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ORIGINAL ARTICLE

Theaflavin-3, 3′-Digallate Attenuates Rheumatoid Inflammation in Mice Through the Nuclear Factor-κB and MAPK Pathways

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Abstract

Rheumatoid arthritis (RA) is a common autoimmune disease which impacts a large number of patients worldwide, and new drugs are required for lower the disease burden. Theaflavin-3, 3′-digallate (TFDG) is polyphenol exhibiting inhibition on inflammatory factors. This study aimed to explore the attenuation of TFDG on RA. The collagen-induced arthritis (CIA) mouse model was established and administered with TFDG. The arthritis score and incidence was recorded to assess the amelioration of TFDG on arthritis. Histopathological change of the mouse joint tissues was evaluated by haemotoxylin and eosin staining. The expression of pro-inflammatory mediators including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 was quantified by ELISA. The activation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) signaling pathways in the synovium were determined by Western blotting. In comparison with the control, administration of TFDG significantly reduced arthritis score and incidence in the CIA mouse model. TFDG significantly suppressed the expression of IL-1β, TNF-α, and IL-6, as well as the levels of MMP-1, MMP-2, and MMP-3 in the synovium. TFDG also showed remarkable inhibition on the activation of NF-κB and the phosphorylation of P38, JNK2, and ERK. This study puts up evidence that TFDG exerts protection on RA via inhibiting the activation of NF-κB- and MAPK-signaling pathways.

Keywords Rheumatoid arthritis · Theaflavin-3 · 3′-digallate · Collagen-induced arthritis model · Nuclear factor-kappa B · MAPK pathway

Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disorder which affects about 1% of the population worldwide (Feldmann et al. 1996). RA is characterized by hyperplastic synovial membrane with ongoing chronic inflammation of the synovial joints and infiltration of the various activated immune cells (Cush and Lipsky 1988). If left untreated, the inflammation can cause pain and swelling, and subsequent joint erosion or progressive disability (Singh et al. 2017). Similar to many other human autoimmune diseases, although there has been progress in its etiology, it remains unknown what initiates the pathogenic process of RA. Due to the attacks of immune system to the joint in RA, biologics or tofacitinib is medications used to reduce joint inflammation to alleviate symptoms (Singh et al. 2017). Conventional disease-modifying antirheumatic drugs (DMARDs) such as non-steroidal anti-inflammatory drugs and corticosteroids has been recognized as effective owing to the attenuation of disease activity and substantial detainment of joint deformity. However, the potency of these pharmacologic therapies is limited in relieving stiffness and pain, but not disease progression (Guo et al. 2018). In recent years, pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been shown to play pivotal roles in the development of RA, and diverse biologic agents targeting these cytokines have proved to be significantly superior to conventional DMARDs (Kwok et al. 2012). Moreover, antibody against receptor activator of nuclear factor-kappa-B ligand (RANKL), anti-CD20 antibody, or Janus kinase inhibitors has recently emerged as new therapeutic strategies (Kim and Moudgil 2017). Despite the increasing varieties of new treatment agents, long-term remission of the symptoms for a large number

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of patients could not be achieved. Therefore, the development of new treatment options is required for lowering the disease burden.

Theaflavin-3, 3′-digallate (TFDG) is a kind of polyphenol extracted from black tea and possesses two galloyl groups from the co-oxidation of (−)-epigallocatechin gallate and (−)-epicatechin gallate during black tea production (Finger 1994). TFDG inhibited the formation and differentiation of osteoclasts via inhibition of matrix metalloproteinases (MMPs), which participate in degeneration of the matrix associated with bone and cartilage (Oka et al. 2012). In addition, TFDG has been shown to suppress the induction of pro-inflammatory mediators and enhances the expression of anti-inflammatory cytokine in a co-culture system comprising adipocytes and RAW 264.7 macrophages (Ko et al. 2014). In a lipopolysaccharide (LPS)-induced acute lung injury mouse model, TFDG suppressed the production of pro-inflammatory cytokines, as well as the phosphorylation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase (MAPK) in macrophages (Wu et al. 2017). Moreover, previous study showed that TFDG significantly inhibited debris-induced osteolysis and prevented bone destruction in a mouse calvarial model by targeting the RANKL-induced extracellular regulated protein kinases (ERK) signal pathway (Hu et al. 2017). Overall, TFDG has been shown potent anti-inflammatory property in different in vitro and in vivo models. The current study aimed to examine the modulation of TFDG in the rheumatoid arthritis mouse model, as well as the underlying mechanism of action.

