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Suppression of miR-21 and miR-155 of macrophage by cinnamaldehyde ameliorates ulcerative colitis

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ABSTRACT

Ulcerative colitis (UC) is a major form of inflammatory bowel disease which involved mucosal immune dysfunction. Cinnamaldehyde (CA) is major active compound from cinnamon, a useful traditional medicine in Asia which shows superior antibacterial and anti-inflammatory activity. In this study, we investigated the effects of CA on UC both in vivo and in vitro. We showed that CA attenuated the symptoms of DSS-induced colitis, including loss of body weights, disease activity index (DAI), shortening of the colon lengths and infiltration of inflammatory cells. Moreover, CA decreased the pro-inflammatory cytokines and NLRP3 inflammasome, miR-21 and miR-155 in colon tissues, in addition, the percentage of macrophages was reduced based on the surface marker F4/80 and IL-10 secretion in CA-treated group, suggesting that the CA ameliorate the UC via activation of macrophage. Herein, the effects of CA on macrophage cells were examined in vitro. We found that CA reduced the level of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, in the activation of RAW264.7, human macrophage-like cells U937, and primary peritoneal macrophages. Furthermore, the suppression of NLRP3 inflammasome, miR-21 and miR-155 was also found in CA-treated LPS-stimulated RAW264.7 cells. CA also reduced the production of reactive oxygen species, the phosphorylation of AKT, mTOR and COX2 protein level in the RAW264.7. Meanwhile, data revealed that transferred miR-21 or miR-155 inhibitor suppressed levels of IL-1β and IL-6, whereas miR-21 or miR-155 mimics increased expressions of these, and CA suppressed these expressions. Our results indicate that CA could ameliorate DSS-induced colitis through inhibition of NLRP3 inflammasome activation and miR-21 and miR-155 levels in colons and macrophage, suggesting that CA might be a potentially effective drug for UC.

1. Introduction

Inflammatory bowel disease (IBD) is an intractable autoimmune disease, leading to a chronic inflammation of the digestive system, which can be classified as either ulcerative colitis (UC) or Crohn's disease (CD), depending on the site and pattern of inflammation [1], and UC is characterized by pathological mucosal damage and ulceration, which extend proximally from the rectum to varying degrees [2]. In the last few decades, the incidence of UC in Western countries and China has risen dramatically and typically presents as a relapsing disorder marked by attacks of bloody mucoid diarrhea that can persist for months, only to recur after an asymptomatic interval of months to years [3]. Detailed pathomechanism of UC is still unresolved, although combination of genetic, environmental and immunological factors has been implicated in the etiology of the disease [4].

Highly activated macrophage, monocyte, and neutrophil counts in the peripheral blood or inflamed gut of UC patients have been reported [5]. The activated macrophages/monocytes produce pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which disrupts the epithelial barrier and induces inflammation in the intestines [6,7]. The production of IL-1β requires converting the inactive precursor, pro-IL-1β, to an active form by caspase-1-dependent cleavage [8]. The activation of caspase-1 is based on the formation of inflammasome, a multiprotein complex consisting of a NOD-like receptor (NLR), the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 [9]. At present, one of the most intensively studied inflammasomes is NLRP3, which is a critical regulator of inflammation and plays an important role in

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infected macrophages [10].

MicroRNA (miRNA) is a small non-coding RNA molecule that regulates gene expression at the post-transcriptional level, which results in translational repression or mRNA degradation [11,12]. Specific miRNAs such as miR-21, miR-155 can be activated by inflammatory mediators and expressed both in DSS induced colitis and macrophages [13–15]. They have been proposed to be involved in the regulation of inflammatory processes in macrophages.

Cinnamon (*Cinnamomum osmophloeum kaneh*) is used extensively as a traditional herbal medicine in Asia, and cinnamaldehyde (CA) is a major bioactive compound isolated from the essential oil of cinnamon leaves. Previous studies have found that CA exhibited anti-hyperglycaemia, antibacterial and anti-inflammatory activities [16–18]. It was reported that CA can inhibit the expression of pro-inflammatory cytokines in macrophages, including TNF-α, IL-1β, and nitric oxide (NO) [19], we also found that CA inhibited PLCγ-1 activation in mucosal mast cells [20]; however, the protective effects of CA on UC remain unknown. Dextran sodium sulfate (DSS), a chemical colitogen with anticoagulant properties, is commonly used to make a model of UC. So we at the first time investigated the effect of CA on DSS-induced colitis in BALB/c mice and the detail mechanism of action.

2. Materials and methods

2.1. Reagent

CA, DCFH-DA, phorbol myristic acid (PMA) and lipopolysaccharides (LPS) were purchased from Sigma (St. Louis, USA), DSS (molecular weight 36,000-50,000) was purchased from MP Biomedicals (Solon, USA). RNAiso plus regent, PrimeScript RT regent kits (RR047A), and SYBR Premix Ex Taq (RR420A) were purchased from TaKaRa Bio (Shiga, Japan). The miRNA detection kits (Magen, R431002) and All-in-one miRNA qRT-PCR reagent kits (GeneCopoeia QP015) were purchased from GeneCopoeia (Rockville, USA). Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin (P/S) were purchased from Life Technologies (Grand Island, USA). Antibodies against cyclooxygenase-2 (COX2) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies against P-AKT, AKT, P-mTOR and mTOR were purchased from Cell Signaling Technology (Beverly, USA). 5-Aminosalicylic acid (5-ASA) was purchased from Macklin (Shanghai, China). Griess reaction kit was purchased from Beyotime Biotechnology (Shanghai, China).

2.2. Cell culture

The mouse macrophage cell line RAW264.7 and human monocytes U937 was purchased from the Shanghai Institute for Biological Sciences (Shanghai, China). RAW264.7 cells were cultured in DMEM, and U937 cells were cultured in RPMI 1640 media containing 10% FBS, 100 U/mL P/S in a constant temperature incubator at 37 °C with 5% CO_2. U937 cells were then incubated with 100 ng/mL PMA to differentiate into macrophage-like cells. After 48 h, the medium was replaced with fresh RPMI 1640 without PMA, and the differentiated cells were incubated for an additional 24 h prior to use.

2.3. Animals

BALB/c mice (male, 6–8 weeks) were obtained from the Shanghai Laboratory Animal Centre of the Chinese Academy of Sciences and raised by the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). The mice were maintained under specific pathogen-free conditions at 18–24 °C and 40–70% humidity, with a 12 h light-dark cycle. Food and drinking water was available ad libitum. All experimental procedures were examined and approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine.

2.4. Murine macrophages

Primary mouse peritoneal macrophages were harvested by peritoneal lavage from BALB/c mice [21]. Briefly, the animals were euthanized and 5 mL of cold PBS was injected into the peritoneal cavity. The abdomen was massaged 30 times. Then, the PBS was recovered by injector and centrifuged at 1300 rpm for 5 min. Cells were seeded into a 24-well plate and cultured in RPMI 1640; 4 h later, the medium was changed to remove the erythrocytes.

2.5. Induction of DSS colitis

Acute colitis was induced by 5% DSS in the drinking water for 7 days. The mice received either drinking regular water (normal) or DSS drinking water (control) in the presence or absence of the positive control anti-inflammatory drug 5-ASA (50 mg/kg) or CA (10 mg/kg). Determination of weight and examination of inflammation-associated rectal bleeding were conducted every day. The mice were sacrificed at day 8 and the length of the colon was determined, which indirectly stipulated the inflammatory index of the colon. The colon specimens were fixed in 10% formalin and embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and examined using a microscope.

