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(+)-Borneol improves the efficacy of edaravone against DSS-induced colitis by promoting M2 macrophages polarization via JAK2-STAT3 signaling pathway

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ABSTRACT

Compound edaravone injection (C.EDA), a compound preparation composed of edaravone (EDA) and (+)-Borneol with the mass ratio of 4: 1, displays a better anti-inflammatory activity than EDA. However, its precise mechanism remains to be further studied. In this work, we investigated whether (+)-Borneol could improve the efficacy of EDA against DSS-induced colitis. We found that C.EDA at 7.5 and 15 mg/kg could significantly relieve the disease activity index (DAI) and reduce the loss of body weight and colon length in a dose-dependent manner, while EDA or (+)-Borneol alone only had moderate effects even at the highest dose. Additionally, ELISA revealed that C.EDA could more dramatically decrease the protein levels of inflammatory cytokines and increase the levels of anti-inflammatory cytokine than EDA or (+)-Borneol alone both in colon tissues and serum. H & E staining and IHC assay also indicated that C.EDA exhibited more prominent effects on increasing the population of M2 macrophages, decreasing M1 macrophages infiltration and protecting intestinal barrier integrity. Furthermore, in vitro studied demonstrated that C.EDA, EDA or (+)-Borneol failed in inhibiting M1 macrophages activation but could specifically induce the activation of M2 macrophages in a STAT3-dependent manner. Knockdown the expression of STAT3 successfully abolished the effect of C.EDA and EDA on promoting M2 macrophages activation. Consistent with in vivo study, C.EDA exhibited a more efficient ability of inducing M2 macrophages polarization and STAT3 activation than EDA or (+)-Borneol alone in vitro. In conclusion, we confirmed that (+)-Borneol improved the efficacy of EDA against DSS-induced colitis by promoting M2 macrophages polarization via JAK2-STAT3 signaling pathway.

1. Introduction

Crohn's disease and ulcerative colitis, also named inflammatory bowel diseases (IBDs) with poor response to clinical treatment, are linked with high mortality and have a great influence on the quality of individual's life due to pain, vomiting, diarrhea, and other socially undesired symptoms. The worldwide incidence of IBDs keeps increasing with a highest prevalence in Europe and Canada [1]. IBDs are structure diseases with underlying physical damages in the gut, often in the end of small intestine or colon, as detected by X-ray, endoscopy, surgery, or biopsy [2]. Although the exact etiology of IBDs remains unknown, it is widely accepted that IBDs are developed in genetically susceptible individuals with abnormal immune responses against the microorganisms in the intestinal flora [3]. Thus, IBDs are a class of autoimmune diseases resulting from the healthy elements of body's digestive system attacked by the excessive activation of their own immune systems [4].

Macrophages are key mediators of immune system and play central roles in maintaining a steady state of intestinal homoeostasis by removing apoptotic cells debris and fighting against pathogens [5,6]. Depending on the environmental signals, macrophages are generally polarized into two functionally opposite forms: classically activated (M1) macrophages and alternatively activated (M2) macrophages [7]. M1 macrophages induced by IFN-γ, lipopolysaccharide (LPS), TNF-a, and granulocyte-macrophage colony-stimulating factor (GM-CSF) could triger Th1 and Th17 responses via producing high levels of inflammatory cytokines including TNF-a, IL-6, IL-1β, IL-12 and IL-23 [8]. While M2 macrophages polarized by IL-4, IL-13 and macrophage colony-stimulating factor (M-CSF) participate in Th2 response, exhibiting anti-inflammation profile through upregulating the expression of IL-10, arginase 1 (Arg-1) and CD206 antigen [9]. Interestingly

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enough, studies has proved that M1 macrophages and M2 macrophages are interchangeably. This switch makes macrophages play a dual role in orchestrating the lasting inflammation and onset of healing and repairing [10]. Although the functions of M1 and M2 macrophages during the initiation and development IBDs have not yet been well identified, scientists found that experimental IBDs could be greatly ameliorated by the induction of intestinal macrophages polarized to M2 macrophages via small molecules or specific antibodies [11].

JAK2-STAT3 signaling pathway in macrophages involves in immunosuppression [12]. Actually, signal transducer and activator of transcription 3 (STAT3) is regarded as a key signaling molecule for macrophage polarization to M2 macrophages [13]. STAT3 signaling pathway is much more highly activated in M2 macrophages than in M1 macrophages [14]. Researches also displayed that STAT3 knockout macrophages stayed on a higher pro-inflammation state via releasing a large amount of pro-inflammatory cytokines, such as TNF-a, IL-6, IL-1β [15]. STAT3 activation promotes production of various anti-inflammatory cytokines including IL-10, which inhibits functional dendritic cells (DC) maturation and increases population of T regulatory cells (Treg) [16].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, EDA) is a free radical scavenger which exhibits promising activities in preventing neuro-inflammation, liver injury and antioxidant, reported by a variety of studies [17]. Compound edaravone injection (C.EDA) is a compound preparation composed of edaravone and borneol with the mass ratio of 4: 1 [18]. C.EDA has been finished Phase III clinical trial for Acute Ischemic Stroke approved by CFDA in 2016 [19]. C.EDA has been reported to display a better activity than EDA alone in fighting against inflammation in multiple mouse models [20]. However, the activity of C.EDA in fighting against colitis has not yet been tested. In this study, we assessed the effects of C.EDA, EDA and (+)-Borneol on a mouse model of experimental colitis induced by dextran sulfate sodium (DSS) administration and the polarization of mouse macrophage RAW264.7 cells. These results revealed that (+)-Borneol could improve the efficacy of EDA against DSS-induced colitis by promoting M2 macrophages polarization in a STAT3-depedent manner. Our study provided a basis for the clinical use of C.EDA in treating IBDs.

2. Methods and materials

2.1. Reagents

C.EDA (10 mg/5 mL), EDA (10 mg/5 mL) and (+)-Borneol were provided by Jiangsu Simcere Pharmaceutical Co., Ltd.. For cell treatment, C.EDA and EDA were diluted to 10 mM with PBS. (+)-Borneol were dissolved in PBS (0.5 mg/mL, 32 mM) and used as 100 ×. CCK-8 and LPS were obtained from Sigma-Aldrich (St Louis, USA). Recombinant murine IFN-γ, IL-4 and IL-13 was purchased from PeproTech (Rocky Hill, USA). Lipofectamine® RNAiMAX was obtained from Invitrogen (Burlington, ON). Antibodies used for Western blotting were described as follows: p-JAK2 (Tyr1008), JAK2, p-STAT3 (Tyr705), STAT3 were purchased from Cell Signaling Technology; GAPDH, Lamin A and Tubulin were bought from Santa Cruz Biotechnology.

2.2. Cell culture

Murine macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (Rockville, MD), and maintained in DMEM supplemented with 10% FBS at 37 ˚C in 5% CO2 in a humidified incubator.

