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Taraxasterol suppresses inflammation in IL-1β-induced rheumatoid arthritis fibroblast-like synoviocytes and rheumatoid arthritis progression in mice

Jianfeng Chen[a], Weibo Wu[b], Miaomiao Zhang[a], Caiming Chen[b,*]

[a] Department of Outpatient Pharmacy, The First People's Hospital of Wenling, Wenling 317500, Zhejiang, China
[b] Department of Pharmacy, The First People's Hospital of Wenling, Wenling 317500, Zhejiang, China

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ABSTRACT

Previous study has indicated that taraxasterol (TAR), one of bioactive pentacyclic triterpenes mainly isolated from Chinese medicine herb Taraxacum officinale, displays considerable anti-inflammatory effects in various kinds of models. However, its effects on rheumatoid arthritis (RA) have still not been elucidated. In this study, we aim to investigate its anti-inflammatory effects and underlying mechanisms of TAR against RA using both interleukin (IL)-1β-stimulated human fibroblast-like synoviocytes rheumatoid arthritis (HFLS-RA) in vitro and collagen-induced arthritis (CIA) mice in vivo. Firstly, our results demonstrated that TRA significantly suppressed the IL-1β-induced expressions of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), IL-6, and IL-8 and productions of matrix metalloproteinases (MMPs), like MMP-1 and MMP-3 in HFLS-RA in vitro. Moreover, TRA alleviated arthritis progressions and prevented inflammatory processes in the joint tissues of CIA mice in vivo. Further mechanism studies indicated that TRA blocked nuclear factor kappa B (NF-κB) activation via modulating inhibitor of kappa B (IκB), IκB kinase (IKK) and transforming growth factor-β-activated kinase 1 (TAK1). Results also demonstrated that TRA suppressed the NOD-like receptor protein 3 (NLRP3) inflammasomes through blocking expressions of NLRP3, apoptosis-associated speck-like protein containing (ASC), and caspase-1 in both IL-1β-induced HFLS-RA and CIA mice. In conclusions, current findings suggested that TRA might one of considerable therapeutic compounds for relieving rheumatoid arthritis progress via suppressing inflammations through modulating NF-κB and NLRP3 inflammasomes pathways.

1. Introduction

Rheumatoid arthritis (RA) is a kind of chronically and systematically inflammatory disorder [1–3]. RA is usually characterized by chronic synovitis and hyperplastic synovial tissue, which ultimately leads to destructions of cartilage, joint and bone [1,2]. The rheumatoid arthritis fibroblast like synoviocytes (FLSs) contributes to the primary population of cells in the invasive pannus [4,5]. Accumulating evidence indicates that FLSs are important modulators of synovial inflammation in RA progression, which are well involved in inflammatory infiltrations, synovial hyperplasia, joint destruction, and various kinds of comorbidity [4,5]. Thus, inhibiting rheumatoid arthritis FLSs-mediated inflammation may be a therapeutic potential for RA treatment.

The inflammatory responses in rheumatoid arthritis FLSs are characterized by excessive productions of inflammatory cytokines, like tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and matrix metalloproteinases (MMPs), e.g. MMP-1 and MMP-3 [6–10]. It is widely reported that inflammation responses in RA-FLSs are mainly modulated by the nuclear factor kappa B (NF-κB) and NOD-like receptor protein 3 (NLRP3) inflammasomes [11–13]. The transcription factor, nuclear factor kappa B (NF-κB) is one of important modulators of different kinds of inflammatory processes. Once activated, NF-κB triggers transcription and expression of pro-inflammatory cytokines mediators [14]. NLRP3 inflammasomes belongs to the NLR family and it consists of NLRP3, apoptosis-associated speck-like protein containing (ASC), and caspase-1 [15–18]. NLRP3 is a component of the innate immune system and its main function is to regulate caspase-1 activation and pro-inflammatory cytokines release, resulting in inflammation [15–18]. Therefore, inhibiting NF-κB/NLRP3 pathways is one of actions of anti-inflammatory compounds and is a promising strategy for the treatment of RA.

Taraxacum officinale (TO), a famous traditional Chinese medicine herb, has been long use for preventions and treatments of various kinds of inflammatory or infectious disorders [19–21], including hepatitis, upper respiratory infections, bronchitis and pneumonia [19,20,22–25].

* Corresponding author at: Pharmacy, The First People's Hospital of Wenling, No. 333 Chuanan Nan Road, Chengxi Street, Wenling 317500, Zhejiang Province, China.
E-mail address: chencaiming8135@163.com (C. Chen).

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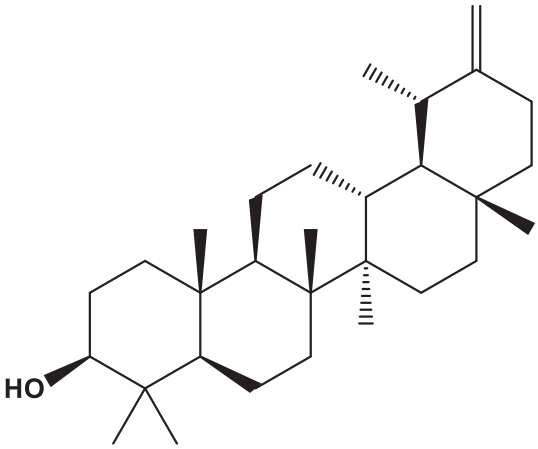

Fig. 1. Chemical structure of taraxasterol.

Recently, taraxasterol (TAR, shown in Fig. 1), one of pentacyclic triterpene isolated from TO, has also been shown to possess significant anti-inflammatory and antioxidant activities [26–29]. TAR could decrease pro-inflammatory cytokines and mediators in LPS-induced murine RAW 264.7 macrophages in vitro and abate ovalbumin-induced allergic asthma and LPS-induced sepsis in mice model [26–30]. However, effects and mechanisms of TAR on synovitis and RA remain unclear. Thus, in this study, we aim to investigate anti-inflammatory actions and underlying mechanisms of TAR in IL-1β-stimulated human fibroblast-like synoviocytes rheumatoid arthritis (HFLS-RA) and in collagen-induced arthritis (CIA) in mice [31].

2. Materials and methods

2.1. Reagents

Taraxasterol (TAR, power, purity HPLC UV: ≥98%, and endotoxin-free) was obtained from Weikeqi Biological Technology Inc. (Si Chuan, China). IL-1β was purchased from PeproTech (Grand Island, NY, USA). Freund's Complete Adjuvant (FCA) and LPS (Escherichia coli O55:B5) were purchased from Sigma (St. Louis, MO, USA). DMEM, FBS, penicillin and streptomycin were purchased from Life Technologies (Grand Island, NY, USA).

2.2. Cells culture and treatments

The primary HFLS-RA were obtained from American type culture collection and maintained in (Manassas, VA, USA) and maintained in DMEM supplied with 10% FBS, 100 U/mL penicillin, and 100 mg/ml streptomycin with a humidified atmosphere 5% CO_2 at 37 °C. Passages between 3 and 9 of HFLS-RA were used in the current study. Experiments were performed at 24 h after cells seeding. TAR and other agents were prepared in DMSO or H_2O as stock solutions and added into the medium.