2.6. Immunohistochemistry (IHC)

Briefly, paraffinembedded slides were deparaffinized, rehydrated, and washed in 1% PBS. After that, they were blocked with 10% goat serum for 1 h at 37 °C. Then, slides were treated with primary antibodies (1:100) overnight at 4 °C. Biotinylated secondary anti-rabbit antibodies were added and incubated at room temperature for 1 h. Streptavidin-HRP was added, and after 40 min the sections were achieved using diaminobenzidine as a chromogen and counter-stained with hematoxylin, Images at 200× magnification were examined with a microscope.

2.7. F4/80 immunofluorescence

The paraffin-embedded colon tissues were cut into 4-μm-thick sections. The sections were treated with 10% normal goat serum for 30 min at room temperature, followed by overnight incubation with the anti-F4/80 antibody (diluted 10:1; Ebioscience; San Diego, CA) at 4 °C. F4/80-positive staining was visualized with diaminobenzidine as a chromogen. Images were captured using a microscope fitted with Zeiss UPlanApo lenses (×10 and ×20 magnification).

2.8. Nitrite assay

RAW264.7 cells were pre-treated with CA (5, 10, or 20 μM) for 2 h and then stimulated with LPS (1 μg/mL) for 24 h. The nitrite accumulation in the supernatant was assessed by a Griess reaction kit, according to the manufacturer's protocol.

2.9. Measurement of reactive oxygen species (ROS) production

ROS production was detected by measuring intracellular ROS formation using the probe DCFH-DA. Briefly, RAW264.7 cells were pretreated with CA (5, 10, or 20 μM) for 2 h and then stimulated with LPS (1 μg/mL) for 6 h to promote ROS generation. Cells were washed twice with PBS and then incubated with DCFH-DA probe (20 μM) for 30 min. Fluorescence staining was visualized using a fluorescence microscope (Olympus, Japan), and the fluorescence was measured with a fluorescence plate reader (BIO-TEK, USA) at an excitation/emission of 485/525 nm.

2.10. Transfection assay

RAW264.7 cells were seeded into each well of a 6-well plate and were maintained in DMEM containing 10% FBS and were treated with either control vehicle. The cells were then transfected with inhibitors of miR-21 (GeneCopoeia AN0316) or miR-155(AN0890), and mimics of miR-21(SN0316) or miR-155(SN0890) mimic at a final concentration of 100 nM using nanoscale micro poly transfection regent, according to the manufacturer's protocol. The transfection reagent was mixed and incubated at room temperature for 10 min. After changing the medium, this mixture was added to each well, and the culture plate was shaken to mix thoroughly, followed by culturing in an incubator containing 5% CO_2 at 37 °C for 48 h.

2.11. Detection of mRNA

Total RNA was isolated from cells and colonic tissues using RNAiso plus reagent, according to the manufacturer's protocol. An equal amount of RNA was transcribed into cDNA using a PrimeScript RT regent kit. Subsequently, mRNA expression for the selected genes was measured using SYBR Premix Ex Taq under standard reaction conditions: 40 cycles at 95 °C for 30 s, 95 °C for 10 s and 60 °C for 30 s. The expression levels in the samples were determined by the relative quantity curve method. Primer sequences as Table 1.

2.12. Detection of miRNA

miRNA was extracted using Hi Pure Universal miRNA kit, according to the manufacturer's protocol. miRNA single-stranded cDNA was synthesized from 100 ng of miRNA using miRNA reverse transcription and was detected using an All-in-one miRNA qRT-PCR reagent kit. The PCR amplification was performed using the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s. Both results were detected using an ABI 7500 Sequencing Detection System (Foster, USA). The data were analyzed by the relative quantity curve method, and all values were normalized to the level of the housekeeping gene U6.

Table 1
Primer sequences for real-time PCR.

Gene	Primer (5′-3′)
Mus-TNF-α-F	CCACCACGCTCTTCTGTCTACTG
Mus-TNF-α-R	CGGCTACGGGCTTGTCACTC
Mus-IL-6-F	CTTCCAGCCAGTTGCCTTCTTG
Mus-IL-6-R	TGGATGCTCTCATCAGGACAG
Mus-Il-1β-F	GAAATGCCACCTTTTGACAGTG
Mus-Il-1β-R	GGTCTGTTGTGGGTGGTATCCTC
Mus-Il-18-F	GACTCTTGCGTCAACTTCAAGG
Mus-Il-18-R	CAGGCTGTCTTTTGTCAACGA
Mus-COX2-F	AGCAACAACATAAGCGTCAT
Mus-COX2-R	CCTCAAACTTGGCAATACTC
Mus-IL-10-F	GCTCTTACTGACTGGCATGAG
Mus-IL-10-R	CGCAGCTCTAGGAGCATGTG
Mus-NLRP3-F	TGCGATCAACAGGCGAGACCT
Mus-NLRP3-R	CCATCCACTCTTCTTCAAGGCTGTC
Mus-Caspase-1-F	CAAGTCTCAAGCTTTGCCCG
Mus-Caspase-1-R	TAATGAGGGCAAGACGGGTG
Mus-ASC-F	GGAGTCGTATGGCTTGGAGC
Mus-ASC-R	ACAAAGTGTCCTGTTCTGGCT
Mus-GAPDH-F	CAAGGTCATCCATGACAACTTTG
Mus-GAPDH-R	GTCCACCACCCTGTTGCTGTAG
Mus-miR-21	CTTGTCGGATAGCTTATCAGAC
Mus-miR-155	CTTAATGCTAATTGTGATAGGGGT
Mus-U6	GCTTCGGCAGCACATATACTA
Homo-TNF-α-F	CACAGTGAAGTGCTGGCAAC
Homo-TNF-α-R	AGGAAGGCCTAAGGTCCACT
Homo-IL-6-F	ATGAACTCCTTCTCCACAAGCG
Homo-IL-6-R	GGCGGCTACATCTTTGGAAT
Homo-Il-1β-F	TGCAGTGAGCGTCAGGAG
Homo-Il-1β-R	CAAGGATTTGCTGTATGGCTGAG
Homo-COX2-F	TGCAGTGAGCGTCAGGAG
Homo-COX2-R	CAAGGATTTGCTGTATGGCTGAG
Homo-GAPDH-F	CTTGCTTGGGTTGGTGGG
Homo-GAPDH-R	CGTTGAGTGGCTGCTGGAT

2.13. Western blot analysis

Total proteins in cells and colonic tissues were extracted with RIPA lysis buffer. Next, the proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% BSA for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies. The membranes were then rinsed and incubated with horseradish peroxide-conjugated secondary antibodies for 1 h at room temperature and rinsed again. The bands were visualized by ECL reagents.