2.3. Macrophage polarization

For M1 macrophages polarization, RAW264.7 cells were treated with 500 ng/mL LPS plus 20 ng/mL recombinant murine IFN-γ and indicated doses of C.EDA, EDA or (+)-Borneol for 24 h. For M2 macrophages polarization, RAW264.7 cells were treated with recombinant murine IL-4 20 ng/mL plus 20 ng/mL recombinant murine IL-4 for 24 h. To assay the ability of C.EDA, EDA and (+)-Borneol in inducing M2 macrophage polarization, RAW264.7 cells were treated with indicated doses of C.EDA, EDA or (+)-Borneol for 24 h.

Table 1
Sequences of STAT3 small interfering RNAs (siRNAs).

SiRNA	Direction	Sequence 5′-3′
siNC	Sense	UUCUCCGAACGUGUCACGU
	Antisense	ACGUGACACGUUCGGAGAA
Si-1	Sense	UGCAUGUCUCCUUGGCUCUUGAGGG
	Antisense	ACGUACAGAGGAACCGAGAACUCCC
Si-2	Sense	CAGGGUGUCAGAUCACAUGGGCUAA
	Antisense	GUCCCACAGUCUAGUGUACCCGAUU
Si-3	Sense	CCUCCAGGACGACUUUGAU
	Antisense	GGAGGUCCUGCUGAAACUA
Si-4	Sense	GGGUCUGGCUAGACAAUAU
	Antisense	CCCAGACCGAUCUGUUGUA

2.4. STAT3 siRNA transfection

The sequences of small interfering RNAs (siRNA) were shown in Table 1 and synthesized by GenePharma (Shanghai, China). RAW264.7 cells were seeded in 6-well tissue culture plates at a density of 3×10^5 cell per well. STAT3 siRNA or control siRNA delivering was conducted by using RNAiMAX following the manufacturer's instructions. After 24 h transfection, the infected cells were treated with indicated doses of C.EDA, EDA or (+)-Borneol for another 24 h.

2.5. Immunofluorescence

3×10^5 RAW264.7 cells grew on the glass lips (WHB, Shanghai, China) and treated with C.EDA, EDA, (+)-Borneol or IL-4 plus IL-13 for 24 h. Then cells were fixed with 4% paraformaldehyde for 20 min and washed twice with PBS. Subsequently, the cellular membrane was permeabilized with 0.2% Triton X-100 for 10 min and blocked with 5% BSA for 30 min. These cells were incubated with the STAT3 primary antibody (Cell Signaling Technology) (1:100) at 4 ˚C overnight, followed by incubation with FITC conjugated secondary antibodies. The nuclei were counterstained with DAPI (50 μg/mL). Finally, the chondrocytes were observed under a confocal microscope (Olympus FV1000, Japan).

2.6. Mice maintenance, induction of colitis and clinical scoring

Female C57BL/6 mice (6 weeks old, 18–22 g) were purchased from Model Animal Genetics Research Center of Nanjing University (Nanjing, China). Acute colitis were induced by adding 2.5% DSS in drinking water for 7 days and C.EDA, EDA or (+)-Borneol were intraperitoneally (ip) administrated with indicate doses for another 10 days. Body weight, stool consistency, and the presence of gross blood in feces and at the anus were recorded every day. The disease activity index was calculated by assigning well-established and validated scores as previously described [21]. Generally, a) diarrhea (0 point = normal, 2 point = loose stool, 4 point = watery diarrhea); b) hematochezia (0 point = no bleeding, 2 point = slight bleeding, 4 point = gross bleeding); the disease activity index scores were summed up with a) and b).

2.7. Histological analysis

For histological analysis, the same parts of the colonic tissues were fixed in 10% buffered formalin and embedded in paraffin. 4-μm sections were stained with H & E according to standard protocols. The

Table 2
Primer sequences for real-time PCR.

Gene	Gene ID		Sequence 5′-3′
STAT3	20848	Forward	CAATACCATTGACCTGCCGAT
		Reverse	GAGCGACTCAAACTGCCCT
IL-10	16153	Forward	GCTCTTACTGACTGGCATGAG
		Reverse	CGCAGCTCTAGGAGCATGTG
Arg-1	11846	Forward	CTCCAAGCCAAAGTCCTTAGAG
		Reverse	GGAGCTGTCATTAGGGACATCA
GAPDH	14433	Forward	TGGCCTTCCGTGTTCCTAC
		Reverse	GAGTTGCTGTTGAAGTCGCA

histological score were obtained from pathologist.

2.8. Cytokine measurement

IL-1β, IL-6, IL-10 and TNF-α in the supernatant, serum and colon tissue homogenates were measured using ELISA kits from Dakewe Biotech Co. Ltd. (Shenzhen, China) according to the manufacturer's instructions.

2.9. mRNA isolation and real-time PCR

Total RNA from cells and tissues was isolated by Trizol (Invitrogen, Carls, CA) and transcribed into cDNA. The real-time PCR was performed with the BioRed CFX96 Touch™ Real-time PCR Detection System (BioRad, CA) using iQTMSYBR1Green supermix (BioRad, CA). The program was described as following: 1 cycle of 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Primers used in this study were shown in Table 2.

2.10. Immunohistochemistry (IHC)

Colon tissues were fixed in 10% formalin, embedded in paraffin, and then cut into 4 μm thick sections. Tissue sections were deparaffinized in xylene and stained with anti-CD11c, anti-CD206 and anti-claudin-1 rabbit polyclonal antibody (1:100 dilution; Proteintech Group, Inc.), as previously described [22].

2.11. Western blotting

Proteins from cells or colon tissues were lysed with lysis buffer and quantitated by BCA assay. Then these proteins were separated in 10% SDS PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA). The aimed bands were cut into appropriate pieces by referring to the size of protein ladder and blocked with 3% BSA for 1 h at room temperature, then incubated with indicate primary antibody overnight at 4 °C. The HRP-coupled secondary antibody was then added. Protein bands were detected by using Western blotting detection system according to the manufacturer's instructions (Cell Signaling Technology, MA).

2.12. Statistical analysis

All the results displayed in this study were expressed as mean ± SEM with > 3 independent experiments. Significance among groups was analyzed by Dunnett's test of one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with vehicle treated group or Control group. Significance between EDA treated groups and C.EDA treated groups was analyzed by two-tailed unpaired *t*-test, $^{\#} < 0.05$, $^{\#\#}P < 0.01$, compared with the equivalent dose of EDA treated groups.