2.3. Experimental animals and protocols

Male DBA/1 mice (18 to 22 g, about 8 weeks) were supplied by IMET Research Inc. (Suzhou, China). The animals were kept under SPF condition with 12 h light and 12 h dark cycles, standard environments (24 ± 1 °C) and relative humidity (55 ± 5%). All animal experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with prior approval from the Institutional Animal Care and Use Committee (FPHW-0036-2017).

The CIA mice model was induced according to protocols previously described with small modifications [32]. In brief, CIA was induced by two times of immunization in mice (secondary immunization is given 12 days after the primary immunization). Animals were randomly divided into to three groups (n = 10): Vehicle, CIA, CIA + TRA group (10 mg/kg, intragastrical administration every other day from day 0 to day 48). Clinical symptoms and score of limbs were observed and recorded by two independently blinded operators every 3 days from day 12 [3]. Clinical arthritis scores were assessed using a range of 0–3 for each limb according to previous reports [3]. On day 48, all animals were euthanized by pentobarbital and samples were collected for further study [3].

2.4. Cell viability

Cell viability was detected using the MTT assay (Invitrogen, Carlsbad, CA, USA). In brief, HFLS-RA cells were seeded in 96-well culture plates (1×10^5 cells/well) and treated with various kinds of agents. After that, cells were incubated with MTT solutions, then the absorbance results (570 nm) were detected.

2.5. Enzyme-linked immunosorbent assay (Elisa)

The TNF-α, IL-6, MMP-1, and MMP-3 released into the conditioned media or supernatants of lysed samples were detected using specific ELISA Ready-SET-Go kits (eBiosciences, San Diego, CA, USA). The levels were quantified according to manufacturer's protocols.

2.6. Quantitative PCR (qPCR) assay

Total RNA extractions were carried out using high-pure-RNA-isolation kits (Roche, Mannheim, Germany) following manufacturer's protocols. The isolated RNA was then reverse-transcribed into cDNA using the transcriptor-first-strand-cDNA-synthesis kits (Roche, Mannheim, Germany). The qPCR assay was performed using fast-start-universal-SYBR-Green-master reagents (Roche) with the Applied Biosystems 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). All samples were analyzed in triplicate; comparative Ct methods were used to calculate relative fold changes normalized against the β-actin. The primer sequences used were listed in the Table 1.

2.7. Reporter gene luciferase assays

In brief, cells were firstly transfected using the pNFκB-luc reporter plasmid obtained from the cignal-NFκB-Reporter (luc) kit, using the attractene-transfection-reagent according to manufacturer's protocols. 24 h after transfection, transfected cells were subjected to further treatments. All samples were then analyzed using the dual-luciferase®-reporter-assay-system, and then the luciferase activity was detected.

2.8. Preparation of whole cell, cytoplasmic, and nuclear protein

Cells were collected and incubated with RIPA lysis buffer containing

Table 1
The primer sequences.

Name	Sequence (5′ to 3′)	
TNF-α	F	GAAAGCATGATCCGGGACGTG
	R	GATGGCAGAGAGGAGGTTGAC
IL-6	F	AAGCCAGAGCTGTGCAGATGAGTA
	R	CTTGGTCACCGACGTCCTGT
MMP-1	F	CAGAGATGAAGTCCGGTTTTTC
	R	GGGGTATCCGTGTAGCACAT
MMP-3	F	CAAAACATATTTCTTTGTAGAGGACAA
	R	TTCAGCTATTTGCTTGGGAA
GAPDH	F	TGGAAGGACTCATGACCACA
	R	AGGGGTCTACATGGCAACTG

PMSF and protease inhibitor cocktail for 45 min on ice. Cell lysates were then centrifuged, and the supernatant was collected. The subcellular fractionation preparation was processed using the nuclear and cytoplasmic protein extraction kits (Beyotime, Shanghai, China). The protein content was assayed using the BCA assay (Invitrogen, Carlsbad, CA, USA).

2.9. Western blot assay

Aliquots protein samples were resolved by SDS-PAGE (7.5 to 12%) and transferred into PVDF membranes (Bio-Rad, CA, USA). After that, blots were incubated with various kinds of primary antibodies: p-TAK-1, p-IKKα/β, t-IKKα, p-IκBα, t- IκBα, p-p65, t-p65, TXNIP, NLRP3, ASC, cleaved caspase-1, Lamin B, GAPDH and β-actin (1:1500), and peroxidase-conjugated secondary antibodies (1:2500, Cell Signaling Technology, Danvers, MA, USA). Finally, bands were visualized using an ECL-plus Western-blotting detection reagents (GE Healthcare, Milwaukee, WI, USA). Membranes were then detected on the Bio-Rad-ChemiDoc-XRS-Imaging System, and the intensity of bands was analyzed using the Bio-Rad-Quantity-One Software.

2.10. Immunofluorescence assay

After treatments, cells were fixed by 3.7% PFA for 15 mins, permeabilized by 0.3% Triton X-100-PBS, and blocked using blocking buffers (0.1% Triton X-100 and 5% BSA in PBS) for another 30 min at room temperature. And then, cells were incubated with primary antibody overnight at 4 °C and Alexa Fluor 488 secondary antibody (1:500 dilution, Invitrogen, Carlsbad, CA, USA) for 45 min at room temperature, respectively. For the nuclei observation, cells were stained with DAPI for 10 min. Samples were mounted with Prolong anti-fade reagent and imaged by a confocal laser scanning microscope (Olympus, Kyoto, Japan).

2.11. Histochemical analysis

Biopsies of hind paws were collected and fixed in 10% neutral buffered formalin solutions. Subsequently, the tissues were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin. The 4-μm-thick sections were prepared and stained with hematoxylin and eosin (H&E) and examined with a BX60 microscope (Olympus, Kyoto, Japan).

2.12. Graphing and statistical analysis

Statistical analyses were conducting using GraphPad Prism software (GraphPad, San Diego, CA, USA), and data were represented as means ± standard error of the mean (SEM). Statistical analysis of differences between two groups was done using the independent-samples *t*-test and one-way or two-way ANOVA with Bonferroni's correction applied was used for comparison of more than two groups. Pearson's correlation coefficient was used for correlation analyses. $P < 0.05$ was considered significant in all analyses.