2.14. Statistical analysis

All data were expressed as the mean ± standard error of mean (SEM) of at least three independent experiments. Data were analyzed by one-way ANOVA procedure of GraphPad Prism 5.0 with the Dunnett's test when more than two groups were compared; otherwise, the Student's *t*-test was used. *P* value of 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of CA on DSS-induced colitis

Oral administration of DSS in mice induces colitis that resembles human UC, and the inflammation is mainly localized to the colon [22]. It has been reported that glucocorticoid steroids and 5-ASA are effective in preventing colitis in animal models; therefore, we used 5-ASA as a positive control [23]. We observed a significant body weight loss and the incidence of bloody diarrhea, a marker of inflammation, in mice receiving DSS from day 4 onward. Administration of CA or positive drug 5-ASA significantly ameliorated the weight loss (Fig. 1A). As shown in Fig. 1B, the disease activity index (DAI) scores were increased at day 7 by 2.63 ± 0.52 in the DSS-treated group compared to the normal group. CA and 5-ASA attenuated the DSS-mediated increase in DAI scores at day 7 by 1.31 ± 0.88 and 1.44 ± 0.68, respectively. After 7 days of treatment with DSS in the drinking water, there was a significant shortening of the colon length compared with the healthy control mice, and this parameter was reversed by CA treatment (Fig. 1C and D). The colon histologic sections from mice exposed to DSS exhibited a discontinuous disruption of the epithelial layer and marked inflammatory cell infiltration within the mucosa and submucosa. These parameters were attenuated in mice after CA was administered (Fig. 1E). Taken together, CA prevented the progression of UC in mice.

3.2. Effects of CA on colonic inflammation

Increased production of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6 and COX2, plays a critical role in DSS-induced UC. In the present study, we also found that the mRNA expression of TNF-α, IL-1β, and IL-6 was markedly decreased in the CA-treated group compared with the DSS model group (Fig. 2A). Moreover, COX-2 mRNA level and protein expression was decreased in the CA-treated groups (Fig. 2B and C). Additionally, as shown in immunohistochemistry analysis (Fig. 2D), the expression of COX2 in colon was increased in DSS-induced colitis and CA decreased this phenomenon, suggesting that CA inhibit the inflammation of colitis.

3.3. Effects of CA on the NLRP3 inflammasome and microRNA level

NLRP3 inflammasome plays a crucial role in occurrence and

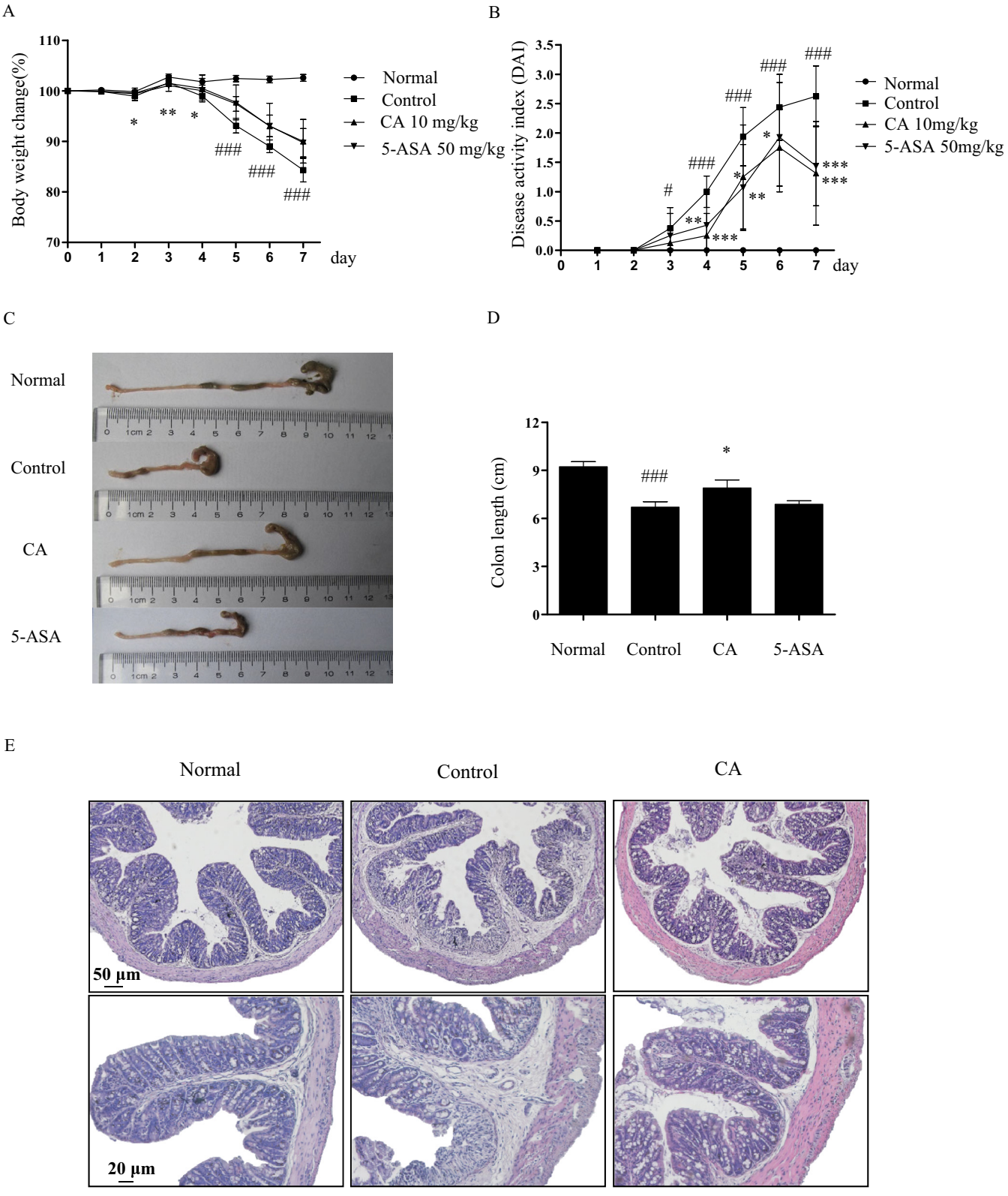

Fig. 1. CA attenuated the development of UC in mice.
The body weight (A) and disease severity (DAI, B) in colitis mice treated with CA or 5-ASA. (C and D) Change in colon length after mice were sacrificed. (E) Colonic tissue sections from each mouse were stained with (HE). The data are presented as the mean ± SEM. $n = 8$; $\#p < 0.05$, $\#\#\#p < 0.001$ vs normal group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs control group, using one-way ANOVA procedure followed with the Dunnett's test.