Materials and Methods

Animals and Collagen-Induced Arthritis Mice Model

DBA/1J mice (15–20 g, 7 weeks) were purchased from Beijing VitalRiver Laboratory Animal Technology Co., Ltd (Beijing, China). The mice were housed in the pathogen-free animal facility with food and water provided at ad libitum. The protocol for animal use was approved by the Animal Care and Use Committee of Daqing Oilfield General Hospital. After 1 week of acclimatization period, the mice were injected 0.1 ml of the emulsion subcutaneously at the base of the tail with 100 mg bovine type II collagen (Chondrex, Redmond, WA, USA) with complete freund's adjuvant (Chondrex). Starting on day 7 after the primary immunization, mice were given TFDG (Sigma-Aldrich, St. Louis, MO, USA) at 10 mg/kg, three times per week by intraperitoneal injections for 9 continuous weeks, and monitored for 10 whole weeks. The mice were visually examined three times per week for the appearance of arthritis, and the arthritis score index for the disease severity was determined according to a previous report (Li et al. 2017). Briefly, it was a 0–16 scale which reflects the severity of erythema and swelling on the midfoot, ankle joint, metatarsal joints and digits of mice. The scoring was executed by two blind observers independently.

Histopathological Assessment of Arthritis

On day 49 post the establishment of the collagen-induced arthritis (CIA) mouse, the mouse joint tissues were removed from four mice in each group, and fixed in 4% paraformaldehyde, decalcified in 10% EDTA bone decalcifing agent, and embedded in paraffin (Leica Biosystems, Buffalo Grove, IL, USA). Seven-micrometer sections were prepared and stained with haemotoxylin and eosin (H&E; ThermoFisher Scientific, Rockville, MD, USA). Histologic scores were evaluated on the basis of infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction, and bone erosions, and the scoring system ranged 1–4 for these parameters, respectively: 1 = normal, 2 = mild, 3 = moderate, 4 = severe (Li et al. 2013b).

Cytokine Measurement by Enzyme-Linked Immunosorbent Assays

The synovium in each group was homogenized using a Polytron tissue homogeniser (Kinematica Inc, Luzern, Switzerland) to extract total protein. The homogenates were centrifuged at 1,000 × *g* at 4 °C for 15 min. Supernatants were transferred to Eppendorf tubes, followed by a centrifugation at 15 000 × *g* for 5 min. Protein levels of cytokines including IL-1β, TNF-α, and IL-6 were quantified by the commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Shanghai, China) according to the manufacturer's instructions. Briefly, samples were diluted and mounted into 96-well plates coated with purified primary polyclonal antibody. The absorbance at 450 nm was recorded by microplate reader (Molecular Device, San Jose, CA, USA), and data are expressed as pg/ml.

Western Blot Analysis

To investigate the mechanisms of the inhibition of TFDG on the inflammation, the relative expression levels of MMPs including MMP-2, MMP-3 and MMP-9 in the synovium tissues were determined by Western Blot. Likewise, the levels of proteins in NF-κB-signaling pathways including p-IκB and p65, as well as the levels of proteins in MAPK-signaling pathways including p-p38, p-JNK, and p-ERK were also detected by Western blot. Briefly, equal amount (20 μg per lane) of total proteins from the synovium homogenates were

separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (ThermoFisher Scientific). The membranes were blocked by 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween-20) buffer for 2 h at room temperature, and incubated with primary antibodies overnight at 4 °C. Membranes were washed five times with TBST and incubated with secondary antibodies for 1 h and detected by enhanced chemiluminescence substrate (Pierce, Rockville, MD, USA). The membranes were imaged by Amersham Imager 600 (GE Healthcare, Chicago, IL, USA). For the detection of MMPs and MAPK-signaling pathways, β-actin was used internal control. For the NF-κB-signaling pathways, lamin B was used as internal control. Primary antibodies to MMPs were purchased from Calbiochem Co. (San Diego, CA, USA). Antibodies against phospho-p38, p38, phospho-JNK2, JNK2, phospho-ERK, and ERK were purchased from Cell-Signaling Technology (Danvers, MA, USA). Rabbit monoclonal antibodies against IκB, p-IκB, β-actin, and Lamin B were purchased from Abcam (Cambridge, MA, USA). Rabbit monoclonal antibody against NF-κB p65 was purchased from Beyotime (AN365, Jiangsu, China). The horseradish peroxidase-conjugated secondary antibodies were purchased from Thermofisher Scientific (Rockville, MD, USA).

Statistical Analysis

Data are expressed as the mean ± SD. Student's *t* test was used for data analysis. All data were analyzed using SPSS statistical software package. Statistical significance was defined as $p < 0.05$.

Results

TFDG-Alleviated Arthritis Score and Incidence in CIA Mice

As shown in Fig. 1a, in the vehicle group, mice started to show arthritis score 1 at week 4, which defined that erythema and mild swelling demonstrated to the midfoot, indicating the initiation of CIA. In contrast, in the TFDG treatment mice, arthritis score 1 started to show at week 5, indicating the delaying of the arthritis commencement by TFDG treatment. Similarly, the arthritis incidence in the vehicle control group reached 20% at week 4, whereas the TFDG-treated group presented the same value at week 5 (Fig. 1b). In addition, the overall arthritis score and arthritis incidence in the TFDG group (arthritis score 5.8 ± 0.45 and arthritis incidence 80%) were significantly lower than the vehicle group (arthritis score 10.6 ± 1.14 and arthritis incidence 100%) across ten weeks observation period ($p < 0.01$),

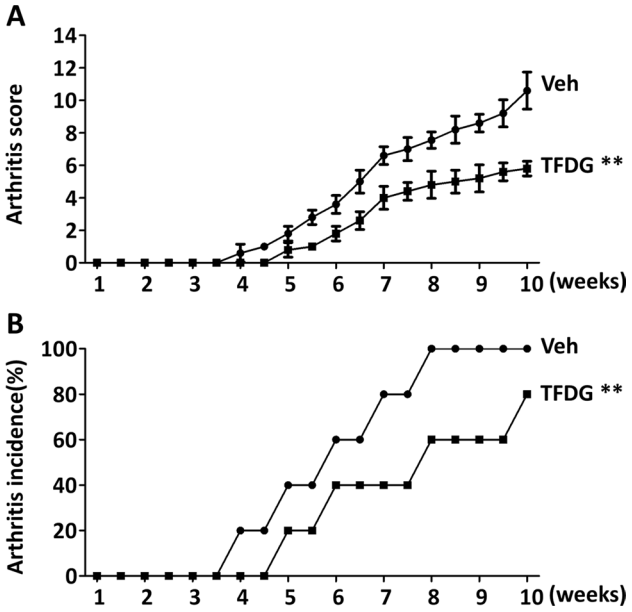

Fig. 1 TFDG-attenuated arthritis score and incidence in CIA mice. A reduction in arthritis score (**a**) and arthritis incidence (**b**) in CIA mice via TFDG. The mice were immunized with CII. 7 days after immunization, the mice were treated with intraperitoneal injections of TFDG (10 mg/kg) or vehicle three times per week for 9 week. The results are shown as the mean arthritis score ± SD, $n = 5$, $^{**}p < 0.01$ vs vehicle (Veh) group

demonstrating that the TFDG treatment alleviated CIA compared with that of vehicle control group.

