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Triptolide, a diterpenoid triepoxide, suppresses inflammation and cartilage destruction in collagen-induced arthritis mice

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Abbreviations:
CIA, collagen-induced arthritis
CII, type II collagen
COX, cyclooxygenase
DAB, diaminobenzidine
DMARDs, disease modifying antirheumatic drugs
IL, interleukin
MMPs, matrix metalloproteinases
NF, nuclear factor
PBS, phosphate buffered saline
PG, prostaglandin

ABSTRACT

Chinese herbal remedy *Tripterygium wilfordii* Hook. f. (TWHF) has been reported to be therapeutically efficacious in the treatment of rheumatoid arthritis (RA), but its *in vivo* actions have not been clarified. The purpose of this study was to investigate the effects of triptolide, a diterpenoid triepoxide extracted from TWHF, on inflammation and cartilage destruction in collagen-induced arthritis (CIA) model mice. Histological examination demonstrated that triptolide significantly reduced the inflammatory responses and cartilage damage in the joint tissues. Interestingly, triptolide interfered with CIA-augmented expression of matrix metalloproteinases-13 and -3, which are considered to be key enzymes in the pathological destruction of cartilage, and simultaneously augmented CIA-reduced tissue inhibitors of metalloproteinases-1 and -2 expression in the joints. Moreover, triptolide inhibited prostaglandin E_2 production via selective suppression of the production and gene expression of cyclooxygenase (COX)-2, but not COX-1. The levels of interleukin (IL)-1β, tumor necrosis factor α and IL-6 were also decreased by triptolide in the joint tissues and sera as well as the suppression of CIA-mediated expression of their mRNAs in the joints. In addition, triptolide treatment *in vivo* was able to reduce an abundance of nuclear factor-κB, the transcriptional factor closely related to the inflammatory process, in articular cartilage and synovium in CIA mice. These results suggest that triptolide exerts novel chondroprotective and anti-inflammatory effects on RA, and the therapeutic action of TWHF on RA is, in part, due to the triptolide activities.

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quantitative real-time RT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction
RA, rheumatoid arthritis
RIA, radio immunoassay
SABC, streptavidin–biotin complex
TIMPs, tissue inhibitors of metalloproteinases
TNF, tumor necrosis factor
TWHF, *Tripterygium wilfordii* Hook. f.

1. Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammation in joints and concomitant destruction of cartilage and bone. Inflammatory mediators, such as prostaglandins (PGs) and proinflammatory cytokines, are closely associated with this pathologic process and play important roles in RA [1,2]. On the other hand, matrix metalloproteinases (MMPs) are involved in the destruction of extracellular matrices in cartilage. In particular, MMP-13/interstitial collagenase-3, which specifically cleaves type II collagen (CII) of hyaline cartilage more efficiently than MMP-1/collagenase-1, and MMP-3/stromelysin-1, which digests proteoglycans and collagen types IX and X, are considered to be key enzymes in the pathological destruction of cartilage [3,4]. It is known that proinflammatory cytokines, interleukin (IL)-1, tumor necrosis factor (TNF) α, and IL-6 are pivotal factors, since they strictly enhance the biosynthesis and secretion of PGE_2 and MMPs from mesenchymal cells at inflammatory sites [5]. Under normal conditions, tissue inhibitors of metalloproteinases (TIMPs) bind to active MMPs in a ratio 1:1 to make an inactive complex. Therefore, an imbalance in the ratio of TIMPs to MMPs, which is generally caused by up-regulation of MMPs, results in continued matrix destruction in RA [6,7].

Currently, most treatments for RA are directly to normalize the immune system and to reduce inflammatory mediators. As therapeutic agents, disease modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs, and steroids are clinically common, and recently, TNFα-neutralizing therapy has been shown to provide sustained clinical benefits [8]. However, DMARD therapy has been impeded by the existence of a large number of nonresponders and by gravely adverse effects with a high frequency [6,9]. Therapy using soluble TNFα receptor or antibody against TNFα entails a high cost, hypersensitivity to medications, and infection due to TNFα blockage [10,11]. The validity of long-term treatment with these medicines has not yet been proven. To our knowledge no drugs have been developed for the purpose of cartilage protection.

Tripterygium wilfordii Hook. f. (TWHF) extracts have been found to be effective in traditional Chinese medicine for the treatment of immune inflammatory diseases including RA [12]. Triptolide, a diterpenoid triepoxide purified from TWHF, has been identified as the major component of TWHF and might account for its therapeutic effects [13]. Previous studies have shown that triptolide possesses both immunosuppressive and anti-inflammatory activities, including inhibition of cytokine gene expression in T cells [14]. These anti-inflammatory actions have been attributed to the inhibition of cyclooxygenase (COX)-2 and PGE_2 production in rheumatoid fibroblasts and other cell types [15]. Recently, we have reported the biological activity of triptolide *in vitro* and found novel evidence that triptolide suppressed the gene expression and production of proMMPs-1 and -3, and augmented those of TIMPs-1 and -2 in human synovial fibroblasts [16]. In addition, triptolide inhibited MMP-13 gene expression in human and bovine chondrocytes [17]. We also reported that triptolide interfered with the gene expression of proinflammatory cytokines in mouse macrophages [16]. These observations encouraged us to investigate the effects of triptolide, *in vivo*, on cartilage destruction and inflammation and its action mechanisms in RA model mice. Although an earlier study showed that prophylactic treatment of triptolide significantly reduced the incidence and severity of arthritis in the collagen-induced arthritis (CIA) rats [18], little detailed cellular or molecular evaluation has been performed to investigate the action mechanism of triptolide in this murine model. We have examined whether daily oral administration of triptolide could exert a therapeutic effect on CIA in mice. Specifically, the effects of triptolide treatment *in vivo* on inflammatory responses and cartilage destruction in this arthritis model were evaluated using immunohistochemistry, *in situ* hybridization, and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

In the present study, we demonstrated that triptolide effectively interferes with cartilage destruction and the inflammatory responses accompanying the direct suppression of MMP gene expression and simultaneous up-regulation of TIMP production in the joints. In addition, triptolide down-regulated the expression of proinflammatory cytokines and COX-2 as well as a common transcription factor nuclear factor (NF)-κB [19,20] which closely participates in their expressions.