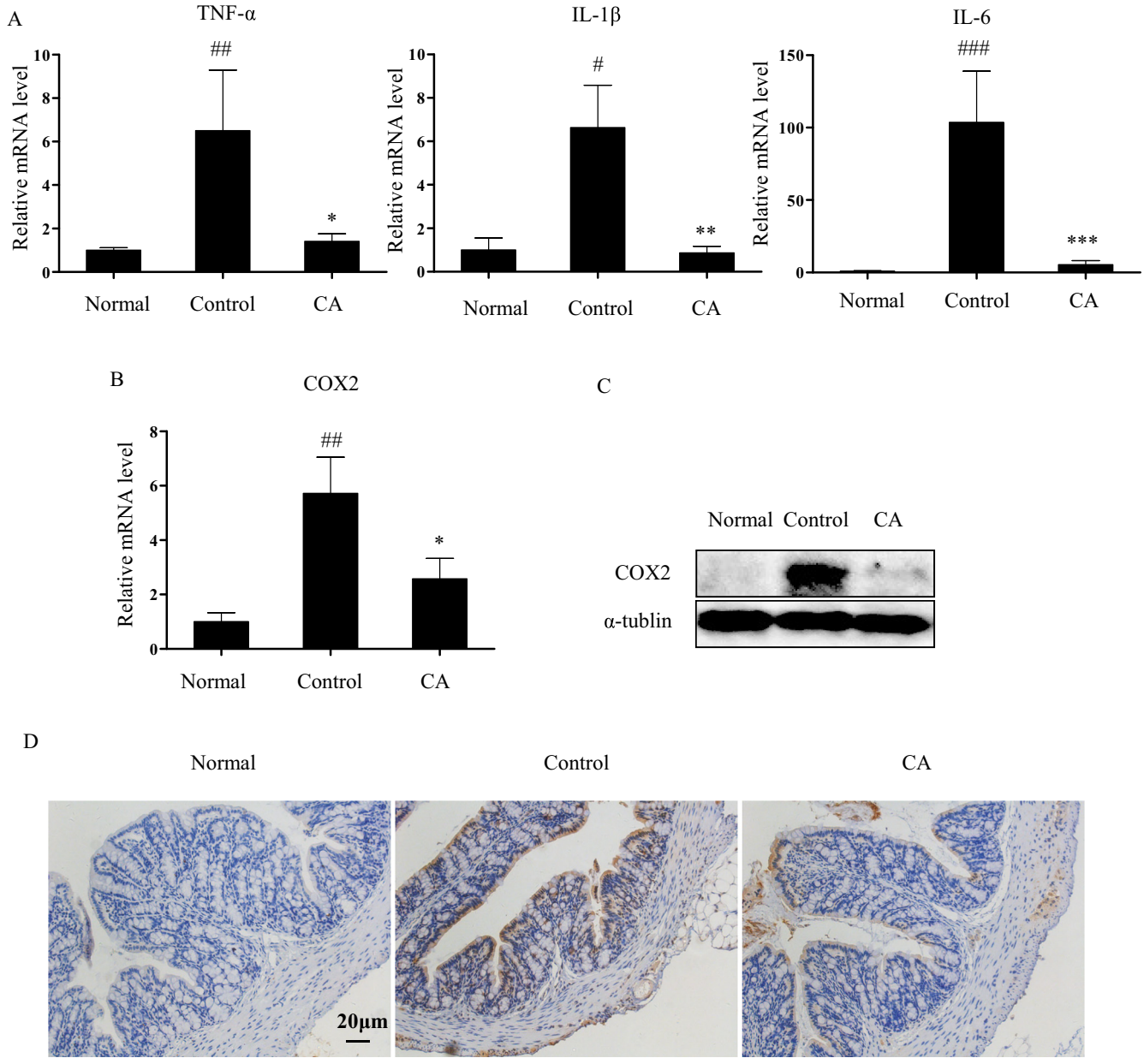

Fig. 2. CA reduced the inflammation factors in colonic tissues.
Administration of CA inhibited inflammation factors. (A-B) Total RNA was collected from the colonic tissues and assayed for TNF-α, IL-1β, IL-6 and COX2 by real-time PCR. The data are presented as the mean ± SEM, $n = 8$. (C) The level of COX2 protein expression was measured by Western blotting. (D) COX2 expression levels in the colonic tissues were examined by IHC at 20× magnification. Scale bar, 20 μm. The data are presented at least three independent experiments. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs normal group; $*p < 0.05$, $**p < 0.01$, $***p < 0.01$ vs control group, using one-way ANOVA procedure followed with the Dunnett's test.

development of colitis, and controls the production of IL-1β [24]. As shown in Fig. 3A–D, CA significantly reduced the mRNA levels of NLRP3, caspase-1, ASC and IL-18 in colons. miR-21, miR-155 can be activated by inflammatory mediators and expressed in DSS induced colitis [25,26]. Therefore, the effects of CA on the microRNA were further detected. CA could inhibit the expression of miR-21 and miR-155 in mRNA level (Fig. 3E and F), suggesting that CA can reduce inflammation via miR-21 and miR-155 inhibition.

3.4. Effects of CA on the macrophage of colon in colitis mice

Macrophages as innate immune cells and fast responders to antigens play a central role in the IBD [27]. As shown in Fig. 3G, the mRNA level of IL-10 was expressed at low levels in the DSS model groups; however, CA reversed this. In addition, the immunofluorescence showed that macrophage marker F4/80 was increased in the DSS model, and CA could decrease the expression of macrophages (Fig. 3H), suggesting that CA inhibited UC through inhibition of macrophage activation of colon.

3.5. Effects of CA on LPS-induced inflammatory response of macrophages

MTT data showed that CA (0–100 μM) treatment for 24 h did not alter the RAW264.7 cell viability (data not shown), suggesting that CA exhibited little cytotoxicity in the macrophages. We found that CA inhibited the LPS-induced expression of TNF-α, IL-1β and IL-6 mRNA (Fig. 4A–C). Furthermore, the increased level of COX2 in the LPS-induced macrophages was significantly reduced by CA treatment (Fig. 4D). CA (20 μM) decreased the NO level (Fig. 4E) but did not