3. Results

3.1. TAR had no effect on cell viability and IL-1β-induced proliferation in HFLS-RA

To investigate the actions of TRA, we firstly detected its potential cytotoxic effects on HFLS-RA in vitro. As shown in Fig. 2A, TRA (0.3 to 30 μM) had no obvious effect on cell viability of HFLS-RA. As we known, the IL-1β-stimulated HFLS-RA model is widely used to discovery agents targeting for RA treatments. In the current study, incubation with IL-1β (10 ng/mL) for 48 h was used to induce the inflammation. As shown in Fig. 2B, we observed that IL-1β stimulation

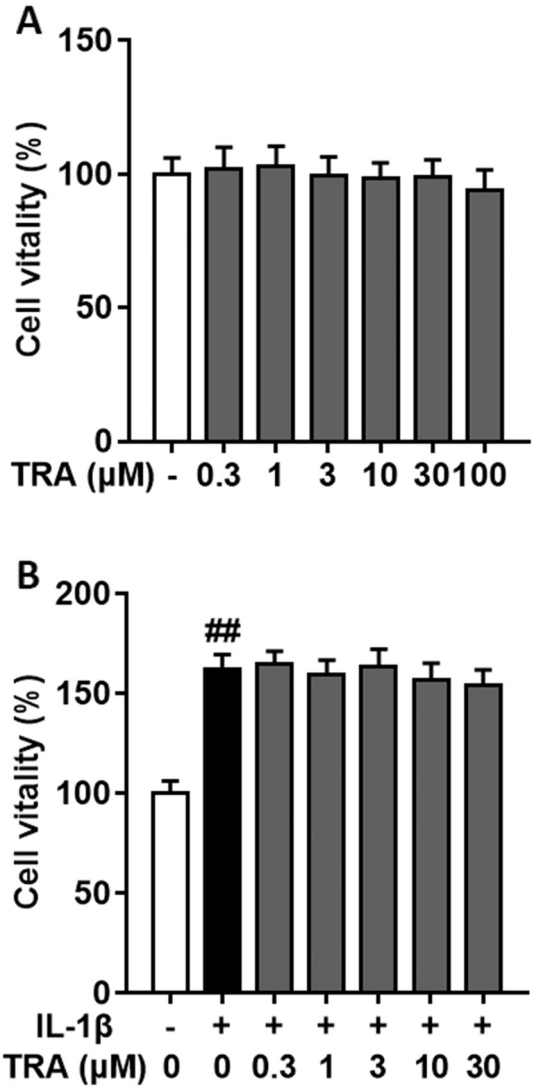

Fig. 2. Effects of TRA on cell viability of HFLS-RA with or without IL-1β stimulation.
(A) HFLS-RA were incubated with TRA for 48 h. (B) HFLS-RA were firstly treated with TRA for 1 h and then incubated with IL-1β (10 ng/mL) for another 48 h. Results are showed as means ± SEM (n = 9). Cell viability was determined by MTT assay. Control group was untreated cells. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus IL-1β-stimulated group.

significantly increased the cell viability of HFLS-RA (compared with the control group, $P < 0.01$), indicating that IL-1β also induce HFLS-RA proliferation. However, TRA (0.3 to 30 μM) showed no inhibition on IL-1β-induced proliferation of HFLS-RA (compared with the IL-1β-stimulated group, $P > 0.05$).

3.2. TRA decreased IL-1β-induced expression of pro-inflammatory cytokines and mediators in HFLS-RA

To investigate the inhibitory actions of TRA on IL-1β-induced inflammation in HFLS-RA, cells were incubated with IL-1β with or without TRA (0.3 to 30 μM). As shown in Fig. 3A–C, we found that TRA (3 to 30 μM) could significantly down-regulate TNF-α, IL-6, and IL-8 proteins increased by IL-1β stimulation in HFLS-RA, in a dose-dependent fashion (compared with the IL-1β-stimulated group, all $P < 0.05$). We also observed that TRA (3, 10 and 30 μM) could significantly down-regulate IL-1β-induced increases of TNF-α, IL-6, and IL-8 mRNA levels

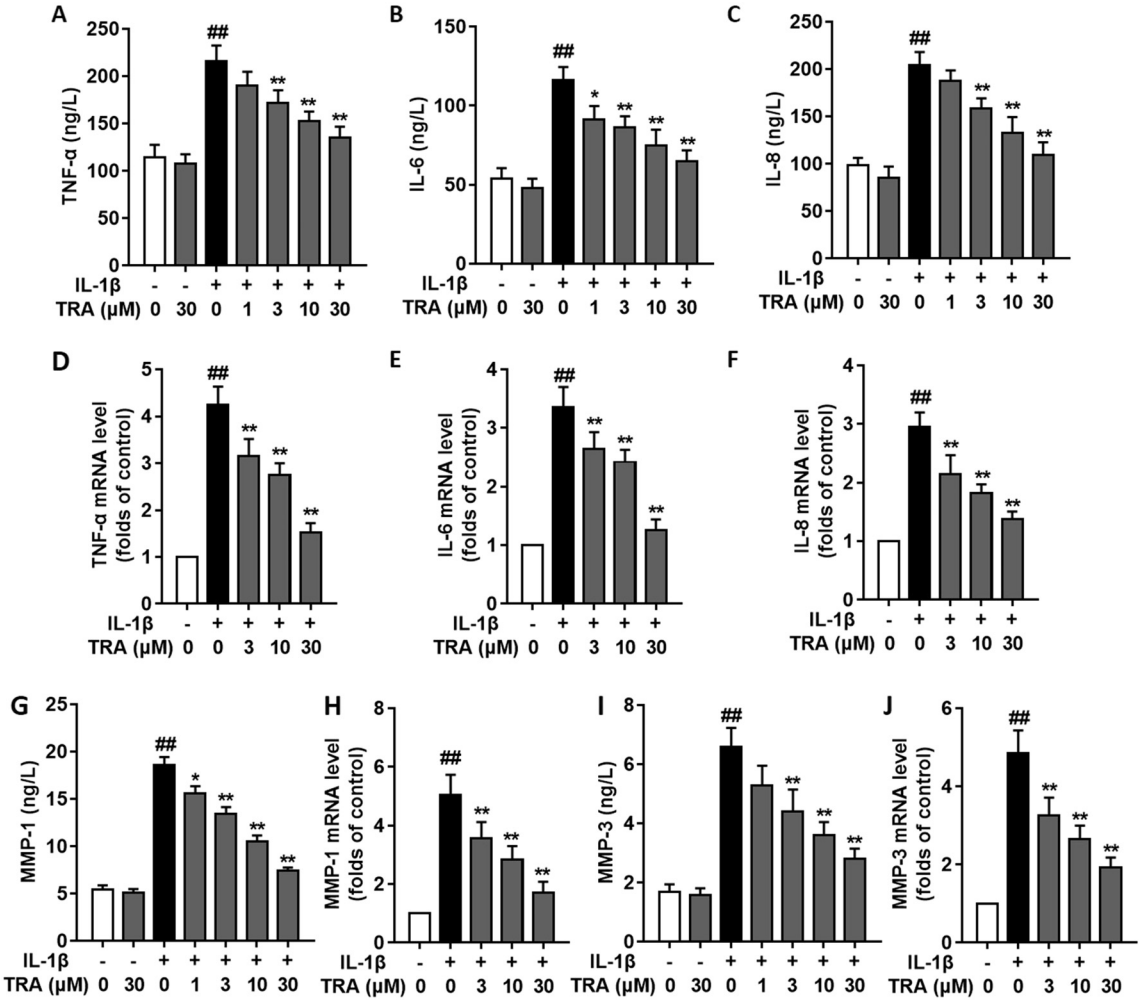

Fig. 3. TRA suppressed the IL-1β-induced expression of pro-inflammatory cytokines and mediators in HFLS-RA.
HFLS-RA were firstly treated with TRA for 1 h and then incubated with IL-1β (10 ng/mL) for another 48 h. (A, B, and C) Culture supernatants were collected, and the protein level of TNF-α, IL-6 and IL-8 was detected via Elisa. (D, E and F) Total RNAs were extracted, and the mRNA level of TNF-α, IL-6 and IL-8 was analyzed using qPCR assay. (G and I) Total proteins were prepared, and protein level of MMP-1 and MMP-3 was detected via Western blot. (H and J) Total RNAs were extracted, and the mRNA level of MMP-1 and MMP-3 was analyzed using qPCR assay. Results are showed as means ± SEM (n = 9). Control group was untreated cells. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus IL-1β-stimulated group.