TFDG-Attenuated Synovial Inflammation and Cartilage-Bone Destruction in CIA Mice

Destruction of cartilage and bone is hallmarks of arthritis, so we used histopathological method to determine the extent of arthritis in the mouse joint tissues. Figure 2a shows the H&E staining results of the mouse joint tissues at week 7 post the establishment of the CIA. In the vehicle control group, we can see severe infiltration of inflammation, obvious synovial hyperplasia, extended cartilage destruction and bone erosion, all of which parameters were significantly attenuated in the TFDG treatment group. As shown in Fig. 2b, the specific histologic scores evaluated on the basis of infiltration of inflammatory cells showed moderate to severe infiltration (2.75 ± 0.50) in vehicle group, whereas the TFDG group showed mild-to-moderate infiltration (1.75 ± 0.50). The histologic scores of synovial hyperplasia, cartilage destruction, and bone erosion in vehicle group were between moderate and severe extents (2.50 ± 0.58, 2.25 ± 0.50 and 2.25 ± 0.50, respectively), whereas these markers were mild in the TFDG group (1.25 ± 0.50, 1.25 ± 0.50 and 1.25 ± 0.50, respectively).

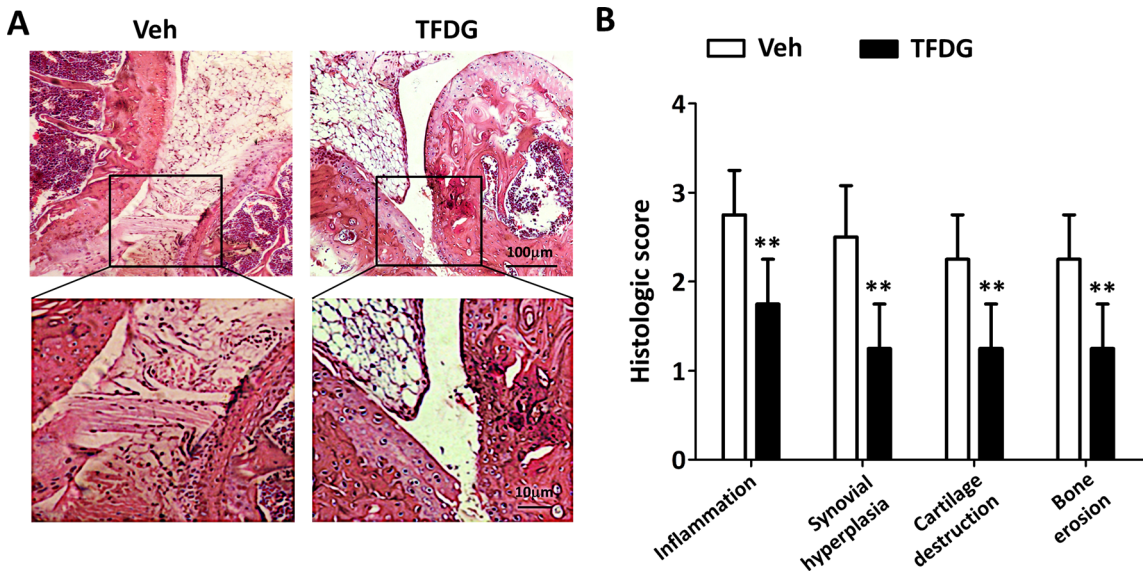

Fig. 2 TFDG-attenuated synovial inflammation and cartilage-bone destruction in CIA mice. The mice were immunized with CII. Seven days after immunization, the mice were treated with intraperitoneal injections of TFDG (10 mg/kg) or vehicle three times per week for 9 week. **a** On day 49 after primary immunization, the joints of hind paws were stained with hematoxylin and eosin to study the degree of synovitis and cartilage-bone erosions. Further magnification of the black-bordered box (top) shows the typical inflammatory injuries (bottom). **b** Histologic score was evaluated from the joints of CIA mice treated with or without TFDG on the basis of infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction and bone erosion. Data are expressed as mean ± SD, $n = 4$, $^{**}p < 0.01$ vs vehicle (Veh) group