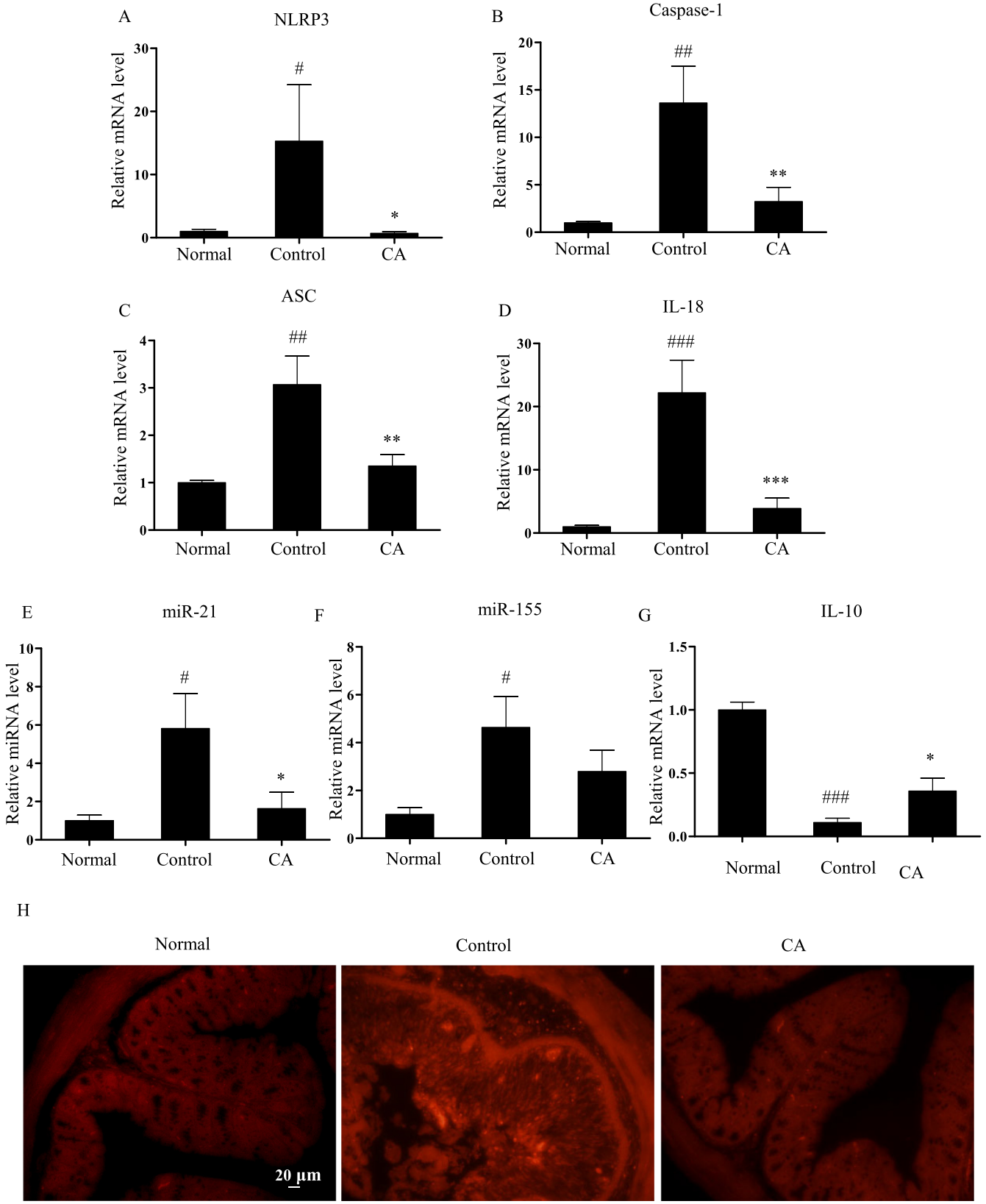

Fig. 3. Effects of on NLRP3 inflammasome, miR-21, miR-155, and macrophage in colons.
Total RNA was collected from the colonic tissues and assayed for NLRP3 (A), caspase-1 (B), ASC (C) and IL-18 (D) by real-time PCR. miRNA was collected from the colonic tissues and assayed for miR-21 (E) and miR-155 (F) by real-time PCR. (G) The mRNA level of IL-10 expression was assayed by real-time PCR. The data are presented as the mean ± SEM. $n = 6$; (H) Immunofluorescence detection of macrophage F4/80 in the colonic tissues at 20× magnification. Scale bar, 20 μm. The data are presented at least three independent experiments. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs normal group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs control group, using one-way ANOVA procedure followed with the Dunnett's test.

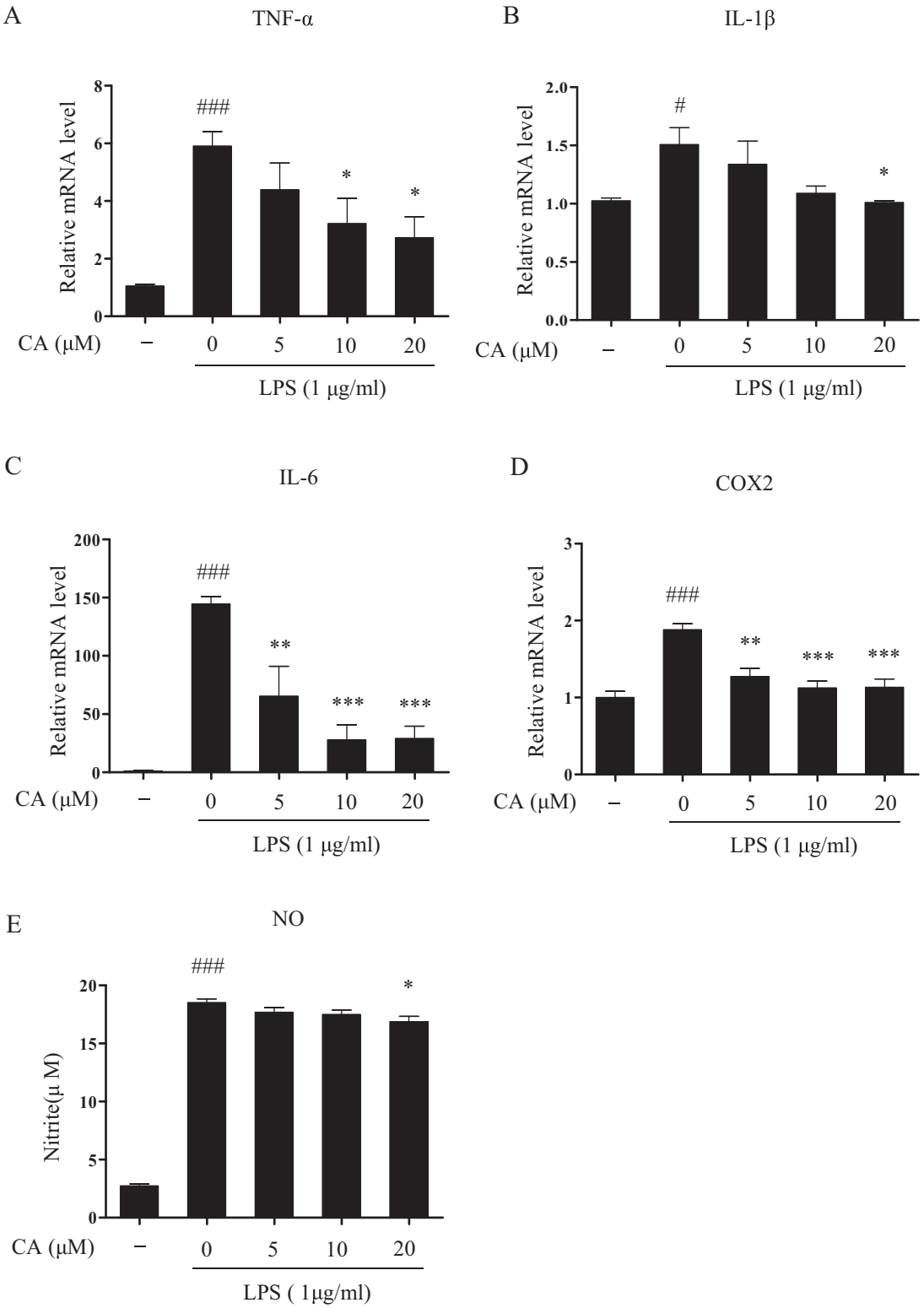

Fig. 4. CA inhibited LPS-induced inflammatory factors in RAW264.7.
RAW264.7 cells were treated with or without CA (5, 10, 20 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h; mRNA levels of TNF-α (A), IL-1β (B), IL-6 (C) and COX2 (D) were then analyzed by real-time PCR. (E) The NO release in the culture media was assayed by a Griess reaction kit. The data are presented as the mean ± SEM. The data are presented at least three independent experiments; $\#p < 0.05$, $\#\#\#p < 0.001$ vs no LPS stimulation group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs LPS stimulation group, using one-way ANOVA procedure followed with the Dunnett's test.

inhibit iNOS mRNA expression (data not shown), which is not consistent with other reports, suggesting that the low concentration of CA may not affect iNOS.