3. Results

3.1. (+)-Borneol improved the efficacy of EDA against DSS-induced colitis

In order to study whether (+)-Borneol could improves the efficacy of EDA on taming colon inflammation, we used a mouse model of DSS-induced experimental colitis to evaluate the therapeutic effects. Mice were challenged with 2.5% DSS in drinking water for 7 days and then treated with indicated does of C.EDA, EDA or (+)-Borneol for another 10 days. The chemical structure of EDA and (+)-Borneol were shown in

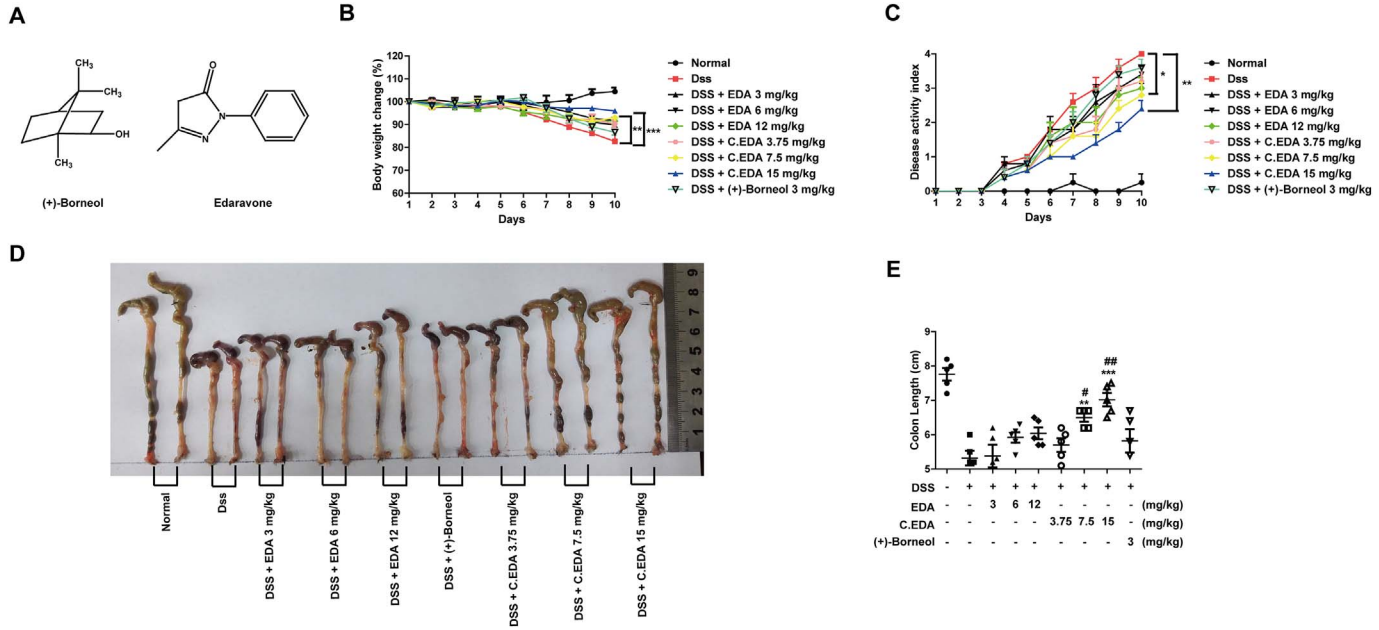

Fig. 1. (+)-Borneol improved the efficacy of EDA against DSS-induced colitis. (A) The chemical structure of (+)-Borneol and EDA. (B-E) Mice were fed with drinking water contains 2.5% DSS for 7 days before intraperitoneally injected with indicate doses of C.EDA, EDA or (+)-Borneol every day for another 10 days. (B) Loss of basal body weight of each group during treatment. (C) Disease activity index (DAI) was calculated. (D) Macroscopic appearance and (E) the length of colons from each group of mice were measured. Data are shown as mean ± SEM. In (B) and (C) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle treated group at the end point. In (E), $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle treated group ($n = 5$ per group). $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ vs. equivalent dose of EDA, respectively.

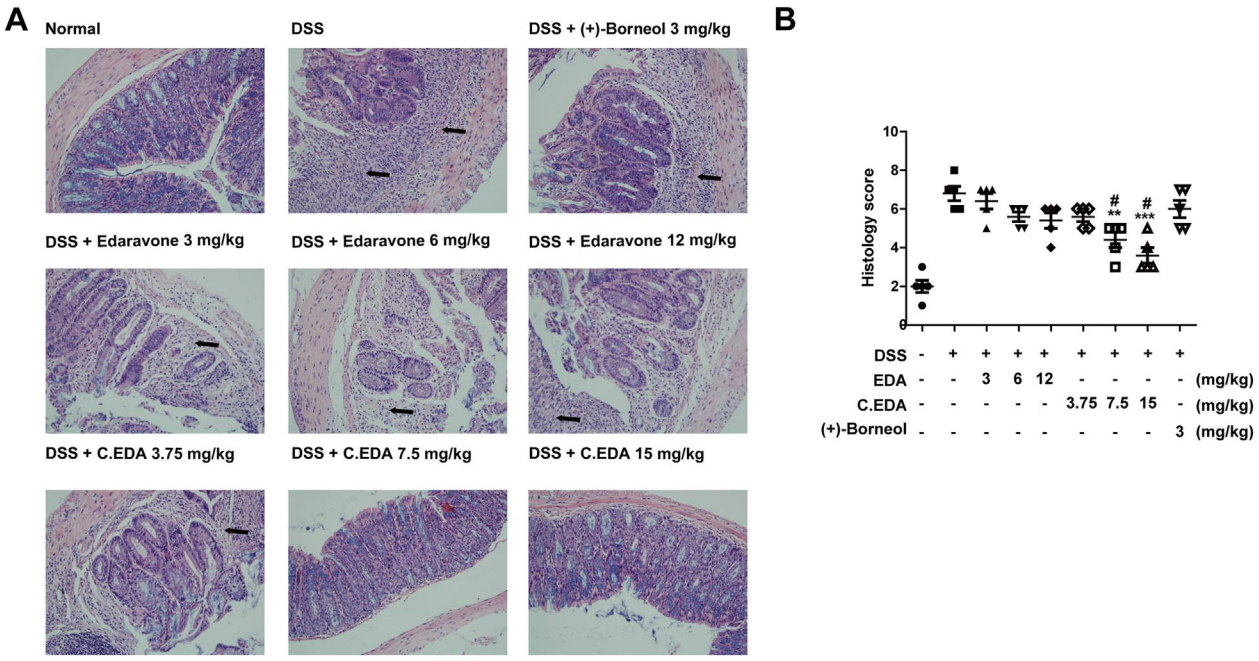

Fig. 2. C.EDA strengthened the protection against DSS-induced histological damage of colon. Sections of colon tissues were separated and stained with H & E (n = 5 per group). The sections of colonic tissues were stained with H & E to observe the damage degrees (A) and the histology scores were also been collected (B). Arrows shows the lesion site and infiltration of immune cells. Formalin-fixed intestine and colonic tissues were subjected to H & E staining to assess: 1) leukocyte infiltration, 2) vascular congestion and erosion, 3) anabrosis of epidermal cells. Each of the three parts was scored from 0 to 4 according to the severity of intestinal inflammation. The histological scores were accumulated from the scores of the three parts. Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle treated group. $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ vs. equivalent dose of EDA, respectively. These photos were observed by confocal laser-scanning microscope, 200 × .