in HFLS-RA (Fig. 3D–F, compared with the IL-1β-induced group, all $P < 0.01$). Moreover, IL-1β significantly enhanced the expression of MMP-1 and MMP-3 proteins (Fig. 3G and H, compared with the control, both $P < 0.01$), whereas TRA treatment reversed the increased level of MMP-1 and MMP-3 in a dose-dependent fashion (compared to the IL-1β group, all $P < 0.05$). Also, as shown in Fig. 3J and K, TRA (3, 10 and 30 μM) obviously down-regulate IL-1β-induced increases of MMP-1 and MMP-3 mRNA level in HFLS-RA (compared with the IL-1β-stimulated group, all $P < 0.05$). Thus, these results demonstrated that TRA provided inhibitory actions against IL-1β-induced expressions of inflammatory cytokines and MMPs in HFLS-RA.

3.3. TRA inhibited IL-1β-induced NF-κB activations via modulating the TAK-1/IKK/IκB in HFLS-RA

We further detected TRA actions on NF-κB pathways in IL-1β-stimulated HFLS-RA. Firstly, we investigated TRA actions on NF-κB-regulated gene transcription and signal transduction using the NF-κB luciferase reporter system. The results indicated that TRA could inhibit the NF-κB-driven gene transcription activity increased by IL-1β stimulation in a dose-dependent fashion (Fig. 4A, all $P < 0.01$, compared with the IL-1β-stimulated group). The ELISA-based EMSA was conducted to assess the binding of NF-κB to DNA, and results showed that IL-1β induced increase of NF-κB binding to DNA (Fig. 4B, all $P < 0.01$, compared with the control). However, TRA could significantly abolish the IL-1β-induced NF-κB-DNA binding activity (Fig. 4B).

Next, we investigated TRA actions on IL-1β-induced nuclear translocation of the NF-κB p65 using Western blot assay. We found that TRA (30 μM) could significantly block the IL-1β-mediated NF-κB p65 nuclear translocation (Fig. 4C–E, all $P < 0.01$, compared with the IL-1β-stimulated group). To further elucidate the roles of TRA on NF-κB pathway, cells were incubated with the NF-κB inhibitor (pyrrolidine-dithiocarbamate ammonium, PDTC, 50 μM) in the presence of TRA. The results showed that compared with the TRA-treated group, PDTC treatment further reduced the IL-1β-upregulated TNF-α and IL-6 in the presence of TRA (Fig. 4F and G, both $P < 0.05$).

In addition, as shown in Fig. 5A–C, we found that levels of phosphorylated IKK and IκB were also significantly increased in IL-1β-induced HFLS-RA. However, TRA obviously attenuated increases of phosphorylated IKK and IκB proteins (5A-5C, all $P < 0.01$, compared with the IL-1β-stimulated group). Moreover, IL-1β inhibited the IκB degradation in HFLS-RA, which was then obviously reversed by TRA treatment (30 μM).

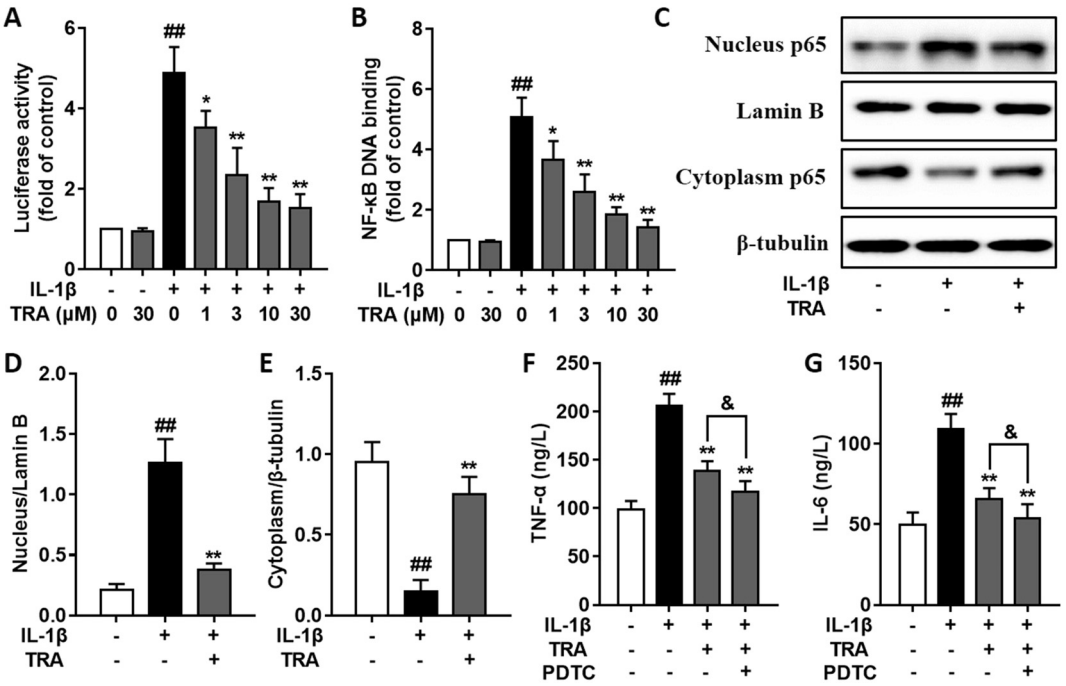

Fig. 4. TRA suppressed the NF-κB activation in IL-1β-stimulated HFLS-RA.
HFLS-RA were firstly treated with TRA for 1 h, and then incubated with IL-1β (10 ng/mL) for another 1 h or 12 h. (A) Cells were firstly transfected with NF-κB reporter luciferase plasmid. The luciferase activity was then measured and then expressed as fold-increase. (B) Nuclear extracts were used and then detected by the ELISA-based DNA binding assay. The absorbance was then measured. (C, D and E) The protein level of nuclear and cytoplasmic p65and p65was measured using Western Blot. (F and G) Cells were treated with 50 μM, and then induced by IL-1β with or without TRA. After incubation, the protein level of TNF-α and IL-6 was detected via Elisa. Results are showed as means ± SEM (n = 9). Control group was untreated cells. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus IL-1β-stimulated group. & indicated $P < 0.05$, between two groups.