We also investigated the effects of CA on the PMA-stimulated U937 as human macrophage-like cells. We found that CA (20 μM) inhibited the LPS-induced mRNA level of TNF-α, IL-1β, IL-6 and COX2 (Fig. 5A–D) in U937-differentiated macrophages. Interestingly, CA pretreatment also significantly decreased the mRNA level of TNF-α, IL-

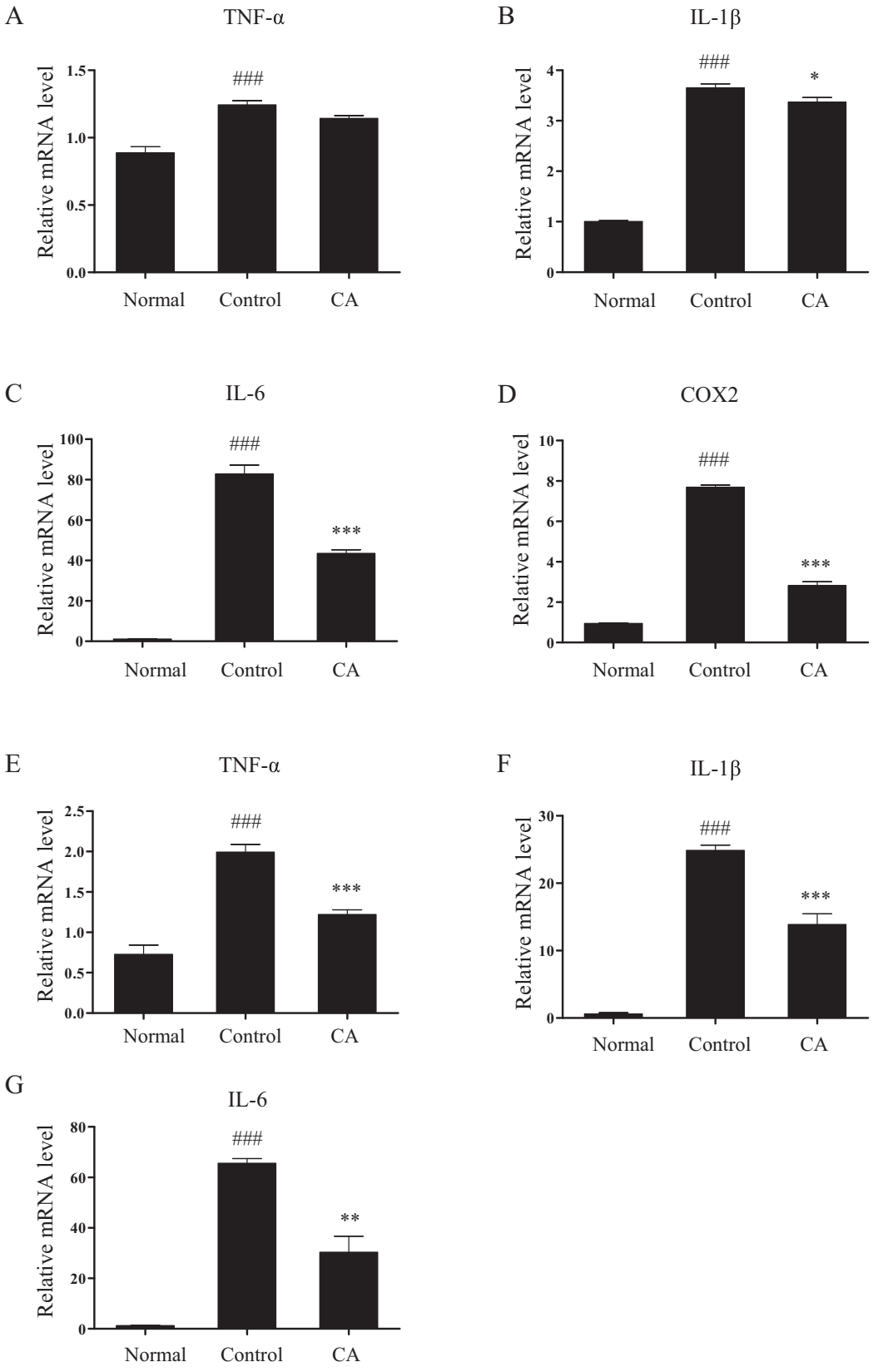

Fig. 5. CA inhibited the activation of U937-differentiated macrophages and peritoneal macrophages.
U937-differentiated macrophage cells were treated with or without CA (20 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h; TNF-α (A), IL-1β (B), IL-6 (C) and COX2 (D) were then analyzed by real-time PCR. Peritoneal macrophages were treated with or without CA (20 μM) for 2 h prior to LPS (1 μg/mL) and IFN-γ (10 ng/mL) stimulation for 24 h, TNF-α (E), IL-1β (F), IL-6 (G) were then analyzed by real-time PCR. The data are presented as the mean ± SEM; $n = 4$. ###$p < 0.001$ vs no LPS stimulation group; *$p < 0.05$, **$p < 0.01$ vs LPS stimulation group, using one-way ANOVA procedure followed with the Dunnett's test.