TFDG Downregulated the Levels of Inflammatory Cytokines and MMPs in Synovium

To determine the anti-inflammatory effects of TFDG treatment in CIA model, the cytokines and MMPs' levels in the mice synovium tissues homogenate were assessed. The protein levels of cytokines IL-1β, TNF-α, and IL-6 in different treatment groups were evaluated by ELISA. As indicated in Fig. 3a–c, compared to the vehicle group, the levels of all these inflammatory factors were significant downregulated in the TFDG treatment group. Specifically, the treatment with TFDG decreased the levels of IL-1β, TNF-α, and IL-6 ($p < 0.01$). As expected, in Fig. 3d, e, Western blot results showed that the treatment with TFDG reduced the protein levels of MMP-2, MMP-3, and MMP-9 ($p < 0.01$).

Effects of TFDG on the Activation of NF-κB-Signaling Pathways in Synovium

To examine whether NF-κB pathway is involved in the antagonism of TFDG on CIA, IκB phosphorylation was evaluated by immunoblotting. The results showed that treatment with TFDG significantly inhibited the phosphorylation of IκB in synovium of the CIA mice (Fig. 4a). Since NF-κB nuclear extract p65 subunit is responsible for the transcriptional activity of NF-κB, we also determined the p65 nuclear accumulation. The level of p65 is also decreased by TFDG treatment (Fig. 4b). As illustrated in Fig. 4c, d, TFDG was able to lower the ratio of p-IκB/IκB and downregulate the ratio of NF-κB p65/Lamin B.

Effects of TFDG on the Activation of MAPK-Signaling Pathways in Synovium

We measured the total expression levels and phosphorylation of MAPKs in the synovium of the CIA mice by Western blot. The results showed that TFDG treatment significantly inhibited the phosphorylation of P38, JNK2, and ERK (Fig. 5a), while the ratios of p-p38/p38, p-JNK2/JNK2, and p-ERK/ERK in the TFDG-treated mouse group were downregulated (Fig. 5b).

Discussion

Rheumatoid arthritis is a common destructive arthropathy which impairs quality of life, even giving rise to morbidity and mortality worldwide (Bortoluzzi et al. 2018). Due to the limitation of the current clinical management for RA, which is restricted to treating or relieving the symptoms, a more comprehensive understanding of the underlying cause of this disease or the development of new therapeutic drugs will shed light to this significant pathologic change. In this study, we investigated the effects of TFDG,