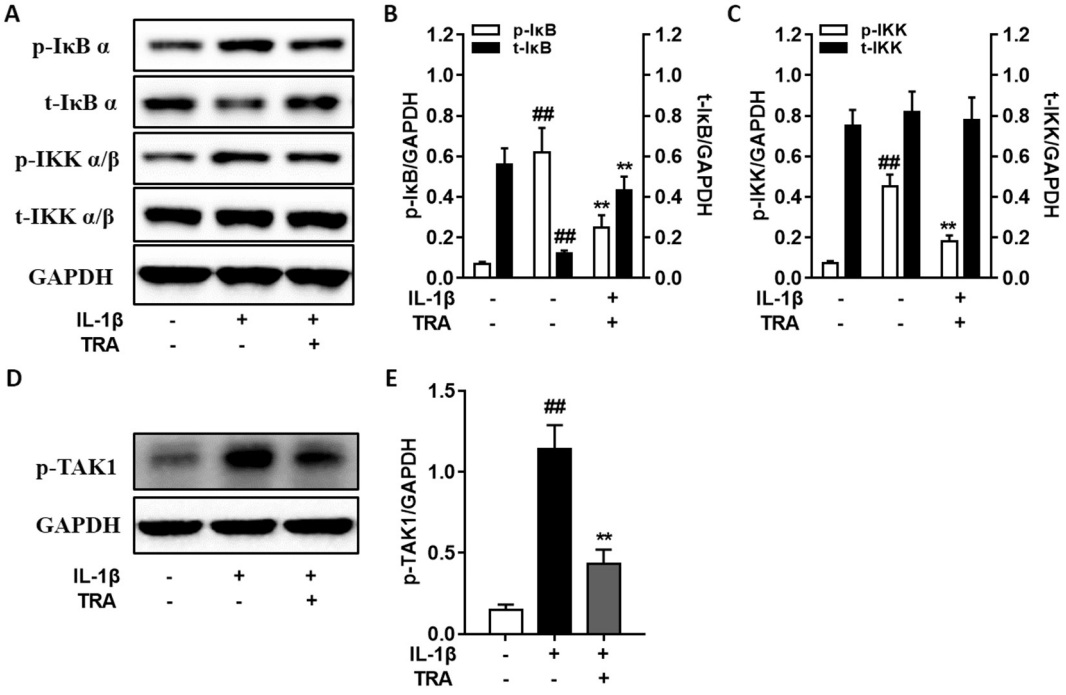

Fig. 5. TRA inhibited IL-1β-induced NF-κB activations via modulating the TAK-1/IKK/IκB in HFLS-RA.
HFLS-RA were firstly treated with TRA for 1 h, and then incubated with IL-1β (10 ng/mL) for another 1 h or 12 h. (A, B and C) After incubation, the protein was prepared, and the levels of total/phosphorylated IκB α and total/phosphorylated IKK α/β were measured via Western blot assay. (D and F) The level of phosphorylated TAK1 was measured via Western blot assay. Results are showed as means ± SEM (n = 9). Control group was untreated cells. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus IL-1β-stimulated group.

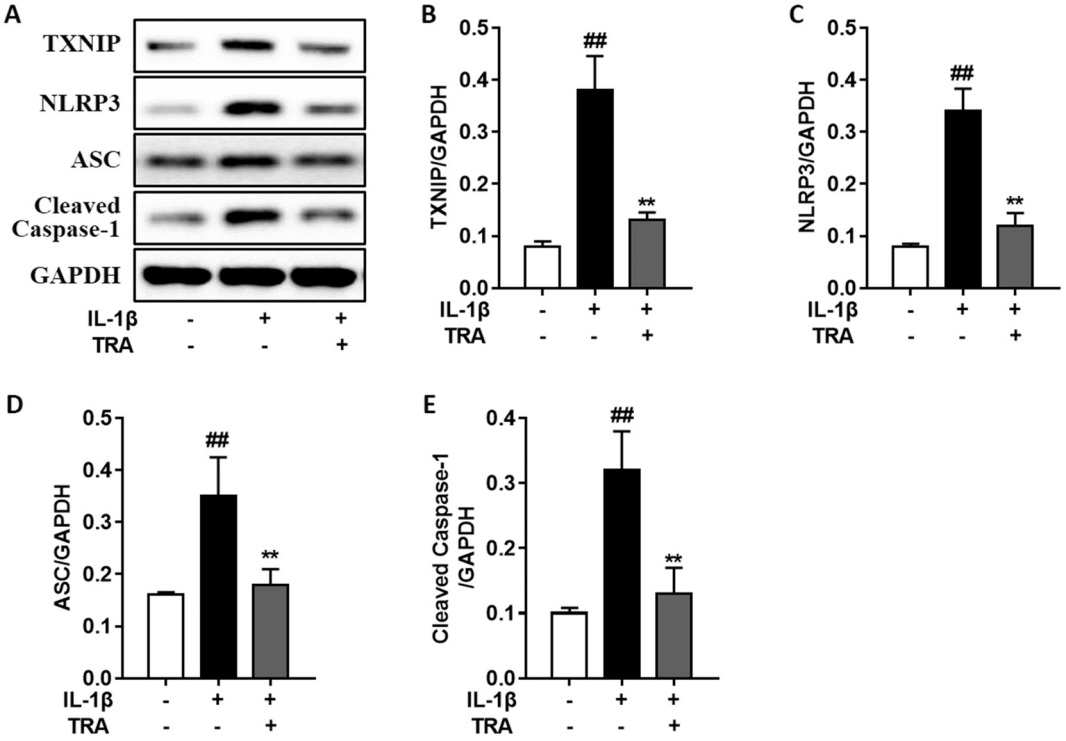

Fig. 6. TRA suppressed IL-1β-induced activation of NLRP3 inflammasome pathways in HFLS-RA
HFLS-RA were firstly treated with TRA for 1 h, and then incubated with IL-1β (10 ng/mL) for another 1 h or 12 h. After incubation, the protein was prepared, and the levels of TXNIP (A and B), NLRP3 (A and C), ASC (A and D), and cleaved caspase-1 (A and E) were measured via Western blot assay. Results are showed as means ± SEM (n = 9). Control group was untreated cells. # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus IL-1β-stimulated group.

Normally, NF-κB activation is also modulated by TAK1 via by interacting with TNF receptor-associated factor (TRAF)-6 and phosphorylating the NF-κB inducing kinase. The phosphorylated level of TAK1 was also measured here, and we also found that TRA treatment (30 μM) could significantly suppress IL-1β-induced phosphorylation of TAK1 in HFLS-RA (5D and 5E, all $P < 0.01$, compared with the IL-1β-stimulated group).

3.4. TRA suppressed IL-1β-induced activation of NLRP3 inflammasome pathways in HFLS-RA

The activation of NLRP3 inflammasomes pathways is well associated with various kinds of inflammatory responses, which also play essential roles in the RA progress. To further explore whether NLRP3 inflammasome pathways were involved in TRA-mediated inhibitory actions against IL-1β-induced inflammation, components of the inflammasome complex were then detected using Western blot. As shown in Fig. 6, IL-1β significantly increased expression of TXNIP, NLRP3 inflammasomes and ASC in HFLS-RA cells (all $P < 0.01$, compared with the control group). However, TRA treatment could suppress IL-1β-induced expressions of TXNIP, NLRP3 and ASC (Fig. 6A–D, all $P < 0.01$, compared with the IL-1β-stimulated group). Furthermore, the maturation of caspase-1 was also markedly increased by IL-1β, which was then reversed by TRA (Fig. 6A and E, $P < 0.01$, compared with the IL-1β-stimulated group).