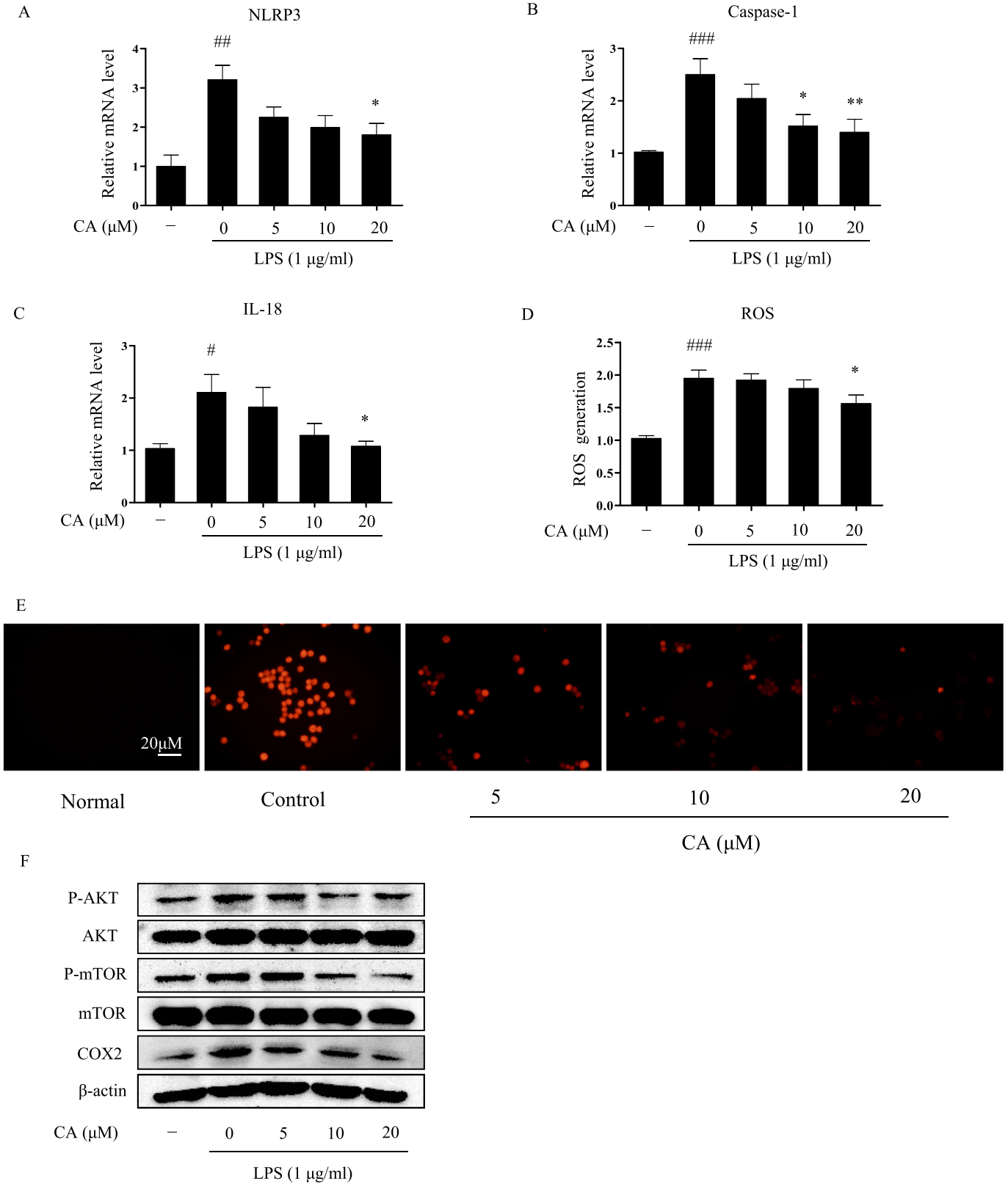

Fig. 6. CA inhibited LPS-induced inflammasomes, ROS, and AKT/mTOR activation.
RAW264.7 cells were treated with or without CA (5, 10, 20 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h; the mRNA levels of NLRP3 (A), caspase-1 (B), and IL-18 (C) were then analyzed by real-time PCR. The data are presented as the mean ± SEM; $n = 4$. The cells were incubated with LPS (1 μg/mL) for 6 h and then incubated with probe DCFH2-DA for 30 min. ROS was measured by fluorescence microplate reader (D) and by fluorescence microscopy (E). (F) The cells were treated with or without CA (5, 10, 20 μM) for 1 h prior to LPS stimulation for 1 h; the levels of phosphorylation of AKT, mTOR and COX2 expression were then measured by Western blotting. The data are presented at least three independent experiments. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ vs no LPS stimulation group; $*p < 0.05$, $**p < 0.01$ vs LPS stimulation group, using one-way ANOVA procedure followed with the Dunnett's test.

1β and IL-6 in peritoneal macrophages with LPS and IFN-γ stimulation (Fig. 5E–G). All data suggested that CA can inhibit the activation of macrophages.

3.6. Effects of CA on NLRP3 inflammasome expression in RAW264.7 cells

We further investigated the effect of CA on the activation of NLRP3 inflammasome in the LPS-induced RAW264.7, CA treatment from 5 to 20 μM notably inhibited the expression of NLRP3 (Fig. 6A) and caspase-1 (Fig. 6B), and also decreased the expression of the NLRP3 downstream cytokines IL-1β (Fig. 4B) and IL-18 in a dose-dependent manner (Fig. 6C). However, the mRNA level of ASC was not affected (data not shown), which is not consistent with in vivo data. Since recent research has demonstrated that activation of NLRP3 inflammasomes requires ROS generation [28], as shown in Fig. 6D, pre-treatment with CA for 2 h suppressed LPS-induced ROS production in a dose dependently manner. Fluorescence staining data (Fig. 6E) also showed that 20 μM CA significantly suppressed LPS-induced ROS generation. Furthermore, CA treatment decreased that the LPS-induced and COX2 protein level (Fig. 6F). CA also inhibited the phosphorylation of AKT, mTOR (Fig. 6F), suggesting that CA suppressed the macrophages activation also through the ROS-AKT/mTOR signaling pathway.

3.7. Effects of CA on miR-21 and miR-155 levels in RAW264.7 cells

As shown in Fig. 7A and B, CA inhibited LPS-induced miR-21 and miR-155 expression in macrophages, suggesting that CA can reduce inflammation via miR-21 and miR-155 inhibition. Furthermore, we also investigated the role of miR-21 and miR-155 in LPS-stimulated RAW264.7.

The miR-21 and miR-155 levels were suppressed from 1 to 0.08-(Fig. 7C) and 0.33-folds (Fig. 7D) with miR-21 or miR-155 inhibitors, whereas miR-21 and miR-155 levels were increased by 5.96- (Fig. 7E) and 13.78- (Fig. 7F) folds with miR-21 or miR-155 mimics, respectively. The level of IL-1β and IL-6 were reduced in the miR-21 or miR-155 deficient cells, and CA also inhibited these productions (Fig. 7G and H), suggesting that CA has the same effect as miR-21 inhibitors or miR-155 inhibitors. Furthermore, we also observed that IL-1β and IL-6 production was substantially enhanced in the miR-21 or miR-155 abundant cells when compared to negative mimic-transfected cells and treatment with CA significantly reduced the mRNA levels of IL-1β and IL-6 in the miR-21 or miR-155-abundant cells (Fig. 7I and J). The data demonstrated that CA could repress the inflammation by targeting miR-21 and miR-155 in macrophages.

4. Discussion

CA and its derivatives have various anti-inflammatory effects, for example, trans-cinnamaldehyde and 2-methoxycinnamaldehyde inhibited the LPS-induced DNA binding activity of NF-κB and NF-κB transcriptional activity in macrophages [29]. In the present study, our results showed that CA significantly improved the intestinal injury induced by DSS administration and inhibited mRNA expression of inflammatory cytokines in the colon tissues, suggesting that CA has an anti-inflammatory effect on the UC.

A previous study revealed that DSS administration triggers inflammation, and recruits M1 pro-inflammatory macrophages [30]. Our data showed that CA ameliorates DSS-induced UC via the reduction of infiltration of inflammatory compounds. Moreover, the F4/80 fluorescence data proved that CA reduced the number of macrophages. In addition, IL-10 as anti-inflammatory cytokine was increased in colonic tissues of the CA-treated mice. Furthermore, we found that CA inhibited the proinflammatory factors, such as TNF-α, IL-1β and IL-6 in RAW264.7, U937 and mouse peritoneal macrophages. These results suggest that CA is an effective anti-inflammatory drug through regulating macrophages in UC.

The evidence from human studies has suggested that the NLRP3 region is implicated in the susceptibility of more common inflammatory diseases such as CD, implying the genetic link between NLRP3 and IBD [31]. Inflammasomes consist of a danger-sensing receptor, the adaptor molecule ASC, the protease caspase-1 and NLR proteins, including NLRP3. ASC subsequently bridges to casepase-1 through CARD-CARD interaction [32,33]. Prior to NLRP3 inflammasome activation, ASC is located in the nucleus of cells completely in order to prevent caspase-1 activation and the subsequent processing and generation of cytokines, such as IL-1β and IL-18 [34]. CA could reduce these expressions in colon tissues, suggesting that inflammasomes are necessary in inflammatory process and CA might protect against DSS-induced colitis by suppressing NLRP3 inflammasome activation. However, from in vitro data, we found that CA could reduce the expression of NLRP3 in LPS induced RAW264.7 cells, but it could not alter ASC (data not shown), which is not consistent with the in vivo data. This difference might relate to the fact that the quantity of ASC and NLRP3 protein expression is not parallel [35]. One previous study has indicated that high concentrations of extracellular [K+]/NLRP3 siRNA down-regulated the expression of NLRP3 and caspase-1 induced by LPS and/or ATP but had little effect on ASC [36].