2. Materials and methods

2.1. Animals

Male DBA/1J mice (Charles River Laboratory Japan, Kanagawa, Japan), age 8–10 weeks, were used for the study of CIA. Filter-top cages were used, with five mice in each cage. During the

course of this experiment, all mice were fed breeder's chow by the Center for Laboratory Animal Care, China Academy of Chinese Medical Sciences.

2.2. Induction of CIA

Bovine CII (Sigma, St. Louis, MO, USA) was dissolved in 0.1 M acetic acid overnight at 4 °C. This was emulsified in an equal volume of complete Freund's adjuvant (Sigma). The mice were immunized intradermally at the base of the tail with 100 μl of emulsion containing 100 μg of CII. On day 21, mice were boosted intraperitoneally with 100 μg CII dissolved in phosphate buffered saline (PBS) [21,22]. This model has been widely used to study disease mechanisms and potential therapies for RA. Indeed, CIA model has many morphological features similar to those of human RA including patterns of synovitis, pannus formation, and erosion of articular cartilage and bone. Moreover, CIA shares with RA many of the cytokines and biological factors in the synovium and cartilage [23].

2.3. Drug treatment

Triptolide (purity > 99.98%) was kindly provided by Professor Sui Lin (Fujian Institute of Medical Sciences, Fuzhou, China), and this is commercially available from Alexix Biochemicals (San Diego, CA, USA). Dexamethasone was purchased from Sigma. Both were dissolved in 0.05% DMSO. In order to assess the effect of triptolide on the established CIA, treatment was commenced from the first day of the onset of the clinical symptoms of arthritis, which was considered to be the day when the first visible signs of erythema and/or oedema were observed in any of the limbs. Mice were randomly selected and assigned to one of the following groups: triptolide (8, 16 and 32 μg/(kg day); $n = 16$, respectively), dexamethasone (1 mg/(kg every 2 days); $n = 16$) or vehicle ($n = 20$). The route of triptolide delivery was oral administration. Treatment was given daily for a period of 21 days. The dosage selection for triptolide (8–32 μg/(kg day)) was based on the results of our previous study (Na Lin, Chunfang Liu: unpublished observations) and corresponded to 0.625–2.5% of LD_{50} for triptolide (1.278 mg/kg). Sixteen age-matched, nonimmunized male mice were used as a normal control.

2.4. Assessment of arthritis

Mice were visually examined for arthritis severity once every 2–3 days throughout the whole period by two independent, blinded observers. Clinical scores expressed arthritis severity on a scale of 0–4 for each paw, according to changes in erythema and oedema as previously described [24]. Briefly, score 0, normal; score 1, detectable arthritis with erythema at least some digits; score 2, significant swelling and redness; score 3, severe swelling and redness from joint to digit; score 4, maximal swelling with ankylosis. The total score was the cumulative value for the four paws, with a maximum of 16 for each mouse. Arthritis was considered to be present if the score for a paw was >2. The incidence of arthritis is defined as the percentage of animals within each group exhibiting any sign of disease regardless of severity.

2.5. Histology

Mice were sacrificed by cervical dislocation on day 22 of arthritis. Both hind limbs including the paws, ankles, and knees, were dissected, fixed immediately for 2 h in 4% paraformaldehyde, decalcified in 10% EDTA for up to 1 month at 4 °C, and embedded in paraffin. Tissue sections (6 μm) were mounted on common slides for staining with hematoxylin and eosin. All sections were randomized and evaluated by a trained observer who was blinded to the treatment groups and the arthritis severity of each mouse. The data were expressed as mean inflammation, pannus, cartilage damage, and bone damage scores. All scores were based on a scale of 0–3, as previously described [25,26].

2.6. Immunohistochemical staining

Paraffin sections (6 μm) of tissue from the knee and ankle joints were mounted on poly-L-lysine-coated slides. Immunolocalizations of COXs-1 and -2, and NF-κB p65 in the joints were carried out with commercial streptavidin-biotin complex (SABC) kits (Boster, Wuhan, China) according to the manufacturer's instructions. The paraffin sections were dewaxed by routine method and incubated for 10 min with 3% H_2O_2. Each section was incubated with normal goat serum for 20 min at room temperature, and then with rabbit polyclonal antibodies against mouse COXs-1, -2 or NF-κB p65 (Boster) respectively at dilution of 1:150 in PBS overnight at 4 °C. After incubation with biotinylated goat anti-rabbit IgG for 20 min at 37 °C, sections were reacted with SABC. The sections were then stained with 3,3-diaminobenzidine (DAB) (Sigma) and counterstained with hematoxylin. For the control staining, PBS was used instead of the primary antibody.

2.7. In situ hybridization studies

The paraffin sections (6 μm) of tissue from the knee and ankle joints as described above, were mounted on poly-L-lysine-coated slides and processed under RNase-free conditions. The sections were then dried overnight at 58 °C, and used immediately or stored at −20 °C.

The gene expressions of MMPs-13 and -3, TIMPs-1 and -2, COXs-1 and -2, and NF-κB p65 were measured using commercial *in situ* hybridization kits (Boster), according to the manufacturer's instructions. Briefly, the paraffin sections were dewaxed by routine method and incubated for 10 min with 3% H_2O_2. Prehybridization included digestion with pepsin in 3% citric acid for 20 min at room temperature followed by fixation in 1% paraformaldehyde for 10 min, and incubation with 20 μl prehybridization solution (40% deionized formamide, 5× SSC, 10% dextran sulfate, 5× Denhardt's solution, 100 μg/ml degenerated ssDNA) of each section for 4 h at 38 °C. Hybridization was performed overnight at 41 °C. DIG-labeled oligonucleotide probe was added in a volume of 20 μl/section. Washes were in 10× SSC solution at 41 °C. After blocking, the sections were incubated with biotinylated mouse antidigoxin antibody for 1.5 h. Each section was reacted with SABC for 30 min followed by biotinylated peroxydase. Development of the peroxidase staining was done with DAB and counterstaining was done with hematoxylin. For the negative control,

hybridization was performed with a prehybridization solution instead of a hybridization solution.