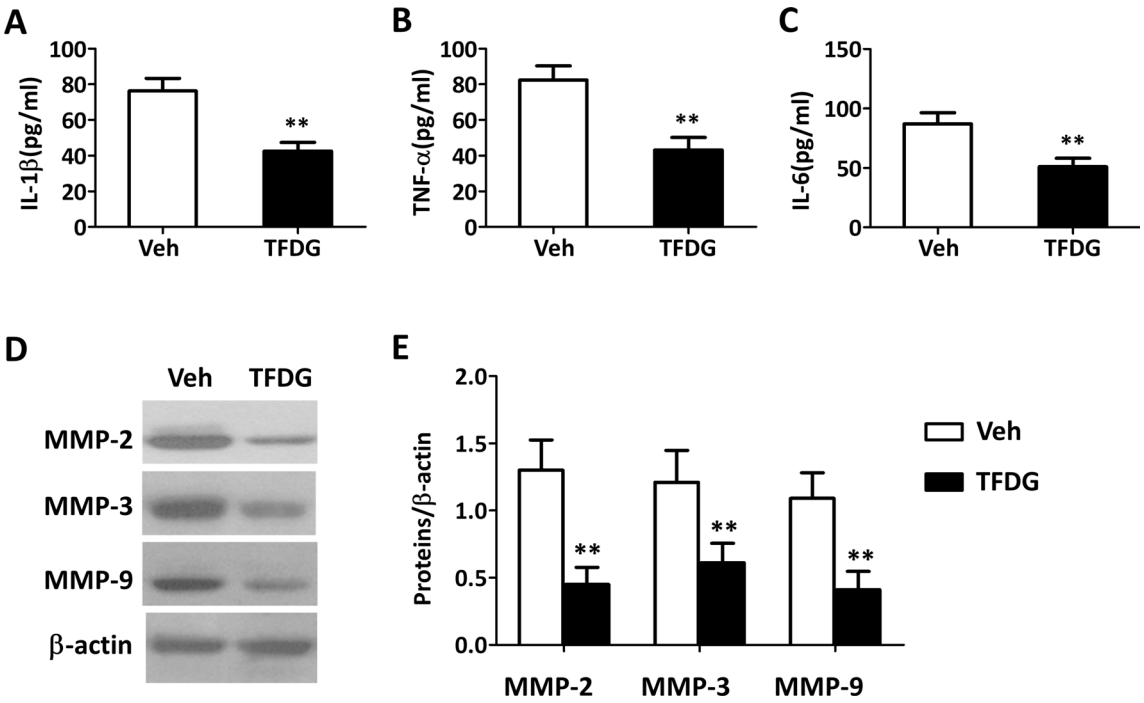

Fig. 3 TFDG downregulated the levels of inflammatory cytokines and MMPs in synovium. The mice were immunized with CII. 7 days after immunization, the mice were treated with intraperitoneal injections of TFDG (10 mg/kg) or vehicle three times per week for 9 week. On day 49 after primary immunization, the synovium was collected. **a–c** Levels of cytokines were tested by ELISA. **d, e** Levels of MMPs were tested by Western blots and quantitation of Western blots with β-actin. Data are expressed as mean ± SD, $n = 5$, $^{**}p < 0.01$ vs vehicle (Veh) group

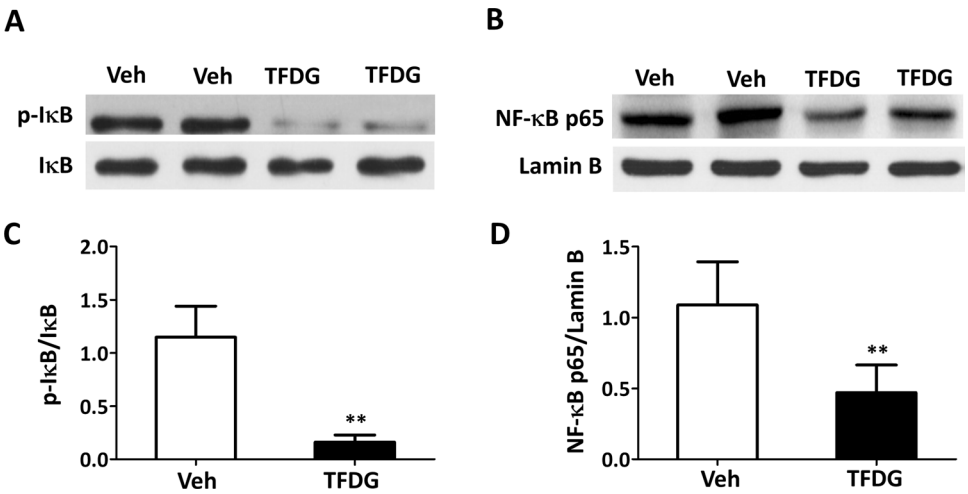

Fig. 4 Effects of TFDG on the activation of NF-κB-signaling pathways in synovium. The mice were immunized with CII. 7 days after immunization, the mice were treated with intraperitoneal injections of TFDG (10 mg/kg) or vehicle three times per week for 9 week. On day 49 after primary immunization, the synovium was collected. **a, b** Levels of p-IκB and p65 were tested by Western blots. **c, d** Quantitation of Western blots of p-IκB and p65. Data are expressed as mean ± SD, $n = 5$, $^{**}p < 0.01$ vs vehicle (Veh) group

an active component in black tea, on the modulation in the CIA mouse model. To our knowledge, there has been no previous report on the treatment of TFDG in the case of RA. To better understand the mechanisms of the alleviation of arthritis by TFDG, we evaluated the expression of the relevant inflammatory factors and the activation of the NF-κB and MAPK-signaling pathways in the synovium of TFDG-treated mice. Our results showed that TFDG significantly alleviated arthritis score and incidence in CIA mice, and specifically delayed the initiation of the arthritis 1 week when compared with vehicle control. Histological analysis showed that TFDG-attenuated synovial inflammation and cartilage-bone destruction in CIA mice, decreasing the extents of synovial hyperplasia, cartilage destruction, and bone erosion from severe in vehicle group to mild.