Fig. 1A. Compared with DSS-treated group, C.EDA at 7.5 and 15 mg/kg could significantly block the loss of basal bodyweight. While, EDA and (+)-Borneol only has a moderate effect (Fig. 1B). Significant features of diarrhea and visible fecal blooding were symptoms displayed by colitis model and often organized as disease activity index (DAI). As shown in Fig. 1C, such symptoms were dose-dependently alleviated by C.EDA at 7.5 mg/kg and 15 mg/kg. EDA and (+)-Borneol only had little effect as suggested. Extensive colon shortening is another feature for indicating severe inflammation in the colon. When compared with EDA or (+)-Borneol, C.EDA also significantly decreased the colon shortening induced by DSS treatment in a dose-dependent manner (Fig. 1D and E). Histological score of colon tissues were calculated by distortion of crypts, loss of goblet cells, infiltration of inflammatory cells, and severe mucosal damage which were observed by H & E staining. Standard pathological assays showed that the serious deterioration of the colons was markedly protected by C.EDA (Fig. 2A and B). Correspondingly, both EDA and (+)-Borneol displayed little effect on these factors. These result indicated that (+)-Borneol could improve the efficacy of EDA against DSS-induced colitis.

3.2. C.EDA is more effective in inhibiting the infiltration of M1 macrophages, promoting the polarization of M2 macrophages and protecting the integrity of colon tissue than EDA and (+)-Borneol alone

It is reported that DSS-induced colitis mainly results from colon inflammation. So, we measured the levels of IL-1β, IL-6, TNF-α and IL-10 in serum and colonic tissues by ELISA. As shown in Fig. 3A-H, C.EDA could significantly decrease the levels of M1 macrophage associated pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and increase the levels of M2 macrophage associated anti-inflammatory cytokine (IL-10) at the dose of 7.5 mg/kg and 15 mg/kg. Although EDA exhibited anti-inflammatory activity, these effects was much weaker than those of C.EDA. Our results suggested that C.EDA improved DSS-induced colitis by inhibiting macrophages activation.

Then we detected the macrophages infiltration in colon from each group by immunochemistry and found that the numbers of $CD11c^{+}$ macrophages (M1 macrophages) which were often located on the lesion site were sharply decreased in colonic samples from C.EDA-treated mice. Conversely, the number of $CD206^{+}$ macrophages (M2 macrophages) responsible for tissue repairing was significantly increased in C.EDA-treated group (Fig. 4 A and B). Furthermore, immunochemistry also showed that the expression of Caudin-1, a tight junction protein which could reflect the integrity of epithelial tissue, was significantly elevated after C.EDA administration (Fig. 4C). Correspondingly, EDA or (+)-Borneol only displayed slightly relieving effect on the population of M1 and M2 macrophages and the expression of Caudin-1.

3.3. C.EDA displayed little effects on M1 macrophages polarization but greatly promoted M2 macrophages polarization in RAW264.7 cells

To further investigate whether C.EDA could modulate macrophages polarization in vitro, we tested the effect of C.EDA, EDA or (+)-Borneol on murine macrophage RAW264.7 cells polarization. As shown in Fig. 5A, both EDA and C.EDA displayed little toxicity even at the dose of 1 mM. Then we induced M1 macrophages polarization by LPS plus INF-γ to evaluate the inhibition efficacy of C.EDA, EDA and (+)-Borneol. As displayed in Fig. 5B-C, C.EDA treatment could only slightly decrease the accumulation of M1 macrophage associated cytokines (IL-6, TNF-α) in the supernatant. Next we treated quiescent macrophages with indicate dose of C.EDA, EDA and (+)-Borneol and found that C.EDA could significantly induce the accumulation of M2 macrophage associated cytokines IL-10 in culture supernatant. While, the effect of EDA on M2 macrophage polarization was much weaker than C.EDA (Fig. 5D). Next, we explored this effect through detecting M1 and M2 macrophage specific markers CD86 and CD206 by flow cytometry. As displayed, C.EDA could selectively promote quiescent macrophages polarize to M2 phenotype (from 10% to 31%) (Fig. 5E).

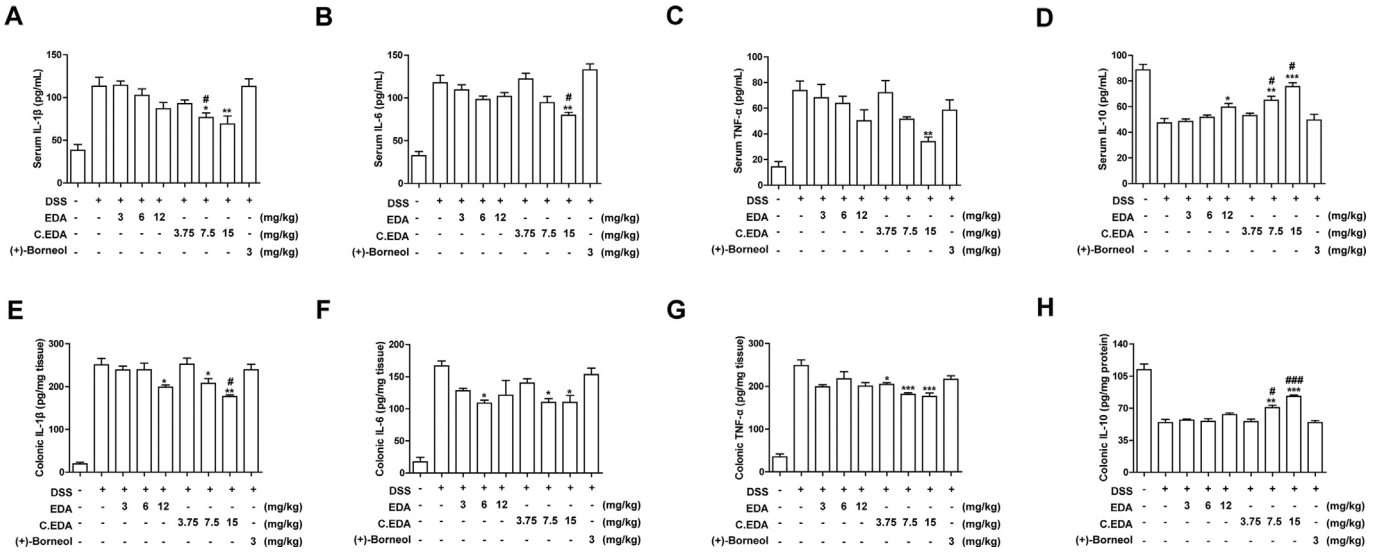

Fig. 3. C.EDA exhibited a stronger ability in decreasing the amount of pro-inflammatory cytokines and increasing the level of anti-inflammatory cytokine in both serum and colon than EDA or (+)-Borneol alone. (A-D) The protein levels of cytokines IL-1β, IL-6, TNF-α and IL-10 in serum were determined by ELISA. (*E*-H) Protein levels of cytokines including IL-1β, TNF-α, IL-6 and IL-10 in colonic homogenates were determined by ELISA. Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle treated group (n = 5 per group); $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ vs. equivalent dose of EDA, respectively.