2.8. Microscopic analysis

Specimens were examined using a Leica image analyzer and analyzed by computer image analysis (Leica Microsystem Wetzlar Gmbh., Wetzlar, Germany) in a blinded manner. To localize and identify areas with positively stained cells, six digital images per specimen of cartilage and/or synovium from a knee or ankle joint were recorded, and quantitative analysis was performed according to the color cell separation. The results are expressed as the mean region of interest, representing the percentage of area covered with positively stained cells per image at a magnification of 200×.

2.9. Assay for the production of PGE_2 and cytokines

Joint tissues were prepared as previously described for measuring the production of PGE_2 [27] and cytokines [28]. Briefly, the left forepaw (including the paw, ankle, and knee) from each mouse was removed and homogenized in 100 mg tissue/1 ml of lysis medium (75% ethanol in 0.1 M sodium acetate, adjusted to pH 3 with HCl for PGE_2, and RPMI 1640 containing 2 mM phenylmethylsulfonyl fluoride and 1 μg/ml of aprotinin, leupeptin, and pepstatin A for cytokines). The homogenates were then centrifuged 3500 × *g* for 15 min at 4 °C. Sera were obtained from the mice on day 22 of arthritis, as described above. Supernatants and sera were stored at −20 °C until use.

PGE_2 concentration was measured with a commercial radio immunoassay (RIA) kit (Amersham Pharmacia Biotech, Amersham, UK) according to the manufacturer's instructions. Commercial enzyme-linked immunosorbent assay kits were used to measure the concentrations of IL-1β (Amersham), TNFα, and IL-6 (Diaclone, Besancon, France) in supernatant and serum, according to the manufacturer's instructions. Results were expressed as pg/ml of serum or supernatant from joint homogenate. The minimum detection limits of the assays were 8 pg/ml for PGE_2, 3 pg/ml for IL-1β, 25 pg/ml for TNFα, and 10 pg/ml for IL-6, respectively.

2.10. Quantification of messenger RNA (mRNA) by real-time RT-PCR

The left hind paws (including the paw and ankle) were dissected from mice, snap-frozen in liquid nitrogen, ground into powder and homogenized. This procedure was done under RNase-free conditions. Total RNA was extracted with TRIzol (Gibco BRL, Gaithershug, MD, USA) from the tissue homogenates according to the manufacturer's instructions. The total RNA (1 μg) was subjected to RT reaction using QuantiTect Reverse Transcription Kit (QIAGEN K.K., Tokyo, Japan) according to the instruction manual. A portion (an equivalent of 25 ng of total RNA for TNFα and IL-6, and an equivalent of 2.5 ng of total RNA for IL-1β and 18S rRNA) of the products of RT reaction was subjected to real-time PCR using QuantiTect SYBR Green PCR Kit (QIAGEN K.K., Tokyo, Japan) and QuantiTect Primer Assay (Cat No. QT01048355 for mouse IL-1β, Cat No. QT00104006 for mouse TNFα, Cat No. QT00098875 for mouse IL-6, and Cat No. QT01036875 for mouse 18S rRNA, QIAGEN K.K., Tokyo, Japan). The amplification cycle was performed at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s using ABI PRISM 7000 Sequence detection system (Applied Biosystems Japan Ltd., Tokyo, Japan). Obtained threshold cycle (C_T) values of each sample were normalized by that of 18S rRNA, and relative expression level was expressed mean value of vehicle group as 1.

2.11. Statistical analysis

Data are presented as the mean ± S.E.M. Except for arthritis incidence and pathological scores, variance analysis was employed and post hoc Bonferroni, LSD or Dunnett's test was used to determine statistical significance. Arthritis incidence was analyzed by chi-square test and pathological score with non-parametric statistics (Kruskal–Wallis test). *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Effects of triptolide on arthritis progression and joint histology

To investigate the effect of triptolide on arthritis, the CIA model in DBA/1 mice was used. Although the disease manifested itself on different days after immunization, we did not observe a relation between clinical response and time of onset of disease. Oral administration of triptolide, once a day started when the first clinical signs of disease were beginning, and continued for 21 days. This first clinical signs of disease were sometime lesser than clinical score 1. Triptolide dose-dependently interfered with increasing of arthritis scores in CIA mice, with significant suppression observed at both the 16 and 32 μg/kg doses (Fig. 1A). Consistent with the clinical scoring, the assessment of arthritis incidence also showed triptolide to be highly effective; in all groups receiving triptolide (8–32 μg/kg), the rate of incidence was markedly reduced from day 7 of arthritis (Fig. 1B).

Following sacrificing of the mice, the ankle and knee joints were evaluated histologically and then semiquantitatively graded for severity of inflammation, pannus, cartilage, and bone damage. In accordance with the clinically observed effects on disease incidence and severity, triptolide (8–32 μg/kg) dose-dependently inhibited the histological damage and cumulative arthritis injury scores, as compared with those of the vehicle control group (Table 1). As shown in Fig. 1C, both cartilage and bone destruction as well as inflammatory responses were inhibited by triptolide in the treated group as compared with that of the vehicle control group.

3.2. Triptolide inhibits MMPs-13 and -3 expression and auguments TIMPs-1 and -2 expression in the joints of CIA mice

To provide insight into the mechanism by which triptolide therapy exerts its inhibition of cartilage destruction, we measured the expression levels of MMPs-13 and -3 and their endogenous inhibitors, TIMPs-1 and -2, in the joints of mice by