Previous research has shown that miR-155 expression can be induced by IL-1β [37]. miR-21 is involved in the pathogenesis of IBD via regulation of relevant gene expression [25] and TNF-α was chosen as a downstream target of it [38]. We found that miR-21 and miR-155 were significantly up-regulated in DSS-induced colitis mice whereas reduced by CA-treatment. A recent study also suggested that NADPH oxidase-derived ROS is essential for the expression and function of miR-21 [39]. Our data also showed that miR-21 or miR-155 is related to the expression of IL-1β and IL-6. Together, these results suggest that microRNAs may play a crucial role in regulating inflammatory and injury responses, in which ROS is activated and NLRP3 participates. A previous study has shown that AKT can mediate ROS regulation of miR-21 expression in prostate cancer cells [40]. On the other hand, miR-21 has been shown to increase ROS generation by decreasing the activity of superoxide dismutase (SOD) 3 or by indirectly inhibiting the SOD2 levels in cancer cells [40], suggesting the existence of a ROS-driven positive feedback loop in cancer cells to promote miR-21 expression. Our data at first time showed that miR-21 or miR-155 is related to the expression of IL-1β and IL-6 in macrophage activation. These results suggest that microRNAs may play a crucial role in regulating inflammatory and injury responses, in which NLRP3 inflammasome is activated and AKT/mTOR and COX2 pathway participates.

In summary, our current study provided evidence that CA could ameliorate DSS-induced colitis through the inhibition of macrophage activation. Furthermore, we have demonstrated that CA inhibited the LPS-induced inflammatory response of macrophages via the NLRP3 inflammasome activation, miR-21 and miR-155. These results suggest that CA might be a new useful therapeutic agent for treating UC.

Author contributions

Shulan Qu carried out the main experiments and statistical analyses and prepared the manuscript. Yunhui Shen designed the study and prepared the manuscript. Mengjie Wang carried out a portion of the animal experiments. Xiaoyu Wang wrote the main protocol, carried out a portion of the animal experiments, and prepared the manuscript. Yifu Yang supervised the study and prepared the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

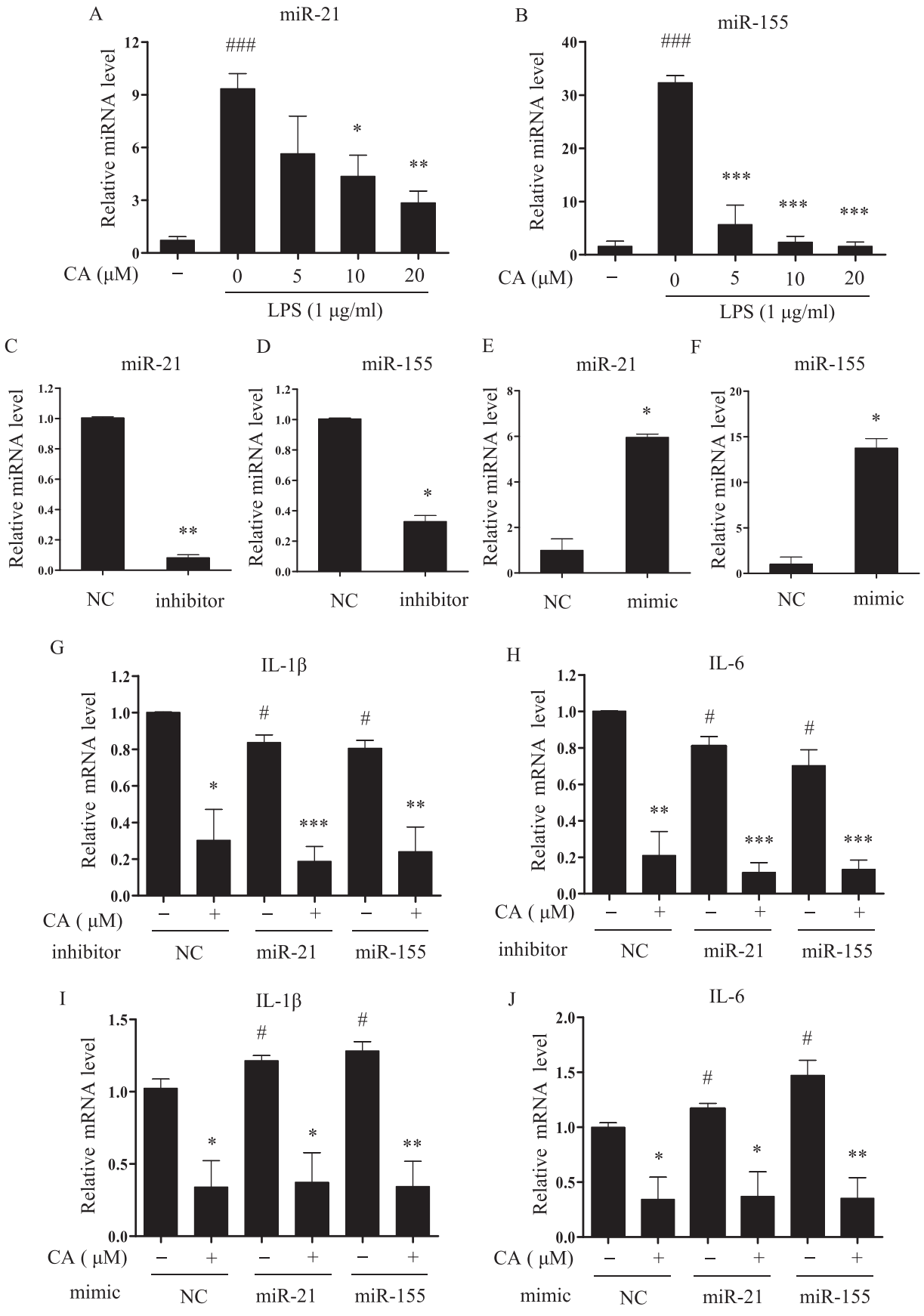

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Fig. 7. CA suppressed cytokine production via the inhibition of miR-21 and miR-155.
RAW264.7 cells were treated with or without CA (5, 10, 20 μM) for 2 h prior to LPS (1 μg/mL) stimulation for 24 h. The relative levels of miR-21 (A) and miR-155 (B) were then analyzed by real-time PCR. The data are presented as the mean ± SEM. ##$p < 0.01$, ###$p < 0.001$ vs no LPS stimulation group; *$p < 0.05$, ***$p < 0.01$ vs LPS stimulation group, using one-way ANOVA procedure followed with the Dunnett's test. Cells were transfected with miR-21 or miR-155 inhibitors (100 nM) for 48 h, miR-21(C) and miR-155 (D) expression were assayed by real-time PCR. Cells were transfected with miR-21 or miR-155 mimics (100 nM) for 48 h, miR-21 (E) and miR-155 (F) expression were assayed by real-time PCR. The data are presented as the mean ± SEM. *$p < 0.05$ vs miRNA negative control (NC) group, using Student's *t*-test. Cells were transfected with miR-21 or miR-155 inhibitors, then treated with 20 μM CA and 1 μg/ml LPS as above, cytokine of IL-1β (G) and IL-6 (H) expression was assayed by real-time PCR. Cells were transfected with miR-21 or miR-155 mimics, then treated with as above and IL-1β (I) and IL-6 (J) expression were assayed by real-time PCR. The data are presented as the mean ± SEM. #$p < 0.05$ vs NC group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ vs NC, miRNA inhibitors or mimics group, using Student's *t*-test. All of the data are presented at least three independent experiments.

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