3.4. C.EDA strengthened the activation of JAK2-STAT3 signaling pathway and promoted STAT3 nuclear translocation

The JAK2-STAT3 signaling pathway plays a vital role in M2 macrophage polarization. Thus we analyzed the phosphorylation levels of JAK2 and STAT3 in RAW264.7 cells by Western blotting. Fig. 6A and B showed that C.EDA could significantly stimulate the phosphorylation of JAK2 and STAT3 in a dose-dependent manner, while EDA displayed

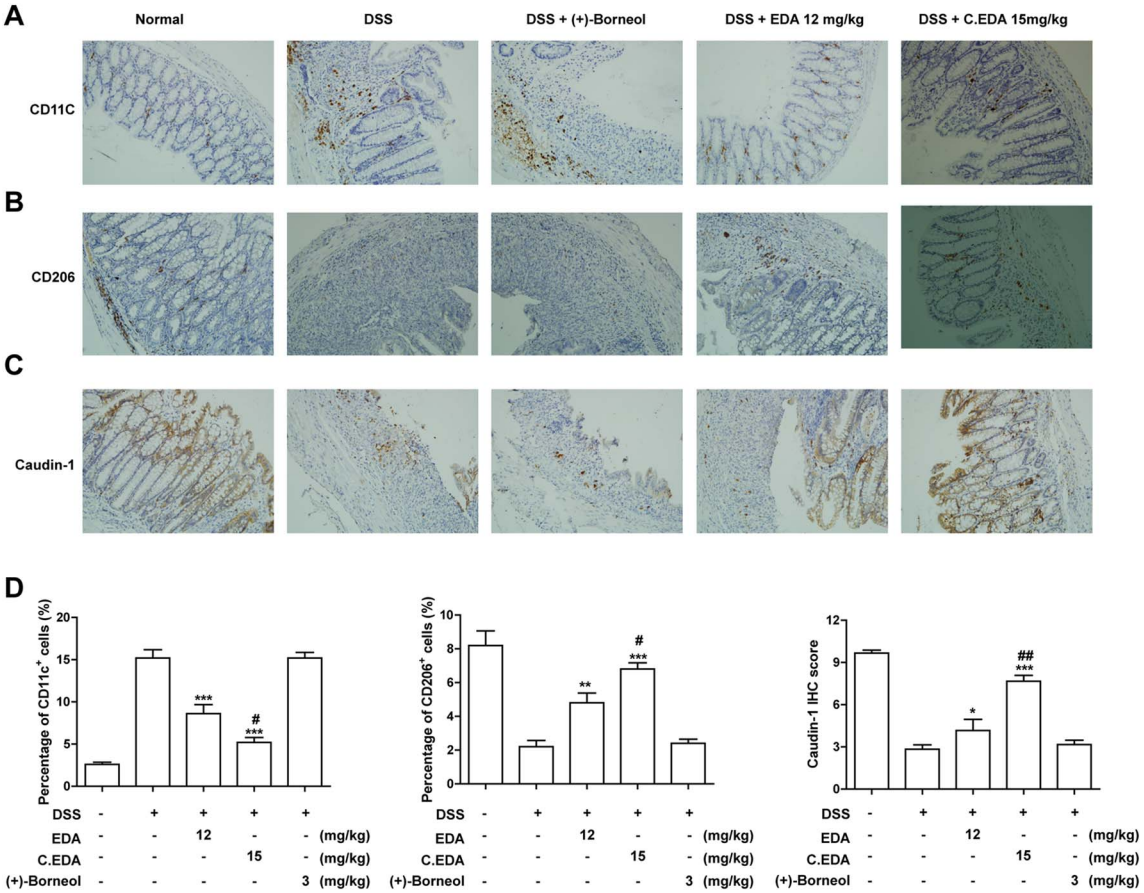

Fig. 4. C.EDA decreased the infiltration of M1 macrophages, increased the infiltration of M2 macrophages and protected the integrity of colon tissues,which were more effective than EDA and (+)-Borneol. Sections of colon tissues were separated and immunostained with anti-CD11c, anti-CD206 and anti-Caudin-1 antibodies, then observed by laser-scanning microscope, 200×. (A) The immunostaining of CD11c, (B) CD206 and (C) Caudin-1, the percentage of CD11c, CD206 positive cells and IHC score were shown in (D). (n = 5 per group). Data are shown as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle treated group; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ vs. equivalent dose of EDA, respectively.