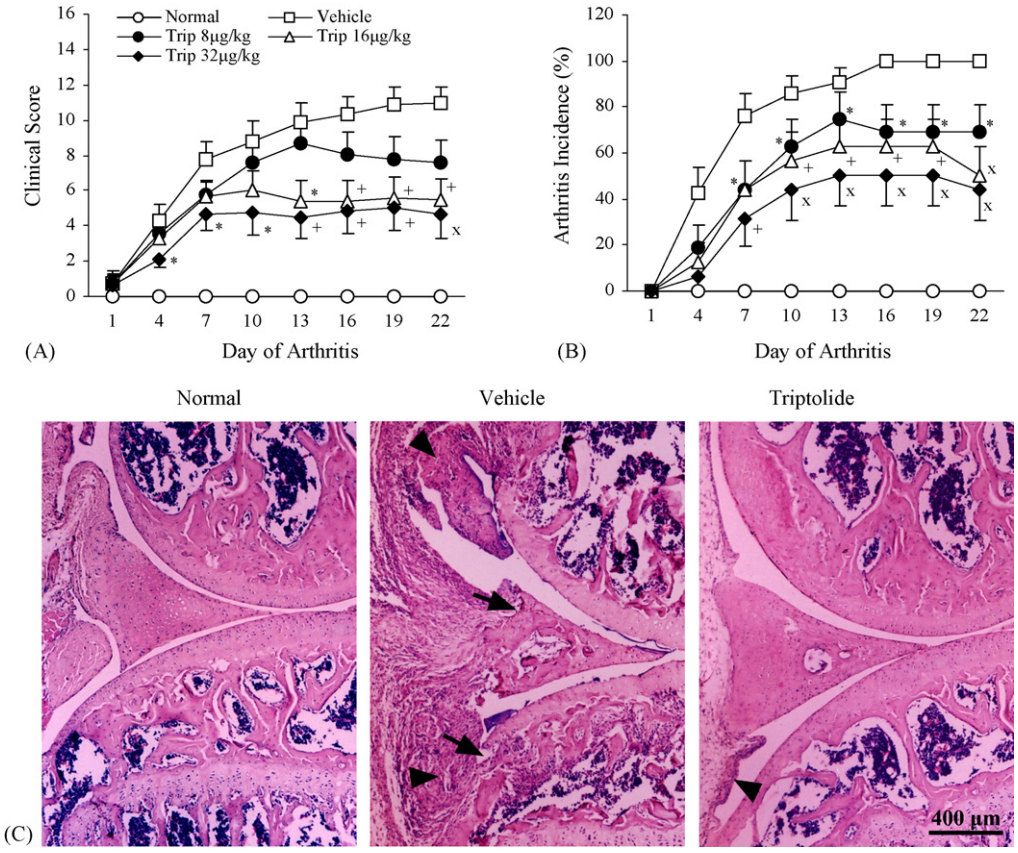

Fig. 1 – Effects of triptolide on arthritis progression and joint histology of CIA mice. Mice were orally administered triptolide (Trip) or vehicle for 21 days from the first day of the onset of the clinical symptoms of arthritis. At the end of the experiment, the clinical onset and histology of the joints were evaluated. (A) indicates clinical score of severity and (B) shows arthritis incidence. Data are represented as the mean ± S.E.M. (n = 16 for normal; n = 20 for vehicle; n = 16 for Trip 8, 16, and 32 μg/kg, respectively). *, +, and × Significantly different from the vehicle control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). (C) displays histological observation of the joints in mice. In normal mice, there is normal knee joint architecture. In vehicle-treated control mice, there is severe arthritis histologically characterized by severe inflammation (histological score 3), pannus (histological score 3), cartilage damage (histological score 3), and bone damage (histological score 1). In Trip (32 μg/kg)-treated mice, there is only mild synovium inflammation (histological score 1). Arrowheads indicate the site of synovium inflammation and arrows indicate the site of cartilage damage. Hematoxylin and eosin staining; original magnification was 40×.

in situ hybridization. As shown in Fig. 2A, in the joints of normal mice, there were certain amounts of MMPs-13 and -3 while their levels were dramatically elevated in the joints of CIA mice. Treatment of triptolide (16–32 μg/kg) significantly reduced the elevated levels of MMPs-13 and -3 to the levels of the age-matched control animals. Unlike MMPs, the expression of TIMPs-1 and -2 was significantly reduced in the joints of CIA mice as compared with that of normal mice (Fig. 2B). The

Table 1 – Histopathology scores in mice with collagen-induced arthritis

Histological parameter	Treatment			
	Vehicle (n = 10)	Trip, 8 μg/kg (n = 8)	Trip, 16 μg/kg (n = 8)	Trip, 32 μg/kg (n = 8)
Inflammation	1.90 ± 0.16	1.31 ± 0.15†	0.53 ± 0.08‡	0.40 ± 0.06‡
Pannus	1.14 ± 0.14	0.65 ± 0.12	0.41 ± 0.07†	0.35 ± 0.08‡
Cartilage damage	1.95 ± 0.15	1.29 ± 0.21	0.64 ± 0.13‡	0.45 ± 0.08‡
Bone damage	1.18 ± 0.11	0.84 ± 0.12	0.69 ± 0.13†	0.43 ± 0.12‡
Total score	6.18 ± 0.56	4.09 ± 0.60	2.28 ± 0.40†	1.64 ± 0.35‡

Mice were orally administered with triptolide (Trip, 8, 16, and 32 μg/kg, respectively) or vehicle for 21 days from the first day of the onset of the clinical symptoms of arthritis. Histopathology changes were scored on a 0–3 scale by an observer blinded to the treatment. Data are represented as the mean ± S.E.M. † and ‡ Significantly different from the vehicle control ($P < 0.05$ and $P < 0.01$, respectively).

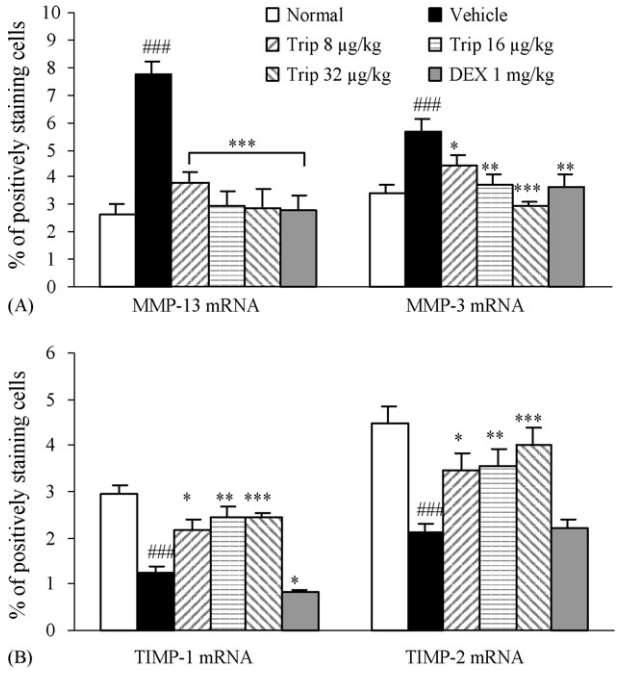

Fig. 2 – Effects of triptolide on mRNA expression of MMPs-13 and -3, and TIMPs-1 and -2 in the joints of CIA mice. Mice were orally administered triptolide (Trip, 8, 16, and 32 μg/kg, respectively), dexamethasone (DEX, 1 mg/kg), or vehicle for 21 days from the first day of the onset of the clinical symptoms of arthritis. At the end of the experiment, paraffin sections of the joints were collected. *In situ* hybridization was performed to determine the expression of MMPs-13 and -3 mRNA (A), and TIMPs-1 and -2 mRNA (B). Quantitative analysis was carried out by PhosphorImager. Data are represented as the mean ± S.E.M. ($n = 6$). ### Significantly different from the normal control ($P < 0.001$). *, **, * Significantly different from the vehicle control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).**