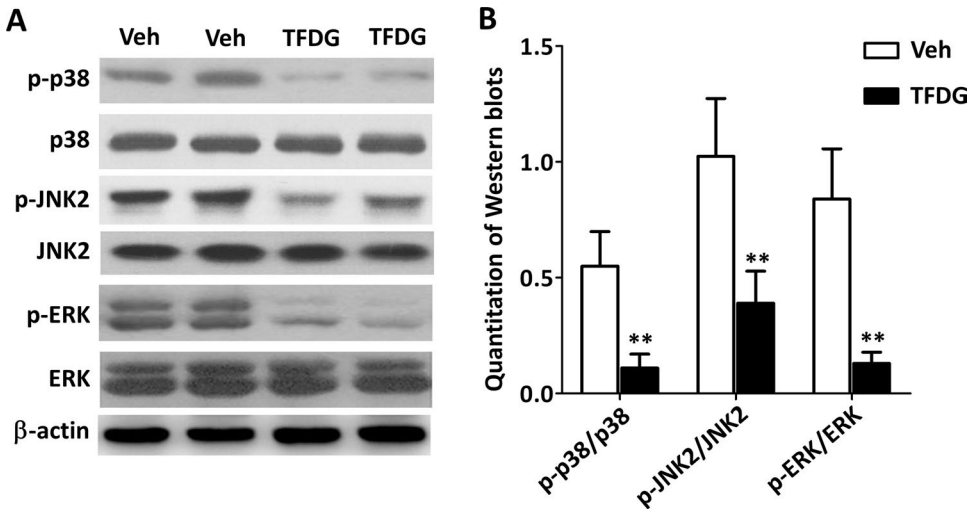

Fig. 5 Effects of TFDG on the activation of MAPK-signaling pathways in synovium. The mice were immunized with CII. 7 days after immunization, and the mice were treated with intraperitoneal injections of TFDG (10 mg/kg) or vehicle three times per week for 9 week. On day 49 after primary immunization, the synovium was collected. **a** Levels of p-p38, p-JNK, and p-ERK were tested by Western blots. **b** Quantitation of Western blots of p-p38, p-JNK, and p-ERK. Data are expressed as mean ± SD, $n = 5$, $^{**}p < 0.01$ vs vehicle (Veh) group

Furthermore, we continued to investigate the effects of TFDG on the regulation of pro-inflammatory cytokines. Cytokines such as IL-1β, TNF-α, and IL-6 have been shown potent pro-inflammatory actions in the pathogenesis of RA (Choy and Panayi 2001). The role of IL-1β on inflammation stimulation and joint erosion has been successfully established in in vitro and animal models (Dayer 2003). Due to similar roles in inflammation regulation, TNF inhibitors in RA have been shown to be effective by sustainably blocking a single cytokine in a human disease (Campbell et al. 2003). Previous report has demonstrated the progressive increase of these three cytokines in the joint of mice with CIA (Marinova-Mutafchieva et al. 1997). Our result showed that the treatment with TFDG significantly decreased the levels of IL-1β, TNF-α, and IL-6 of the CIA mice, indicating the anti-inflammatory effects of TFDG. Given the proven clinical efficacy of cytokines-based therapies in RA (Joosten et al. 2008), the attenuation of TFDG on the RA contains the value to be closer studied for the mechanism of action.