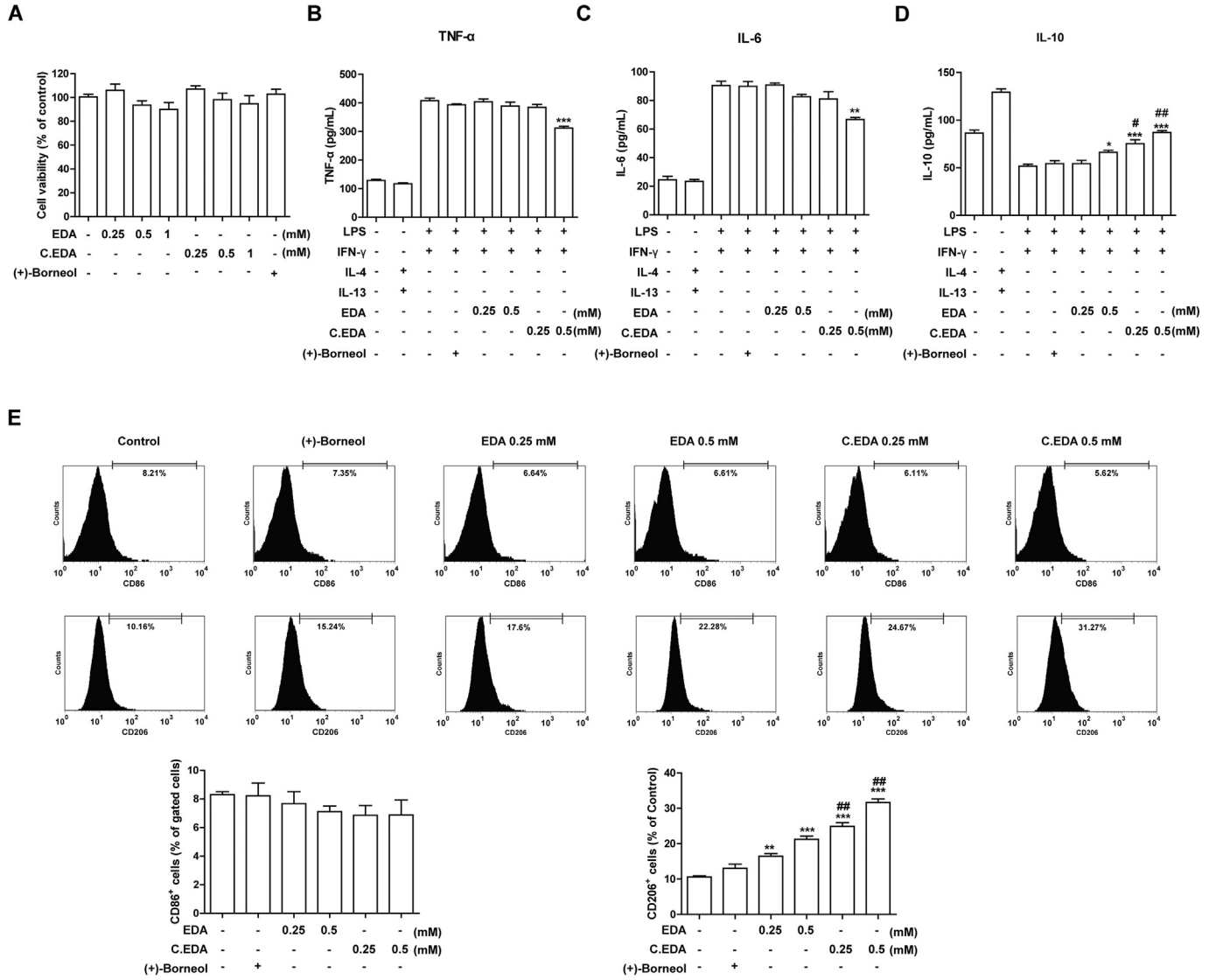

Fig. 5. C.EDA had no effect on M1 macrophage polarization, but could promote M2 macrophage polarization in vitro. (A) RAW264.7 cells were treated with indicate doses of C.EDA, EDA and (+)-Borneol for 24 h, then cell viability were detected by cck-8 reagent. (B-D) M1 macrophages were treated indicate doses of C.EDA, EDA and (+)-Borneol for 24 h during the polarization induced by 500 ng/mL LPS plus 20 ng/mL IFN-γ. Amount of released TNF-α (B), IL-6 (C) and IL-10 (D) in the supernatant of RAW264.7 cells were detected by ELISA. (E) RAW264.7 cells were treated with indicate doses of C.EDA, EDA and (+)-Borneol for 24 h, then expression of M1 macrophage marker CD86 and M2 macrophage marker CD206 was detected by flow cytometry. Data are shown as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated group; #P < 0.01, ##P < 0.01 vs. equivalent dose of EDA, respectively.

little effect on these processes. Furthermore, in a time course experiment we found that 0.5 mM C.EDA induced the expression of p-JAK2 and p-STAT3 as early as 5 min post exposure, with a steady increasing up to 60 min. While, EDA and (+)-Borneol only has little effects (Fig. 6C and D). In vivo study also revealed that C.EDA also possessed a stronger ability in stimulating the phosphorylation of STAT3, contributing to the expression of anti-inflammation cytokine IL-10 than EDA (Fig. S1). STAT3 would dimerize after phosphorylation and these dimeric STAT3 translocate to the nucleus, binding to consensus STAT3 binding sequences within the promoter region of target genes, thereby activating their transcription, resulting in M2 macrophage activation. Furthermore, we investigated whether C.EDA could promote STAT3 translocation. Immunofluorescence assay displayed that both C.EDA and EDA could significantly promote STAT3 nuclear translocation, while C.EDA was much more powerful than EDA (Fig. 6E). The same result was detected when we separated the fraction of nucleus and cytoplasm (Fig. 6F).

3.5. STAT3-specific RNA interference abolished the effects of C.EDA on inducing M2 macrophages polarization

To further confirm that STAT3 activation plays an important role in C.EDA mediated M2 macrophages polarization, RAW264.7 cells were treated with C.EDA, EDA or (+)-Borneol respectively in the presence of siRNA control (siNC) or STAT3-specific RNA interference (siSTAT3). As shown in Fig. 7A, STAT3-specific RNA interference could significantly decrease the STAT3 protein levels. Western blotting showed that siSTAT3 siRNA significantly blocked STAT3 phosphorylation induced by C.EDA, EDA or IL-4 plus IL-13 (Fig. 7B). The result of Flow cytometry reflected that the ability of C.EDA and EDA in promoting M2 macrophage polarization was totally abolished without a difference by STAT3-specific RNA interference (Fig. 7C and D). Furthermore, the expression of STAT3 target genes Arg-1 and IL-10 which involved in M2 macrophages polarization were also inhibited after STAT3-specific RNA interference treatment (Fig. 7E and F). Finally, our data suggested that C.EDA promoted M2 macrophages polarization mainly through JAK2-STAT3 signaling pathway. The graphical abstract of this effect was