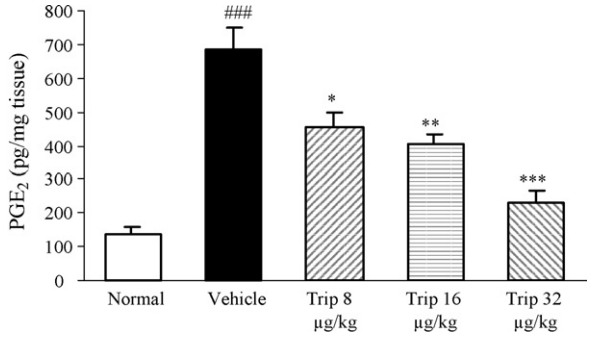

Fig. 3 – Triptolide interferes with PGE_2 production in the joints of CIA mice. Mice were orally administered triptolide (Trip, 8, 16, and 32 μg/kg, respectively) or vehicle for 21 days from the first day of the onset of the clinical symptoms of arthritis. At the end of the experiment, the joints were collected and homogenized. Radio immunoassay was performed to determine the production of PGE_2 in supernatants of the joints. Data are represented as the mean ± S.E.M. ($n = 6$). ### Significantly different from the normal control ($P < 0.001$). *, **, * Significantly different from the vehicle control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).**

administration of triptolide (8–32 μg/kg) dose-dependently increased the TIMPs-1 and -2 gene expressions with the treatment of 32 μg/kg showing levels comparable with those of the normal animals. The most interesting findings were that dexamethasone (1 mg/kg) significantly inhibited the elevated expression of MMPs-13 and -3, whereas the levels of TIMP-1 in CIA mice was significantly suppressed (Fig. 2). These results suggest that triptolide prevents the destruction of connective tissues via the simultaneous augmentation of TIMP production and interference with MMP production at inflammatory sites in RA.

3.3. *Triptolide interferes with PGE_2 production via selective inhibition of COX-2 expression in the joints of CIA mice*

Because RA is characterized by chronic inflammation in the synovial joints, the level of PGE_2, an inflammatory mediator, was measured in the joints of CIA mice by RIA. As shown in Fig. 3, tissue PGE_2 levels in the joints were significantly elevated as compared to those of the normal control. Triptolide effectively inhibited CIA-induced PGE_2 production in a dose-dependent manner. We further examined whether the inhibition by triptolide of PGE_2 production was due to the suppression of CIA-augmented COXs-1 and -2 transcripts. Immunohistochemical analysis indicated that in the joints of CIA mice, triptolide predominantly interfered with the CIA-mediated production of COX-2 but not COX-1 in a dose-dependent manner (Fig. 4A). The selective suppression of COX-2 production by triptolide was further supported by *in situ* hybridization, which showed that triptolide selectively interfered with COX-2 transcript (Fig. 4B and C).

3.4. *Effects of triptolide on gene expression and production of proinflammatory cytokines in CIA mice*

To obtain insights into the mechanisms of triptolide-mediated beneficial effects, local and circulating cytokine levels were measured in the joint tissues and sera. As shown in Fig. 5A, there were relatively small amounts of IL-1β, TNFα, and IL-6 mRNA transcripts in the joints of normal mice as determined by quantitative real-time RT-PCR, however, amounts of these transcripts were dramatically increased in the joints of the CIA mice. Triptolide treatment effectively reduced the levels of IL-1β mRNA (Fig. 5A) and IL-6 mRNA (Fig. 5C), but the suppression of TNFα mRNA (Fig. 5B) was lesser than that of IL-1β or IL-6 in the joints of CIA mice. Moreover, the protein levels of IL-1β in the joints (Fig. 5D) and TNFα in the sera (Fig. 5E) were also reduced by triptolide in a dose-dependent manner (8–32 μg/kg), and the similar results were observed for IL-6 (data not shown). The suppressive effect of dexamethasone on the gene expression and production of these proinflammatory cytokines in the CIA mice was similar to that of triptolide (Fig. 5).