NF-κB is a transcription factor involved in numerous pathologies in many cell types (Viatour et al. 2005). Activated NF-κB is able to combine with specific gene promoter regions of inflammatory factors and regulated genetic transcription of TNF-α, IL-1 and IL-6 (Xu et al. 2018). Phosphorylated IκB is rapidly ubiquitinated, subsequently leads to the expression of NF-κB. To explore the function of TFDG on the NF-κB-signaling pathway in RA, we first examined the synovium of the CIA mice for the correlation between TFDG and P65 dimer, which is the most common form of NF-κB, and often used as a marker of NF-κB activation. We demonstrated that TFDG reduced the phosphorylation of IκB, as well as the production of NF-κB p65 subunit. This result suggested that TFDG could directly augment the transcriptional activity of the NF-κB complex, indicating that TFDG had a significant impact on the RA synovial inflammation progression via the NF-κB pathway.

It has been known that autocrine and paracrine secretion of NF-κB promotes the aggregation of extracellular matrix and induces the synthesis of related MMPs (Lepenies et al. 2011). We also checked the regulation of TFDG on the expression of MMPs. MMPs belong to endo-proteinase that is responsible for the cartilage degradation and destruction of extracellular matrix components in RA, causing ligament or cartilage damage (Almodovar et al. 2014; Yamanishi and Firestein 2001). In RA, MMP-1 contributes to erode joint tissue which evolves the transformation of normal synovial into a hyperplastic, invasive tissue pannus (Iwamoto et al. 2008). Previous study showed that chemokine RANTES/CCL5 induced MMP-1 expression in cultured human RA synovial fibroblasts (Agere et al. 2017). Other group demonstrated that the overexpression of MMP2 is important in promoting the migration and invasion of fibroblast-like synoviocytes (FLSs) in RA in the presence of both IL-17A and hypoxia (Li et al. 2013a). Moreover, high baseline levels of MMP-3 could be an important risk factor for structural damage including bone erosions in early RA (Prodanovic et al. 2018). Our result clearly showed that TFDG administration significantly lower the expression of all three MMPs including MMP-1, MMP-2, and MMP-3, indicating the protection of TFDG on the cartilage degradation and joint tissue erosion.

It has been known that IL-1 induces rapid phosphorylation of ERK and p38 in RA FLS (Yamanishi and Firestein 2001), and JNK activation was unique for RA FLS (Okamoto et al. 1997), we also evaluated the effects of TFDG on the MAPK-signaling pathway, which is involved in diverse cellular processes including inflammation, angiogenesis, and proliferation. TNF-α treatment stimulated the Ras/p38 MAPK-signaling pathway in cultured human RA synovial cell line (Yang et al. 2018). In addition, p38 MAPK signaling has been reported as new mechanisms mediating serum amyloid A-induced angiogenesis event in RA (Hong et al.

2015). The regulation of chemokine CXCl16 on the expression of NF-κB ligand in RA FLS was also related to the p38/MAPK-signaling pathway (Li et al. 2016). Sudachitin, which is a type of polymethoxy flavonoid, has been shown to block LPS-induced bone destruction and osteoclast differentiation via inhibiting the activation of ERK and JNK (Ohyama et al. 2018). Our result showed that TFDG treatment significantly inhibited the phosphorylation of P38, JNK2, and ERK, whereas the expression levels of these three proteins showed no significant change. These findings supported that TFDG had a significant impact on the RA synovial inflammation processes via MAPK-signaling pathway.

Nevertheless, there were some limitations of our study. First, in the present study, solely CIA mice model was used, which represents active immunization strategy. In the future, other well-established arthritis animal models such as zymosan-induced arthritis, collagen-antibody-induced arthritis could be utilized to verify the efficiency and safety of TFDG.

In conclusion, the present study showed that TFDG administration inhibited arthritis score and incidence in CIA mice model suppressed the expression of inflammatory cytokines and MMPs. The modulation may be mediated by the NF-κB- and MAPK-signaling pathways, supported by the reduction of the downregulated expression and phosphorylation of the key components in these two pathways.

Funding Not applicable.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed Consent Not applicable.

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