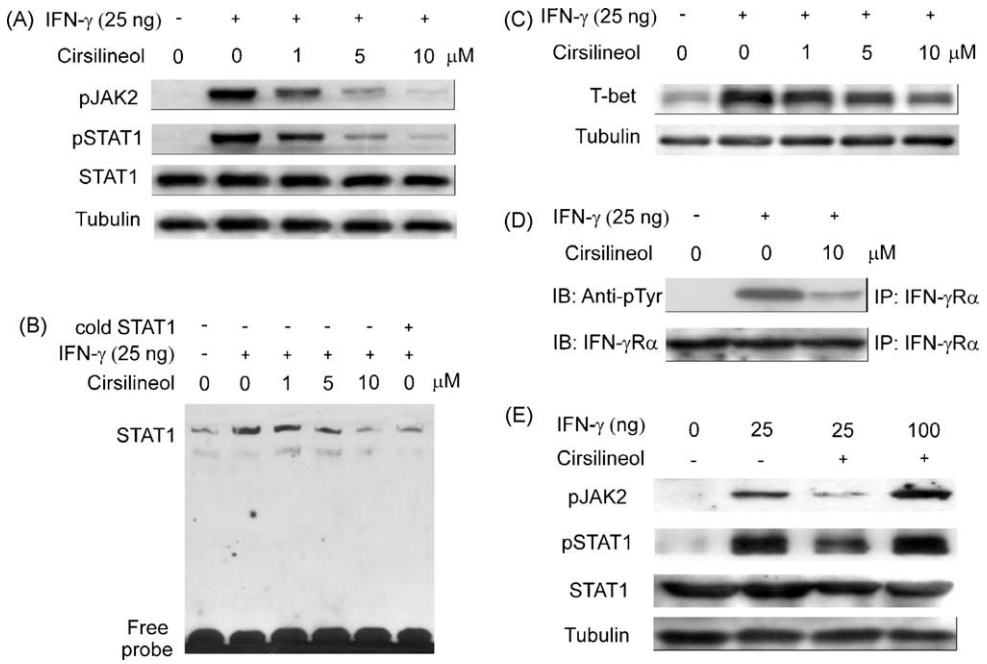

Fig. 6. Effect of cirsilineol on IFN-γ signaling in mouse $CD4^+$ T cells in vitro. (A) Splenic $CD4^+$ T cells isolated from BALB/c mice were cultured with cirsilineol for 3 h, then treated with murine IFN-γ for 30 min. After the incubation, proteins were extracted and assessed by Western blot analysis. (B) Splenic $CD4^+$ T cells were cultured with cirsilineol for 3 h, then treated with murine IFN-γ for 30 min. After the incubation, nuclear proteins were extracted and assessed by EMSA analysis. (C) Splenic $CD4^+$ T cells were cultured with cirsilineol for 3 h, then treated with murine IFN-γ for 12 h. After the incubation, proteins were extracted and assessed by Western blot analysis. (D) Splenic $CD4^+$ T cells were cultured with cirsilineol for 3 h, then treated with murine IFN-γ for 30 min. Total protein extracts were prepared and immunoprecipitated with a monoclonal anti-IFN-γ receptor subunit α antibody using protein A/G-agarose. The immunoprecipitated protein was loaded on a SDS-PAGE, and after blotting, tyrosine phosphorylation of IFN-γ receptor subunit α was analyzed using an anti-phosphotyrosine antibody. (E) Splenic $CD4^+$ T cells were cultured with cirsilineol (10 μM) for 3 h, then treated with various concentrations of IFN-γ for 30 min. After the incubation, proteins were extracted and assessed by Western blot analysis. All data are one of three independent experiments with similar results.

$CD4^+CD25^+FoxP3^+$ T cells. Although the production of IL-10 and TGF-β were up-regulated in cirsilineol-treated mice, the proportion of mucosal $CD4^+CD25^+$ T cells decreased (Fig. 4A), suggesting that the increased functional activity rather than the quantity of regulatory T cells upon cirsilineol treatment contributed to the ameliorative effect in TNBS-induced colitis. These data collectively provide a coherent model illustrating that these regulatory actions of cirsilineol work in concert to contribute to dampened autoreactive T-cell responses in cirsilineol-treated colitis mice.

STAT1 is a key intracellular effector molecule of IFN-γ-mediated signaling. There is compelling evidence that the expression and activation of STAT1 are predominantly heightened in inflammatory bowel diseases and may therefore play an important role in the pathophysiology of colonic inflammation [14,42]. Results from Fig. 5C and D revealed that cirsilineol-treated colonic lamina propria $CD4^+$ T cells from colitis mice exhibited the dampened STAT1 phosphorylation and impaired T-bet expression determined by immunoblotting and intracellular protein staining, indicating that IFN-γ/STAT1/T-bet signaling was closely involved in the underlying molecular mechanism of action induced by cirsilineol. Furthermore, cirsilineol acted to down-regulate IFN-γ-mediated JAK2 activation in mouse $CD4^+$ T cells in vitro, as the result of the impaired tyrosine phosphorylations of both JAK2 and IFN-γ receptor subunit α. Subsequently, the phosphorylation and DNA-binding activity of STAT1, as well as the expression of the downstream molecule T-bet were also abrogated in mouse $CD4^+$ T cells. It was worthy noting that the inhibitory effect of IFN-γ/STAT1 signaling in $CD4^+$ T cells by cirsilineol was reversible in the presence of high level of IFN-γ, together with non-cytotoxic immunosuppressive activity, suggesting the safety to some degree of cirsilineol. In addition, there was no significant difference in the weight and cell numbers of lymphoid tissues (thymus, spleen and lymph nodes) between the mice intraperitoneally injected with cirsilineol (30 mg/kg) daily for 7 days and the mice treated with normal saline (data not shown), suggesting cirsilineol was not toxic for administration to mice in vivo. In this study, these results strongly indicated that cirsilineol had unique anti-inflammatory property and therapeutic potential for TNBS-induced experimental colitis. It should be noted that cirsilineol treatment induced a beneficial effect also in a murine model for dermatitis, picryl chloride-induced contact hypersensitivity, in our parallel experiments (data not shown), because both these inflammatory disorders share many similarities involving the role of IFN-γ. Recently, a plant steroid isolated from the gum resin of the Commiphora mukul tree, a traditional Ayurvedic medicine, named guggulsterone has been proven effective for the treatment of chronic intestinal inflammation in mice through regulating the function of effector T cells [43]. In this view, seeking some effective candidate compounds from natural herbal medicines may be a useful strategy in exploring the value of traditional medicine for the treatment of autoimmune disease and inflammation. More importantly, the emphasis of the present study was to elucidate the novel regulatory mechanism induced by cirsilineol and to probe its specific interactions with potential signaling molecule, which were responsible for its treatment effect in colitis. In this regard, these detailed characterizations collectively suggest that the therapeutic potential of cirsilineol in colitis is associated primarily with its selective inhibition of IFN-γ through the JAK2/STAT1/T-bet pathway in colonic lamina propria $CD4^+$ T cells.

In conclusion, cirsilineol is effective in ameliorating the macroscopic and microscopic manifestations in TNBS-induced experimental colitis, possibly due to its novel immunoregulatory activity with selective inhibiting IFN-γ/STAT1/T-bet signaling in colonic lamina propria $CD4^+$ T cells. In view of its unique property, these results warrant further evaluation of cirsilineol treatment for human inflammatory bowel disease, in particular, for Crohn's disease.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.08.014.

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