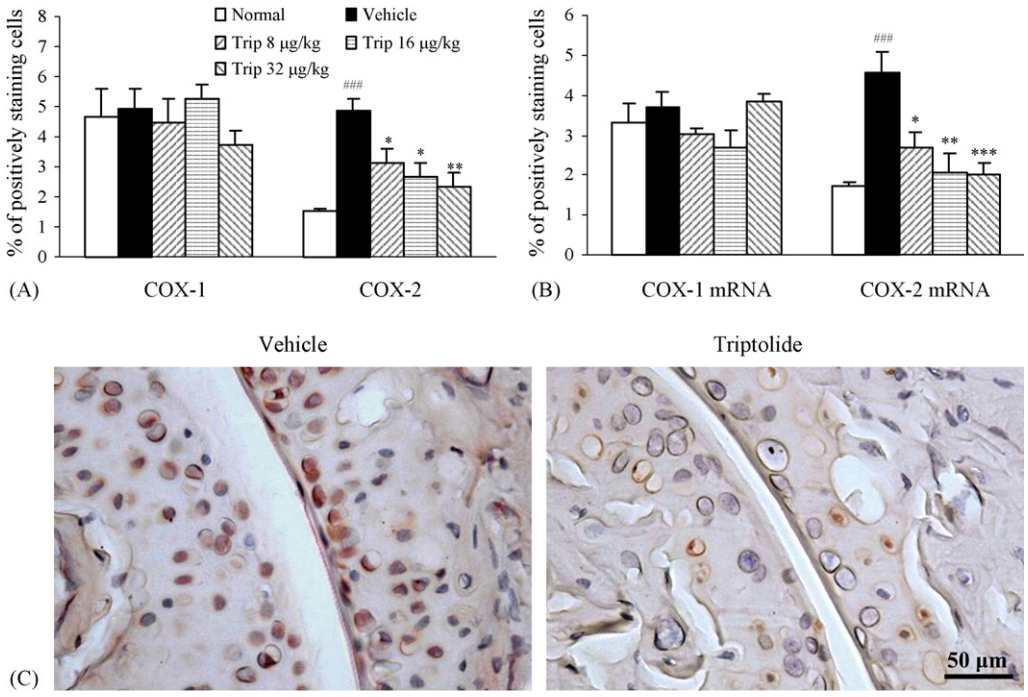

Fig. 4 – Effects of triptolide on the expression of COXs-1 and -2 in the joints of CIA mice. Treatment of the mice was the same as in Fig. 3. At the end of the experiment, paraffin sections of the joints were collected and subjected to immunohistochemical staining and *in situ* hybridization. (A) represents immunohistochemical study of the joints with rabbit polyclonal anti-murine COXs-1 and -2 antibodies. (B) shows *in situ* hybridization of COXs-1 and -2 mRNA. Quantitative analysis was performed by PhosphorImager. Data are represented as the mean ± S.E.M. ($n = 6$). ### Significantly different from the normal control ($P < 0.001$). *, **, * Significantly different from the vehicle control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively). Panel (C) displays *in situ* hybridization sections of the tibial astragaloid joints from the vehicle-treated control mice and triptolide (Trip, 32 μg/kg)-treated mice. The COX-2 mRNA positive cells were stained brown (hematoxylin counterstained). Original magnification was 400×.**

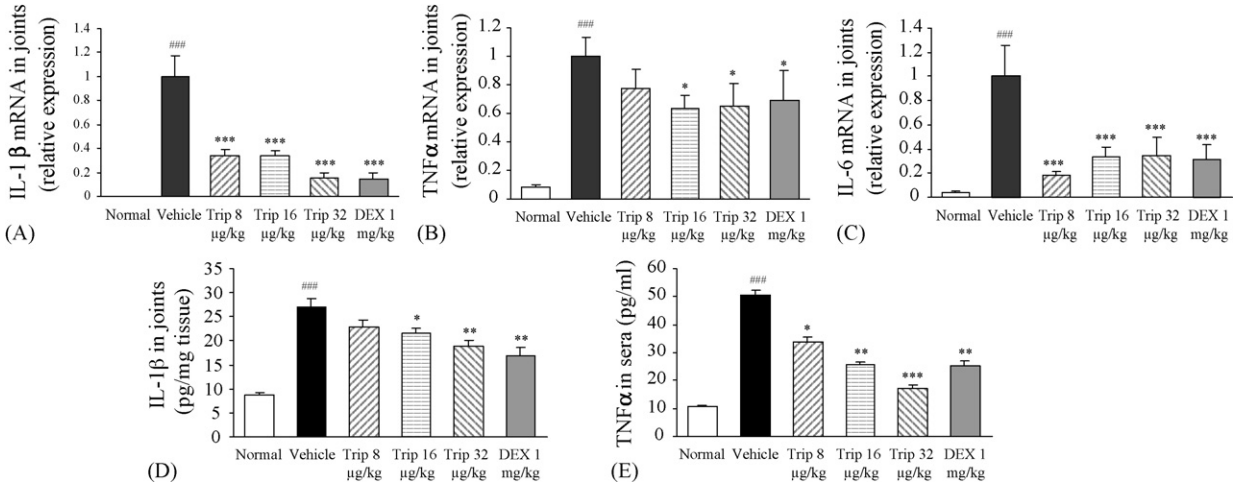

Fig. 5 – Effects of triptolide on the levels of IL-1β, TNFα, and IL-6 in the joints and sera of CIA mice. Treatment of the mice was the same as in Fig. 2. At the end of the experiment, the joints and sera were collected. Panels A–C display the results for the mRNA expression of IL-1β, TNFα, and IL-6, respectively. Relative mRNA expression levels of IL-1β, TNFα, and IL-6 were determined by quantitative real-time RT-PCR. 18S rRNA was employed as an internal standard. C_T values of each sample were normalized by that of 18S rRNA. Relative expression level was expressed mean value of vehicle group as 1. Groups: normal ($n = 12$); vehicle ($n = 12$); triptolide (Trip) 8 ($n = 8$), 16 ($n = 8$), and 32 μg/kg ($n = 6$), respectively; dexamethasone (DEX) 1 mg/kg ($n = 8$). Panel D shows a histogram of IL-1β in the joints and panel E indicates the protein levels of TNFα in sera measured by enzyme-linked immunosorbent assay, respectively. Data are represented as the mean ± S.E.M. ($n = 6$). ### Significantly different from the normal control ($P < 0.001$). *, **, * Significantly different from the vehicle control ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).**