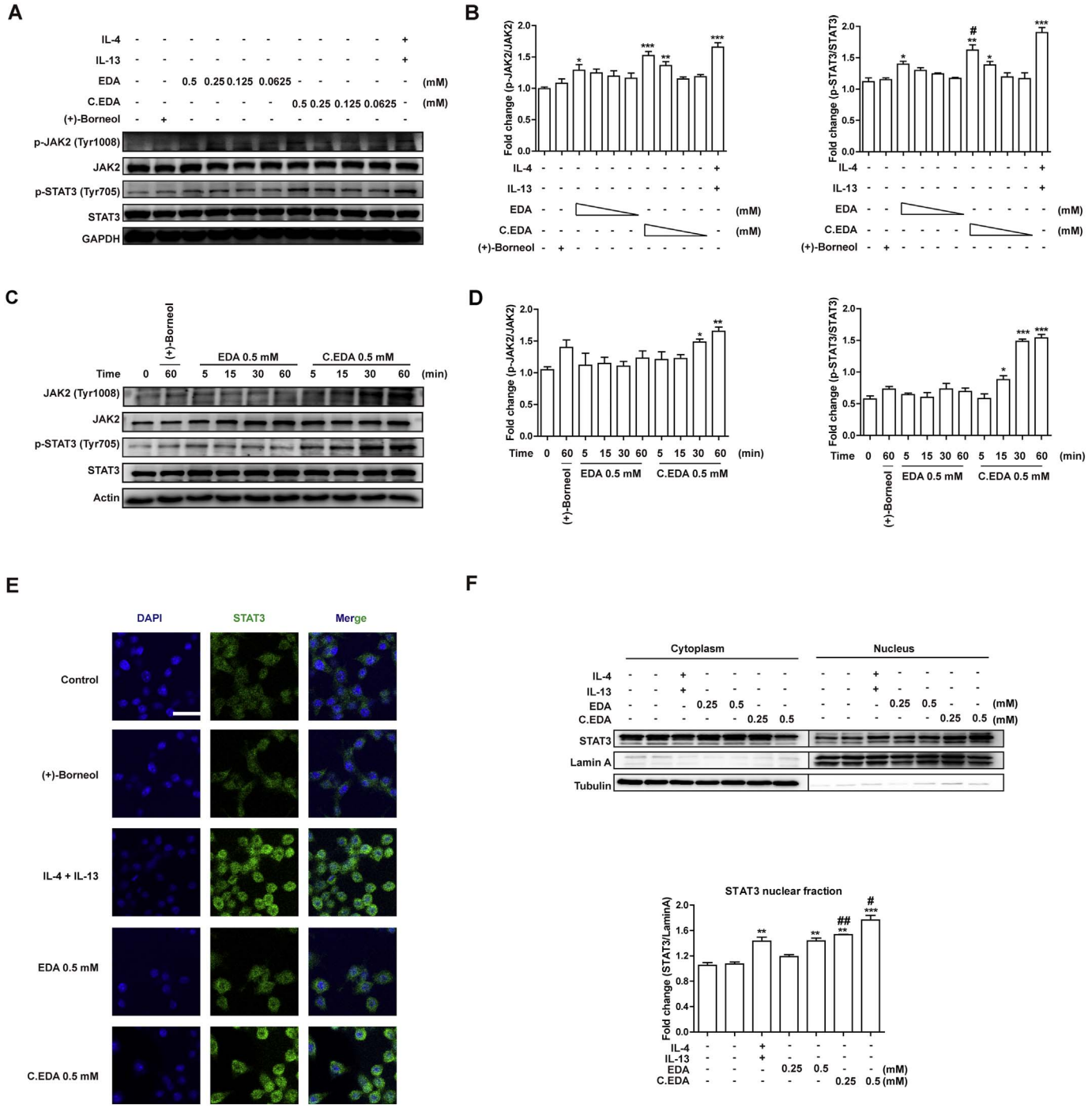

Fig. 6. C.EDA reinforced the activation of JAK2-STAT3 signaling pathway. RAW264.7 cells were treated with indicate doses of C.EDA, EDA and (+)-Borneol for 24 h, then the phosphorylation levels of JAK2 and STAT3 were detected by Western blotting (A) and phosphorylation of JAK2 and STAT3 was normalized to total protein levels (B). RAW264.7 cells were treated with 0.5 mM C.EDA, EDA or (+)-Borneol for 5, 15, 30 and 60 min, then the phosphorylation levels of JAK2 and STAT3 were detected by Western blotting (C) and phosphorylation of JAK2 and STAT3 was normalized to total protein levels (D). RAW264.7 cells were treated with indicate doses of C.EDA, EDA and (+)-Borneol for 24 h, then the nucleus translocation of STAT3 were detected by immunofluorescence (E) and western blotting (F). Bar = 20 μm. Data are shown as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated group; #P < 0.01, ##P < 0.01 vs. equivalent dose of EDA, respectively.

shown in Fig. 8.

4. Discussion

Nature Borneol, only contains (+)-Borneol, is a traditional Chinese medicine often used to increase the therapeutic effect of various drugs by improving their permeability and increasing drug concentrations in the brain [23,24]. C.EDA is a compound preparation composed of edaravone and borneol with the mass ratio of 4: 1 and has been finished Phase III clinical trial for Acute Ischemic Stroke approved by CFDA in 2016, having a better activity than EDA alone in fighting against sepsis. However, whether the combination of (+)-Borneol and EDA has a synergistic effect on treating colitis is remaining unknown. Furthermore, the precise mechanism of C.EDA in fighting against inflammation still needs to be further studied. In this study, we first found that (+)-Borneol improved the efficacy of EDA against DSS-induced colitis

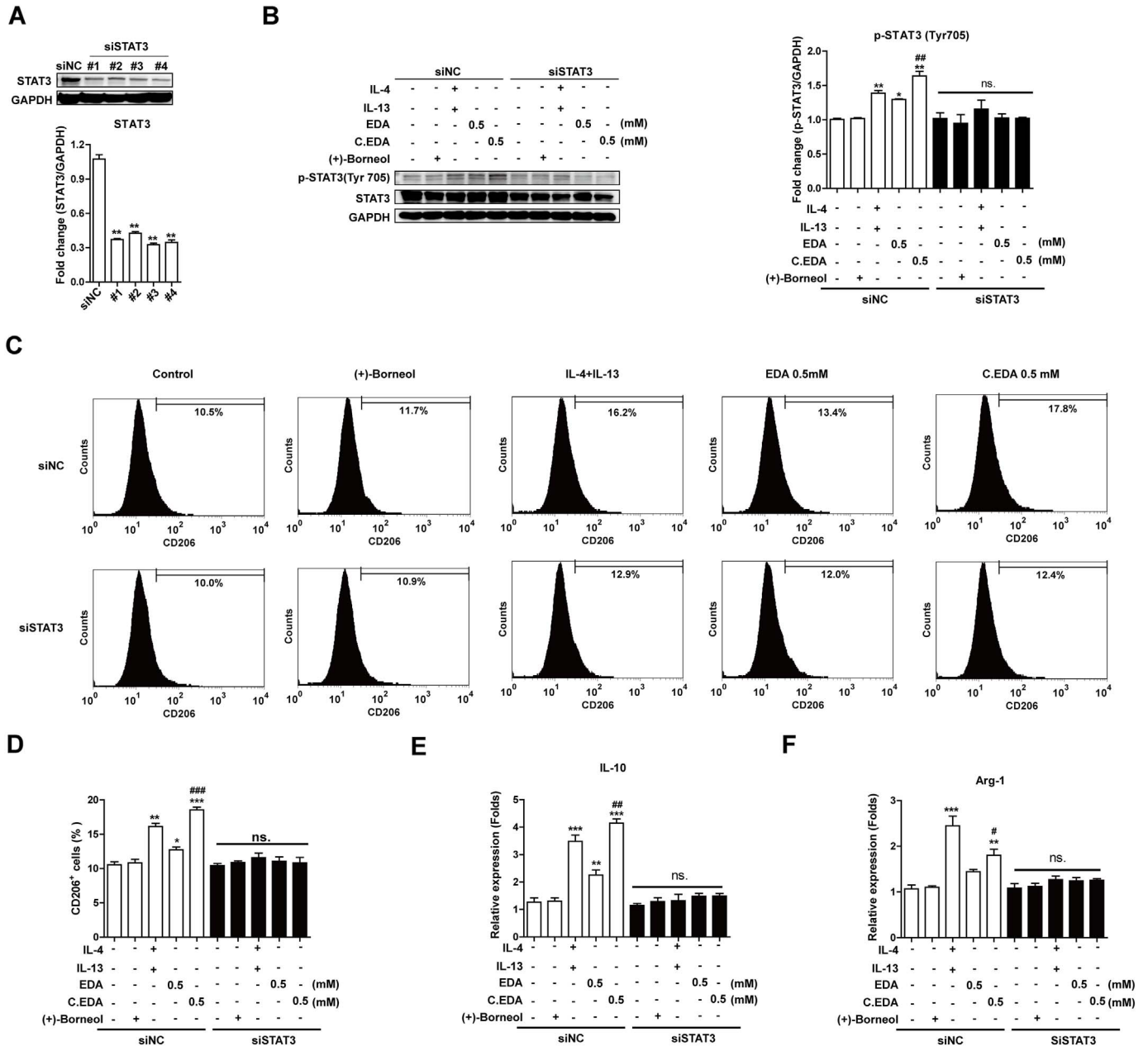

Fig. 7. C.EDA promoted M2 macrophage polarization mainly through JAK2-STAT3 signaling pathway. (A) RAW264.7 cells were transfected with STAT3 siRNA or control siRNA for 48 h and the interference efficiency were detected by western blotting and normalized to total protein levels. (B–F) RAW264.7 cells were transfected STAT3 siRNA or control siRNA for 24 h, then the infected cells were treated with indicated doses of C.EDA, EDA or (+)-Borneol for another 24 h. The phosphorylation of STAT3 were detected by western blotting and normalized to total protein levels (B). The population of M2 macrophages was analyzed by flow cytometry (C and D). The expression of IL-10 (E) and Arg-1 (F) was detected by real-time PCR. Data are shown as mean ± SEM, *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated group; #P < 0.01, ##P < 0.01 vs. equivalent dose of EDA, respectively.

by promoting M2 macrophage polarization via JAK2-STAT3 signaling pathway.