3.5. TRA prevented arthritis progression and alleviated synovial inflammation in CIA mice

To further detect the potential therapeutic effects of TRA on RA in vivo, the CIA-induced RA mice model was used in the current study. As illustrated in Fig. 7A, TRA obviously decreased the clinical arthritis score when compared to the vehicle-treated group ($P < 0.01$). In addition, histopathological results also indicated that TRA alleviated inflammatory cell infiltration, ureterostenosis, synovial hyperplasia, and cartilage destruction in ankle joints of CIA mice (Fig. 7B).

Pro-inflammatory cytokines play a key role in the pathogenesis of RA. Thus, pro-inflammatory cytokines in joint tissues were further determined by Elisa. As shown in Fig. 7C–E, TRA significantly decreased the expression of TNF-α, IL-6 and IL-8 in joint tissues ($P < 0.01$, compared with the vehicle-treated group). In addition, the actions of TRA on MMPs expressions were also investigated. We found that TRA could also suppress the expression of MMP-1 and MMP-3 (Fig. 7F and G), when compared to the vehicle-treated group (all $P < 0.01$). Therefore, the current results demonstrated TRA inhibited arthritis progression and suppressed synovial inflammation in CIA mice.

3.6. TRA modulated the NF-κB and NLRP3 inflammasome pathways in joint tissues of CIA mice

To further investigate the actions of TRA on the NF-κB pathway in vivo, NF-κB p65 nuclear translocation in joint tissues of CIA mice were then detected. As shown in Fig. 8A and B, CIA resulted in significant NF-κB p65 nuclear translocation in joint tissues ($P < 0.01$, compared with the vehicle-treated group). However, TRA (10 mg/kg) treatment obviously blocked the NF-κB p65 nuclear translocation, when compared to the vehicle-treated group (Fig. 8A and B, $P < 0.01$). Moreover, TRA treatment also significantly decreased increases of IKKα/β and IκBα phosphorylation and reversed CIA-induced decreases of IκBα degradation (Fig. 8D–F, $P < 0.01$, compared with the vehicle-treated group). Results also showed that TRA treatment could block the phosphorylation of TAK1 increased by CIA stimulation (Fig. 8G and H, $P < 0.01$, compared with the vehicle-treated group).

In addition, as shown in Fig. 9, CIA stimulation significantly increased expression of TXNIP, NLRP3 inflammasome and ASC in joint tissues of mice (all $P < 0.01$). However, TRA treatment could suppress

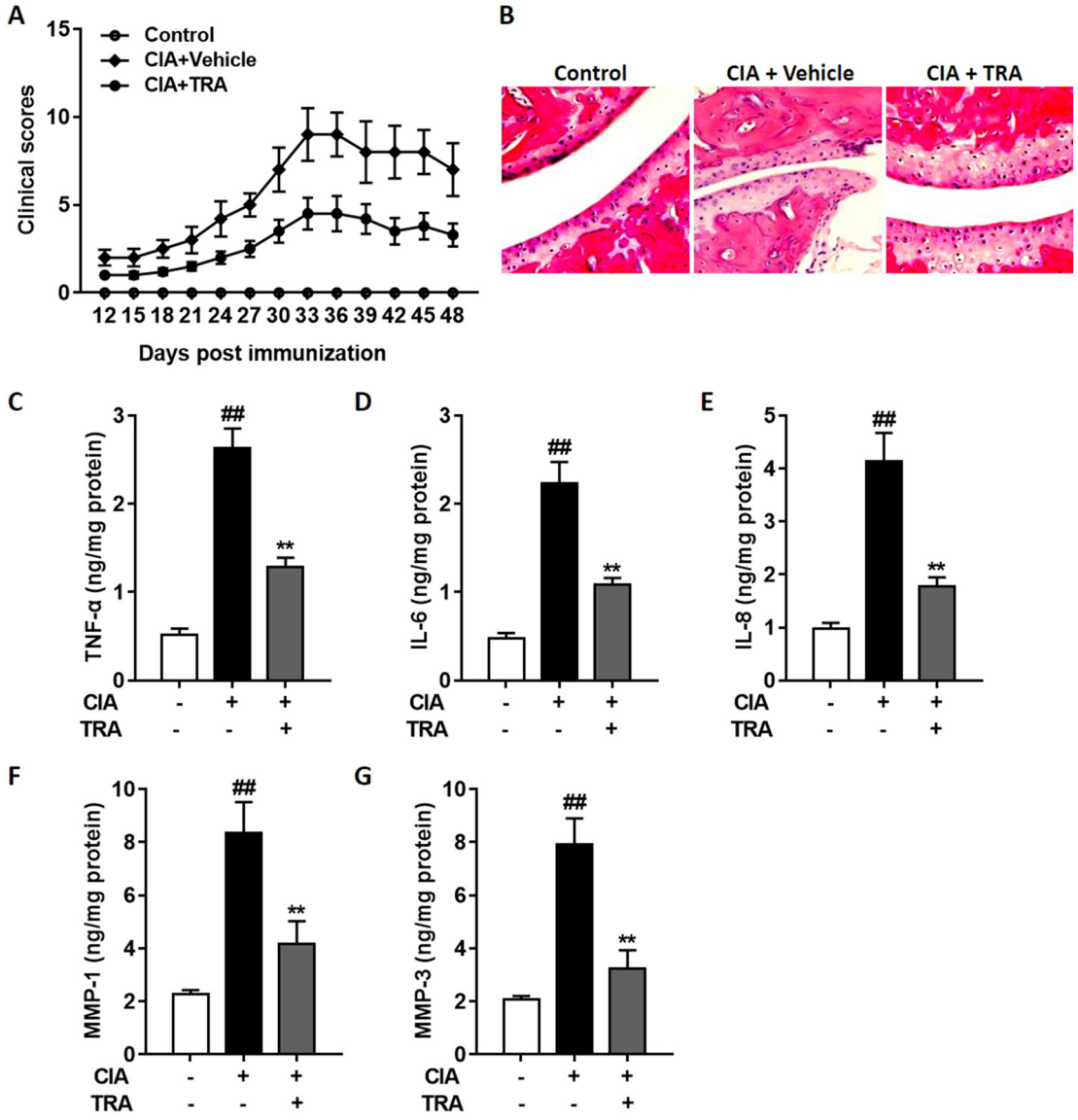

Fig. 7. Effects of TRA on arthritis progression and alleviated synovial inflammation in CIA mice.
CIA was induced by two times of immunization in mice (secondary immunization is given 12 days after the primary immunization). Animals were administrated with vehicle or TRA (10 mg/kg) on every other day from day 0 to day 48. Clinical symptoms and score of limbs were observed and recorded from day 12. On day 48, animals were sacrificed, and synovial tissues were collected. (A) Effects of TRA on arthritis scores in CIA mice. (B) Representative histological assessment of joint in CIA mice on day 48 (400×). (C and D) The level of TNF-α, IL-6, IL-8, MMP-1, and MMP-3 proteins in joint homogenates was measured by Elisa. Results are showed as means ± SEM (n = 9). # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus the vehicle-treated group.