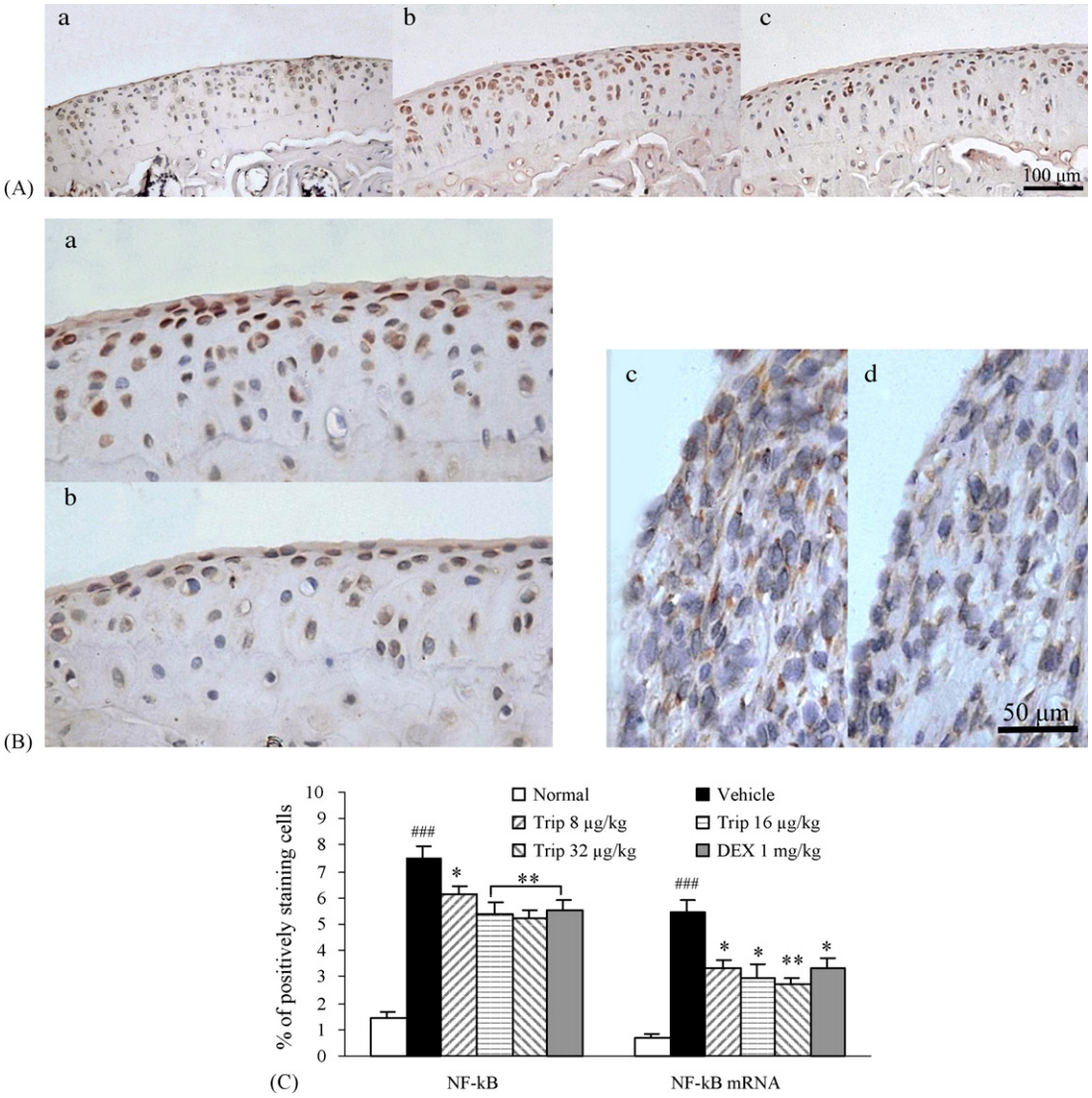

Fig. 6 – Inhibition by triptolide of the expression of nuclear factor NF-κB in the joints of CIA mice. Treatment of the mice was the same as in Fig. 2. At the end of the experiment, paraffin sections of the knee joints were collected and subject to immunohistochemical study and *in situ* hybridization to determine the presence of NF-κB p65 in cartilage and/or synovium. (A) displays immunohistochemical staining of NF-κB-positive cells in cartilage from three groups of normal (a), vehicle-treated (b), and triptolide (Trip, 32 μg/kg)-treated (c) mice. (B) shows NF-κB p65 mRNA in cartilage (a and b) and synovium (c and d) from two groups of vehicle-treated (a and c) and Trip (32 μg/kg)-treated (b and d) mice. The NF-κB-positive cells were stained brown (hematoxylin counterstained). Original magnification was 200× in panel A and 400× in panel B. (C) represents percentage of area covered with NF-κB-positive cells out of cartilage and synovium region was determined by immunohistochemical study and *in situ* hybridization. Quantitative analysis was performed by PhosphorImager. Data are represented as the mean ± S.E.M. ($n = 8$). ### Significantly different from the normal control ($P < 0.001$). *, ** Significantly different from the vehicle control ($P < 0.05$ and $P < 0.01$, respectively).

3.5. *Effect of triptolide on expression of NF-κB in the joints of CIA mice*

NF-κB has been shown to be active in RA and to be of functional relevance since it regulates the expression of proinflammatory cytokines and MMPs at inflammatory sites [19,20]. The effect of triptolide on NF-κB expression in the joints of CIA mice was immunohistochemically examined. As shown in Fig. 6A, NF-κB p65-positive cells, mostly chondrocytes, were observed in both the superficial and deep layers of the articular cartilage of the CIA mice (Fig. 6A-b), while in normal mice there were few positive signals for NF-κB p65 (Fig. 6A-a). In addition, the articular cartilage of CIA mice treated with triptolide showed limited and weak cytoplasmic staining for NF-κB p65 (Fig. 6A-c). This suppressive effect of triptolide on NF-κB was further supported by *in situ* hybridization, which showed that triptolide effectively reduced the NF-κB p65 transcript in the cartilage (Fig. 6B-a and -b). Triptolide also reduced the NF-κB p65-transcript as well as total cells in synovium of CIA mice joints (Fig. 6B-c and -d).

Fig. 6C shows triptolide to be highly effective; in all groups receiving triptolide (8–32 μg/kg), both protein and gene expression of NF-κB were markedly reduced in a dose-dependent manner, indicating that the inhibition of NF-κB production by triptolide closely correlates with the changes in steady-state level of its mRNA. This is also true for dexamethasone.

4. Discussion

Data presented in this study indicate that purified triptolide from the anti-rheumatic herb, TWHF, can potently suppress the inflammatory responses and cartilage destruction in the CIA model. Several mechanisms may account for this effect. Triptolide directly down-regulates the expression of MMPs and simultaneously up-regulates that of TIMPs in the joints. Triptolide also interferes with the gene expression of proinflammatory cytokines and PGE_2 production. Moreover, triptolide inhibits NF-κB expression in the articular cartilage and synovium. Thus, the observed beneficial anti-arthritic effects of TWHF may be due to its active triptolide component. This study shows for the first time that besides its known anti-inflammatory and immunosuppressive activities, triptolide has the potential to protect cartilage integrity *in vivo*.