IBDs in human are closely associated with chronic inflammation in gastrointestinal tract and proposed to increase the risk of developing various types of cancers including intestinal, bladder, gastric, prostate and ovarian tumors [25,26]. To investigate whether (+)-Borneol can improve the efficacy of EDA against the symptoms of IBD, we established a mouse model colitis induced by DSS [27]. We found that 2.5% DSS induced acute colitis including basal body weight loss, bloody stools and shortened colon length in mice, which were consistent with previous study. Evidence has shown that C.EDA was more powerful in improving the symptoms of DSS induced colitis than EDA at the same dosage. While, (+)-Borneol alone had little activity. Hence, our results revealed that the combination of (+)-Borneol and EDA had a synergistic effect against colitis and (+)-Borneol improved the efficacy of EDA against DSS-induced colitis.

A hallmark of DSS-induced colitis is the increase production of pro-inflammatory cytokines. Among these cytokines, IL-1β, IL-6 and TNF-α play central roles in intestinal inflammation [28,29]. In our study, C.EDA successfully decreased the high levels of IL-1β, IL-6 and TNF-α in both serum and colon tissues in a dose-dependent manner. Meanwhile, EDA and (+)-Borneol only have slightly effects. Furthermore, we also found that C.EDA could increase the levels of anti-inflammatory cytokines, such as IL-10. IHC analysis also revealed that C.EDA increased the infiltration of M2 macrophages, decreased the infiltration of M1 macrophages and protected the integrity of colon tissue, which were more markedly than EDA at the same dose. Our results indicated that (+)-Borneol reinforced the effects of EDA on modulating macrophage

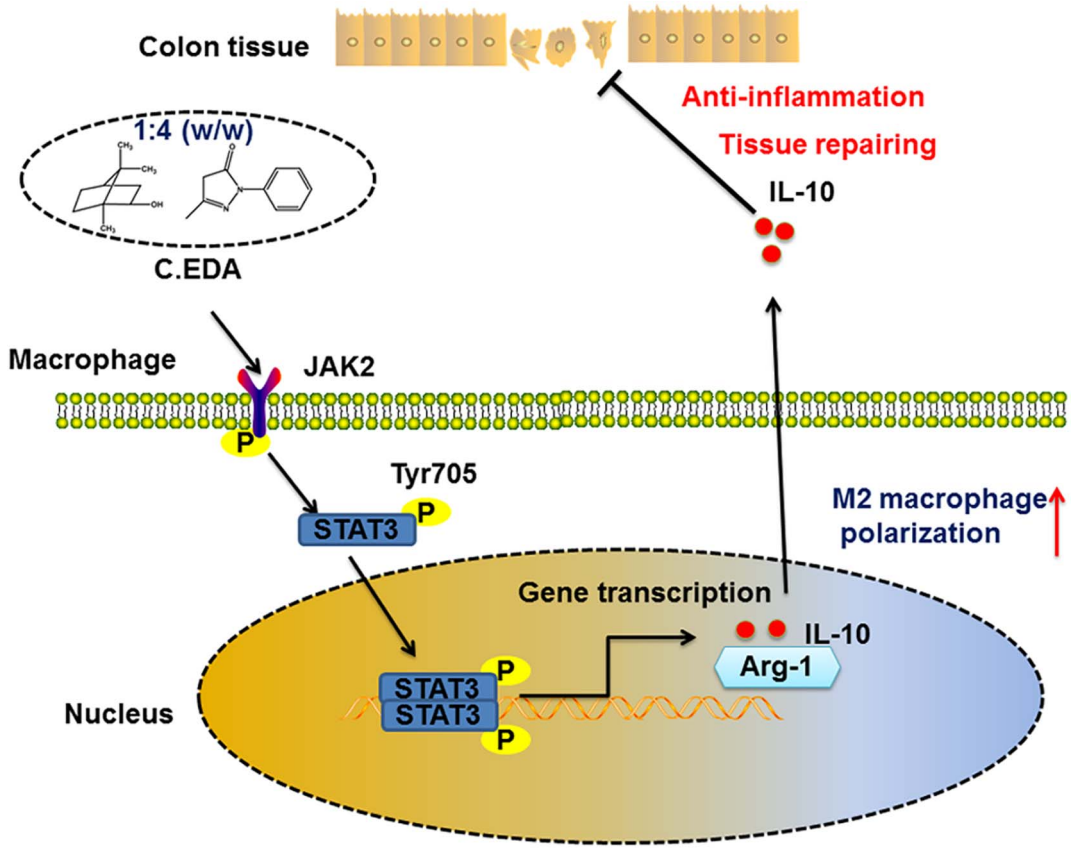

Fig. 8. A proposed scheme representing that C.EDA promotes M2 macrophage polarization.

polarization.

Switching from a pro-inflammatory response (M1) to an inflammation-resolving anti-inflammatory response (M2) during the progression of inflammation is the most important mechanism for controlling immune homeostasis [11,30–32]. Our in vitro study further demonstrated that C.EDA had little effect on M1 macrophage polarization but could increase M2 macrophage polarization. Thus, the inhibition of pro-inflammation cytokines expression and rebuilding of intestinal epithelial construction in DSS-induced colitis by C.EDA might result from M2 macrophages activation. According to the studies, STAT3 is one of the primary transcription factors for macrophage polarization toward M2 phenotype [33,34]. The phosphorylation of STAT3 by JAK2 promotes the nucleus translocation of STAT3 and activates the expression of M2 macrophages related anti-inflammatory factors, such as IL-10 and Arg-1 [35,36]. In this study, we found that C.EDA could significantly promote the phosphorylation and nucleus translocation of STAT3, compared with Control or EDA treatment. Inhibiting the activation of STAT3 by STAT3 specific siRNA could totally abolish the ability of C.EDA and EDA in promoting M2 macrophage polarization and producing of anti-inflammation factors IL-10 and Arg-1. Indeed, these results showed that C.EDA promoted the activation M2 macrophage polarization mainly through JAK2-STAT3 signaling pathway.

Collectively, our work discovered that (+)-Borneol improved the efficacy of EDA against DSS-induced colitis by promoting M2 macrophage polarization via JAK2-STAT3 signaling pathway. Our findings defined a new potential use of C.EDA in fighting against colitis.

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Conflict

The authors declare that they have no conflicts of interest concerning this article.

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Author contribution

Xiong Zhang and Fang Xu contributed equally. Study conception and design: X. Wu and Q. Xu; acquisition, analysis and/or interpretation of data: X. Zhang and F. Xu; drafting/revision of the work for intellectual content and context: X. Wu; final approval and overall responsibility for the published work: X. Wu and Q. Xu.

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