CIA-induced expressions of TXNIP, NLRP3 and ASC (Fig. 9A–D, all $P < 0.01$, compared with the vehicle-treated group). Furthermore, the maturation of caspase-1 was also markedly increased by IL-1β, which was then reversed by TRA (Fig. 9A and E, $P < 0.01$, compared with the vehicle-treated group).

4. Discussion

In the present study, we demonstrated that TRA significantly suppressed inflammation in 1β-stimulated HFLS-RA in vitro and alleviated arthritis progressions and cartilage damages in CIA mice in vivo. Furthermore, TRA blocked activations of NF-κB and NLRP3 inflammasome pathways, which might contribute to its protective actions against inflammation in RA models. Thus, our findings suggested that TRA, targeting NF-κB/NLRP3 inflammasomes-mediated synovial inflammatory processes, might be develop as a novel agent for RA treatment.

FLSs are important modulators of synovial inflammation in RA progression [1,2]. FLSs could secrete pro-inflammatory cytokines and mediators, which modulate growth factor expressions and trigger more FLSs activations as a feedback [6–10,33–35]. Pro-inflammatory cytokines, especially TNF-α, IL-6, and IL-8, play important roles in synovial inflammatory responses in RA [3,33–35]. Thus, development of anti-inflammatory agents is considered as the main way for RA treatment [3]. As a first step towards the application of TRA, we investigated TRA actions on inflammatory cytokines in IL-1β-stimulated HFLS-RA. It is well reported that IL-1β could induce activation of HFLS-RA and trigger significant increases of pro-inflammatory cytokines in this cell lines. In our study, we also confirmed that TRA could suppress expressions of TNFα and IL-6 in IL-1β-stimulated FLSs-RA and CIA mice.

Elevated expressions of MMPs are well involved in the development of RA [6–10]. IL-1β stimulation is well known to induce MMPs secretions in FLSs-RA, and these inductions are mainly modulated by the transcriptional and translational levels [36,37]. It is well known that

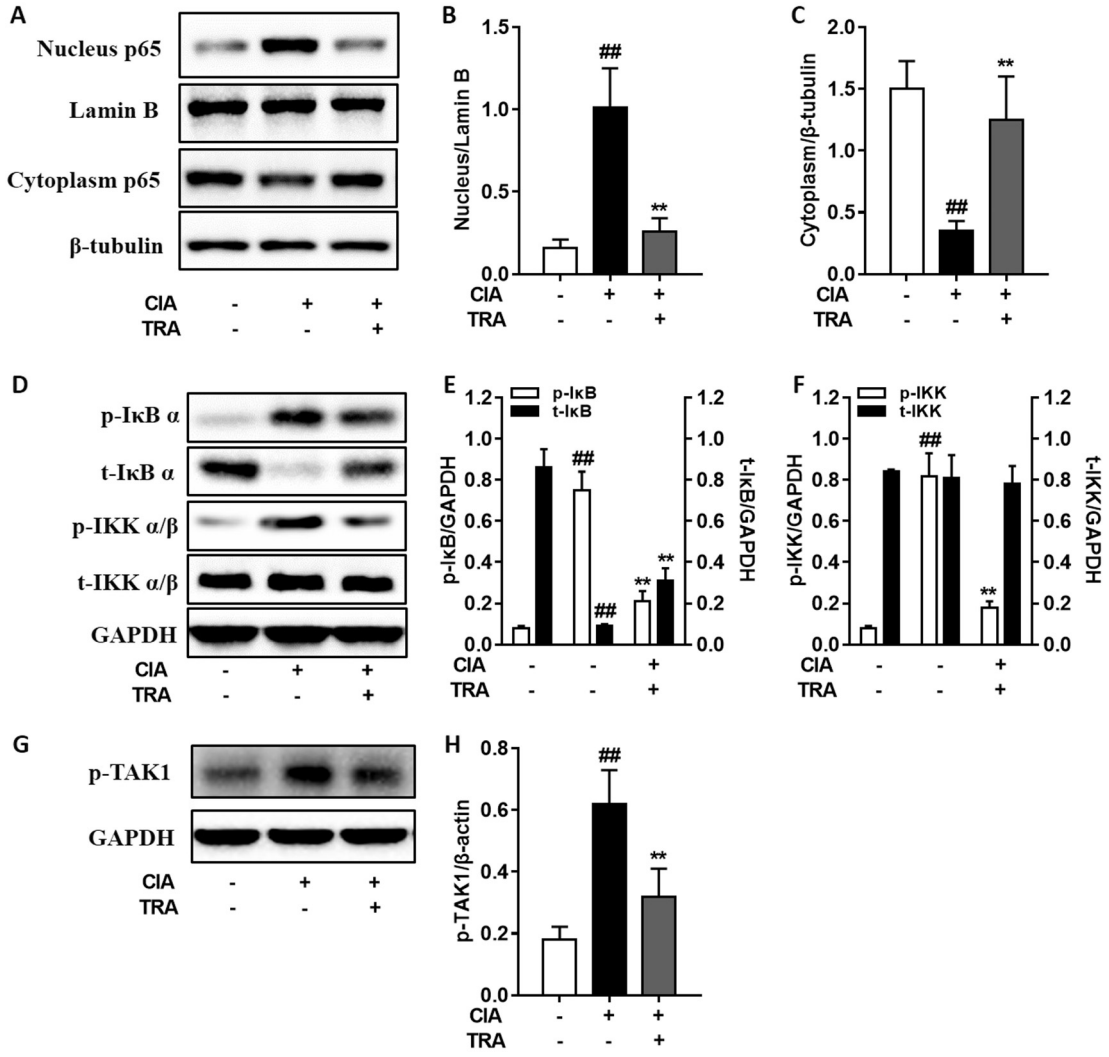

Fig. 8. Effects of TRA on the NF-κB signaling pathway in joint tissues of CIA mice.
On day 48, animals were sacrificed, and tissues were collected for Western blot assay. (A, B, and C) The nuclear protein was prepared, and the level of nuclear p65 was measured using Western blot assay. (D, E, and F) The total proteins were prepared, and levels of total/phosphorylated IκB α and total/phosphorylated IKK α/β were measured via Western blot assay. (G and H) The total proteins were prepared, and level of phosphorylated TAK1 were measured via Western blot assay. Results are showed as means ± SEM (n = 9). # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus the vehicle-treated group.

MMP-1 and MMP-3 are two important gelatinases in the MMP family. These two MMPs could not only efficiently and rapidly cleave unfolded collagen but also cleave other matrix and non-matrix components [38–40]. MMPs are usually activated by pro-inflammatory cytokines and have been found to be down-regulated in response to the anti-TNF therapy [38,39]. In our study, IL-1β-stimulated human FLSs-RA showed increases in both MMP-1 and MMP-3. However, MMPs secretions were decreased by TRA in FLSs-RA. Thus, it is likely that TRA blocked cartilage damages via mediating MMPs in inflamed joints of RA.