It has been recognized that MMPs-13 and -3 play important roles in the degradation of connective tissue components in cartilage with RA, and high levels of MMPs-13 and -3 are found in synovial tissues and fluid from RA patients [29,30]. The expression and production of MMPs are regulated at the transcriptional levels by various cytokines and other stimuli including IL-1 and TNFα [4]. Moreover, the enzymatic activities of MMPs at inflammatory sites are controlled by their endogenous inhibitors called TIMPs [4]. Therefore, it is likely that the imbalanced production of MMPs and TIMPs causes the destruction of connective tissue of the joints. In the present study, we demonstrated that triptolide significantly interfered with the CIA-augmented gene expression of MMPs-13 and -3, while augmented that of TIMPs-1 and -2 in the inflamed joints of CIA mice. These results are supported by our previous study where IL-1-augmented expression of MMP-1/collagenase-1 as well as MMP-3 is inhibited by triptolide in synovial fibroblasts [16]. A recent study has also showed that the induction of MMPs-13 and -3 by inflammatory cytokines was inhibited by triptolide in chondrocytes *in vitro* [17]. Thus, triptolide can block cartilage degradation mediated by MMPs in the inflamed joints of RA.

At inflammatory sites of RA, proinflammatory and immunomodulatory cytokines such as IL-1, TNFα, and IL-6 are known to exert various actions during progress in inflammation [31,32]. Both IL-1 and TNFα induce and/or enhance the production of PGE_2 as well as MMPs including MMPs-1, -3, and -13 in many mesenchymal cell species like synovial cells, chondrocytes, and macrophages [4,33–36]. IL-6 is also characterized as an inflammatory factor because it synergistically augments the inflammatory actions of IL-1 in human synovial cells [37]. We have previously reported that triptolide has an inhibitory effect on the production of proinflammatory cytokines by lipopolysaccharide-stimulated mouse macrophages [16]. In this study, we further confirmed that oral administration of triptolide resulted in reduced concentrations of IL-1β, TNFα, and IL-6 at both the local and systemic levels, which correlated with the changes of their mRNAs in the joints. The actions of triptolide toward the production of cytokines are similar to those of the crude extract of TWHF; ethyl acetate-extract suppresses cytokine production by monocytes, IgG secretion by B cells, and IL-2 and IL-4 production by lymphocytes [38].

Since inducible COX-2 is a key enzyme for PGE_2 production at sites of inflammation in humans and CIA mice [27,39], the effects of triptolide on PGE_2 production and COXs induction in the joints of CIA mice were examined. Triptolide inhibited PGE_2 production in the inflamed joints through selective suppression of gene expression and production of COX-2, but not COX-1. It is recognized that COX-1, a constitutively expressed enzyme, participates in the production of physiological levels of PGs in tissues, such as in the mucus of the stomach [40]. Therefore, an anti-inflammatory drug that selectively inhibits the production and/or activity of COX-2 is very desirable. On the other hand, COX-1 and COX-2 overexpression by stable transfection has been shown to induce membrane type 1-MMP (MT1-MMP), which activates proMMP-2/progelatinase A [41] suggesting possible interaction between COX and MMPs.

Recently, the transcription of MMPs and proinflammatory cytokines has been shown to be dependent on NF-κB, as is the expression of COX-2 [19,20]. We, therefore, investigated the effects of triptolide on NF-κB expression, and demonstrated that triptolide remarkably suppressed the gene expression and production of NF-κB in the cartilage and synovium of CIA mice, this suppression of triptolide was supported in part by the latest study [42]. Consistent with these results are previous observations indicating that triptolide inhibits IL-2 gene expression in T cells via interference with transcriptional activation of NF-κB [14]. In addition, an ethanol extract of TWHF has been shown to suppress proinflammatory cytokine-induced MMPs-13 and -3 gene expression partly by inhibiting the DNA binding capacity of activating protein-1 and NF-κB [43]. Taken together, it is therefore highly possible that NF-κB is a precise target molecule for triptolide-mediated novel chondroprotective and anti-inflammatory effects in RA.

It is of interest that the bifunctional, anti-inflammatory and immunosuppressive effects of triptolide are very similar to those of anti-inflammatory corticosteroids. Previous studies have indicated that active components of TWHF and dexamethasone selectively inhibit COX-2-mediated PGE_2 production [15,16], and suppress the IL-2 transcription and production of interferon γ along with a similar spectrum of anti-inflammatory and immunosuppressive activities [44]. Therefore, it is of concern whether these actions of triptolide appear via glucocorticoid receptor in synovial cells. In this point of view, Maekawa et al. [45] have reported an interesting fact that one of the extracts from TWHF, as well as dexamethasone selectively suppresses the IL-1β-induced expression of COX-2 in human synovial cells, whereas the extract does not act as a glucocorticoid agonist. This was further supported by our findings that triptolide down-regulated the gene expression of MMPs-13 and -3, while

up-regulated those of TIMPs-1 and -2. In contrast, dexamethasone coordinately suppressed the gene expression of both MMPs and TIMPs [16]. Taken together, these results indicate that the anti-inflammatory action of TWHF, especially triptolide, is clearly distinguished from that of dexamethasone.

Throughout the present study, triptolide was well-tolerated in the dose used (0.625–2.5% of LD_{50}) with no evidence of drug toxicity and no damage to viscera including the kidneys and liver of mice. Additionally, in our previous *in vitro* study, triptolide was found not to exert any cytotoxic effect on mouse macrophages or human synovial fibroblasts [16]. Therefore, it is likely that triptolide selectively exerts its suppressive effects on arthritis in RA.

In conclusion, we have demonstrated for the first time that the therapeutic *in vitro* effects of triptolide so far reported were also confirmed in our *in vivo* RA model; *i.e.* triptolide effectively interfered with the destruction of cartilage and inflammatory responses in CIA mice. These therapeutic effects resulted in part from the direct suppression of the production of MMPs and the simultaneous up-regulation of TIMPs production in the joints. Interference by triptolide with the gene expression of proinflammatory cytokines and PGE_2 production was also highly effective. The suppression of MMP and cytokine production is in part due to the inhibition of NF-κB. Therefore, these results suggest that triptolide exerts novel chondroprotective and anti-inflammatory effects in RA, and indicate that the therapeutic action of TWHF on RA is, in part, due to actions of triptolide.

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