Accumulating evidence shows that among transcriptional factors in modulating inflammatory genes expressions, the NF-κB is considered as one of important signaling factors in regulating inflammation, synovial hyperplasia, and matrix degenerations [11–13,41,42]. Thus, NF-κB has been widely reported in the pathogenesis of RA, and NF-κB inhibitions have also been explored as a therapeutic approach to RA [11–13,40–42]. Recent reports indicated that TRA inhibited the NF-κB pathway in LPS-stimulated mouse peritoneal macrophages [26–29,40]. Therefore, we postulated that TRA might also inhibit NF-κB pathway in HFLS-RA. As expected, results indicated that TRA suppressed NF-κB activation in IL-1β-stimulated HFLS-RA. In response to IL-1β stimulation, NF-κB is translocated into a nucleus through IκB degradation [40]. In our study, we found that TRA obviously inhibited IL-1β-induced IκB degradations and nuclear translocations of p65 in HFLS-RA. Furthermore, TRA also obviously blocked NF-κB activations in HFLS-RA. Normally, NF-κB activation is also modulated by TAK1 via by interacting with TNF receptor-associated factor (TRAF)-6 and phosphorylating the NF-κB inducing kinase [11–13]. In current study, we also found that TRA could modulate the TAK1 activation, indicating that TAK1 might be one of upstream modulator of TRA-mediated NF-κB pathways [11–13]. These findings suggested that TRA might provide anti-inflammatory actions by inhibiting NF-κB activations via modulating TAK1/IκB/IKK pathway.

The activation of NLRP3 inflammasome pathways are another common modulator of the inflammatory processes in RA progress [15–18]. Interacting with an adaptor protein ASC, the NLRP3 inflammasome mediates precursor caspase-1 and IL-1β into cleaved caspase-1 and mature IL-1β, respectively [15–18]. The active caspase-1 and mature IL-1β were then released into the extracellular environment and contribute to inflammatory responses [15–18,43]. Our results demonstrated that TRA could block the expression of NLRP3 inflammasomes as well as its modulators, including TXNIP and ACS in HFLS-RA and CIA mice. Moreover, cleaved caspase-1 was also decreased by TRA,

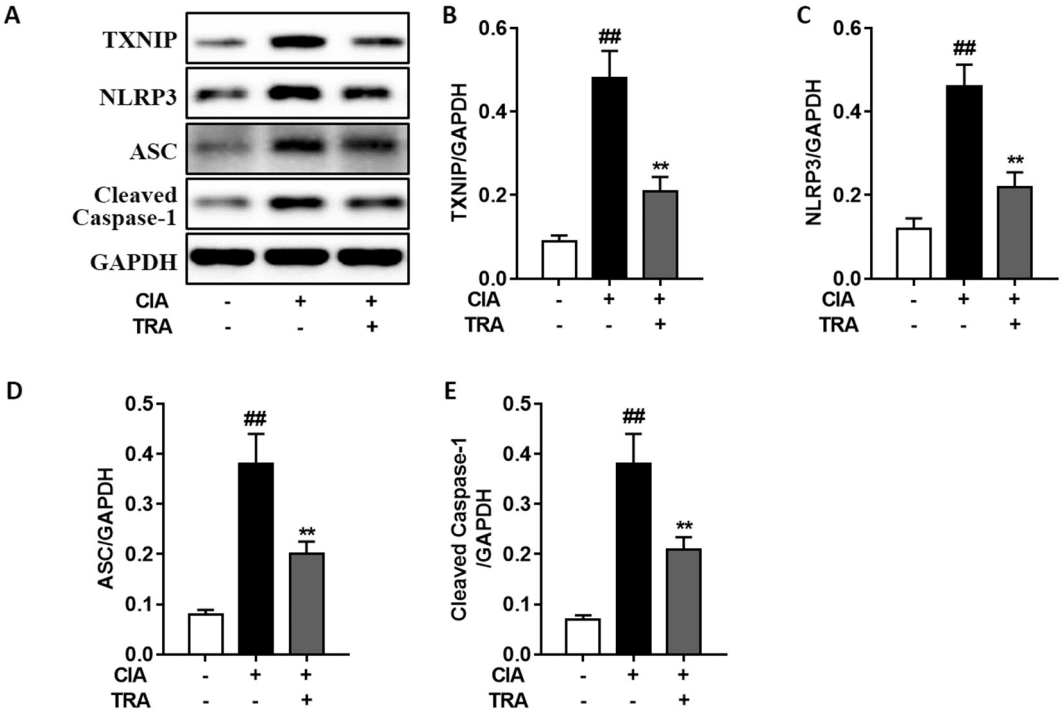

Fig. 9. Effects of TRA on NLRP3 inflammasome pathways in the joint tissues of CIA mice.
On day 48, animals were sacrificed, and tissues were collected for Western blot assay. The protein was prepared, and the levels of TXNIP (A and B), NLRP3 (A and C), ASC (A and D), and cleaved caspase-1 (A and E) were measured via Western blot assay. Results are showed as means ± SEM (n = 9). # $P < 0.05$ and ## $P < 0.01$, versus control group; * $P < 0.05$ and ** $P < 0.01$, versus the vehicle-treated group.

indicating that TRA also provided inhibitory actions on downstream of NLRP3 inflammasomes. Accumulating evidence indicates that the NLRP3 inflammasome expression is well associated with the NF-κB signals transduction [15–18]. Thus, we suggested that the effects of TRA on NF-κB activation might be involved with the in the inactivation NLRP3 inflammasomes. These findings suggested that TRA might provide anti-inflammatory actions by inhibiting NLRP3 inflammasomes signaling.

It is well reported that CIA is one of common models of RA and CIA has been widely applied in various kinds of studies to explore RA pathogenesis and discovery therapeutic targets for RA [44–48]. To further investigate the actions of TRA, we treated CIA DBA/1 mice with TRA (10 mg/kg). Treatments of arthritic animals with TRA obviously decreased clinical scores and alleviated synovial hyperplasia as well as inflammatory responses. Consistent with results of HFLS-RA, TRA significantly reduced expressions of TNF-α, IL-6, IL-8, MMP-1 and MMP-3, as well as down-regulated NF-κB in CIA mice in vivo. In conclusion, our study provided evidence to support the notion that TRA, by inhibiting inflammatory reprocesses and alleviating joint destructions, have therapeutic effects on the development and progression of CIA in mice.

In conclusion, this study provided the first evidence that TRA attenuated IL-1β-induced synovial inflammations in HFLS-RA and RA progression in CIA mice. The underlying mechanisms of TRA on RA might be well associated with inhibition of inflammation via suppressing NF-κB and NLRP3 inflammasome pathways. These evidences indicated that TRA might be develop as a novel agent for treatment of inflammation-associated RA.

Acknowledgments

None.

Conflict of interests

The authors have declared no conflicts of interest.

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