Madecassoside impedes invasion of rheumatoid fibroblast-like synoviocyte from adjuvant arthritis rats via inhibition of NF-κB-mediated matrix metalloproteinase-13 expression

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[ABSTRACT] Fibroblast-like synoviocytes (FLS) play a pivotal role in Rheumatoid arthritis (RA) pathogenesis through aggressive migration and invasion. Madecassoside (Madec), a triterpenoid saponin present in Centella asiatica herbs, has a potent anti-inflammatory effect. In the present study, Madec exerted an obvious therapeutic effect in reversing the histological lesions in adjuvant-induced arthritis (AIA) rats. To recognize the anti-rheumatoid potentials of Madec, we further investigated whether Madec interfered with FLS invasion and metalloproteinase (MMP) expression. In cultures of primary FLS isolated from the AIA rats, Madec (10 and 30 μmol·L⁻¹) was proven to considerably inhibit migration and invasion of FLS induced by interleukin 1β (IL-1β), but exhibiting no obvious effect on cell proliferation. Madec repressed IL-1β-triggered FLS invasion by prohibiting the expression of MMP-13. Additionally, Madec suppressed MMP-13 transcription via inhibiting the MMP-13 promoter-binding activity of NF-κB. Our results further showed that Madec down-regulated the translocation and phosphorylation of NF-κB as demonstrated by Western blotting and immunofluorescence assays. In conclusion, our results suggest that Madec exerts anti-RA activity via inhibiting the NF-κB/MMP-13 pathway.

[KEY WORDS] Rheumatoid arthritis; Fibroblast-like synoviocytes; Madecassoside; Adjuvant-induced arthritis; Matrix metalloproteinase-13; Invasion


Introduction
Rheumatoid arthritis (RA) is a systemic, progressive, and inflammatory autoimmune disease characterized by synovial fibroblast hyperplasia and bone erosion [1-2]. Environmental and cellular stresses can influence the articular cartilage, such as mechanical loading, endoplasmic reticulum stress, and inflammatory cytokines [3]. Extreme loading is harmful to joint tissues. It has been well known that the abundant generation of the inflammatory cytokines plays essential roles in RA formation; especially, IL-1β and TNF-α exhibit a fundamental role in RA development [4-5]. Moreover, IL-1β-driven elevation of matrix metalloproteinases (MMPs) is important in the irreversible collapse of cartilage matrix by degrading collagens [6].

The MMP family includes over 20 members that differentially mediate the digestion of each component of extracellular matrix. Cytokine-triggered fibroblast-like synoviocytes (FLS) generate MMPs, which proficiently degrade the collagenous components of cartilage and bone, finally contributing to joint deformity and causing much pain in RA patients [7]. One of the most principal collagenases in damaging the articular cartilage is matrix metalloproteinase 13 (MMP-13, collagenase 3). MMP-13 is a unique mammalian enzyme acknowledged for its capability to cleave the triple helical domain of fibrillar collagen types I, II, and III. As an abnormal collagenase subtype, MMP-13 has also been found in higher levels in the synovial fluids of RA patients [8]. IL-1β is known to be a prime inducer of MMP-13 through pathways mainly involving NF-κB [9]. To maintain arthritic joint tissues, abolishing degenerative functions of MMP-13 is imperative.

Current RA therapies are limited, since potential side effects remain one of the major problems for long-term drug use for treating RA. There is a strong need to develop safe and effective drugs for long-term use. Phytochemicals serve as suitable candidates for RA therapy [10]. Herbal medicines have drawn the attention of the whole world for its well-toler-
rurance, safety, and effectiveness [11-12]. Not only widely used in Asian countries, herbal medicines are also suggested as dietary supplements or alternative therapies in USA and European countries [13]. Some effective compounds extracted from traditional Chinese medicine, such as norisoboldine, celastrol, and madecassoside (Madec), have shown potent anti-arthritic effects. As the highest amount of triterpene ingredient in C. asiatica, Madec possesses a wide range of pharmacological effects. Madec has been proven to prompt apoptosis of keloid fibroblast and keratinocyte in a mitochondrial-dependent pathway [14], and repress LPS-induced TNF-α production in cardio-myocytes via blocking ERK, p38, and NF-κB activities [15]. Moreover, it alleviates infiltration of inflammatory cells and synovial hyperplasia in type II collagen-induced arthritis in mice [16-17]. But the molecular mechanism of Madec remains unknown. Madec has also been reported to inhibit the migration of keloid fibroblast and suppress MMP-13 protein levels.

The present study was carried out to verify the anti-arthritis effect of Madec in adjuvant-induced arthritis rats and investigate whether Madec could inhibit the invasion of FLS by scratch−wound-closure and transwell membrane assays. Since NF-κB signaling is greatly involved in the activation of MMP-13, we further explored the function of MMP-13 and NF-κB signaling in FLS invasion.

Materials and Methods

Induction, assessment and treatment of AIA rats

Adjuvant-induced arthritis model was established by an intradermal injection at the hind paw and the tail of the rats with 0.1 mL mycobacterium butyricum in Freund’s complete adjuvant (10 mg·mL−1) (Becton Dickinson, Franklin Lakes, NJ, USA) [18]. Fourteen days after first injection, the rats were randomly divided into four groups: normal control group (0.9% saline), model group (0.9% saline), Madec (Baoji Herbest Bio-Tech Co., Ltd., Baoji, Shaanxi, China) group (25 mg·kg−1), and dexamethasone (DEX) (Sigma–Aldrich, Saint Louis, MS, USA) group (0.5 mg·kg−1). The chemical structure of Madec is shown in Fig 1. The rats were administered by oral gavage with Madec and DEX respectively for 13 days, from Day 14 to Day 26. Body weights were recorded. The volume of hind paws, as an indicator of paw swelling, was monitored every three days by blinded examiners with the plethysmometer, A previously reported scoring system was used to assess the polyarthritis index [19]. Ankles were decalcified, sectioned, embedded, and subjected to H&E staining.

Primary culture of FLS

The FLS were isolated from synovial tissues of the AIA rats. Briefly, synovium was washed twice with PBS containing antibiotics, cut into pieces, and then attached to the flask with drops of DMEM on the sample pieces at 37 °C, 5% CO2. FLS would grow around the small pieces. After FLS in flasks reach 70% confluence, the cells were split in 0.25% trypsin and collected. The cells were cultured with 10% FBS-DMEM (Keygen Biotech. Co., LTD., Nanjing, China) supplemented with 100 U·mL−1 penicillin and 100 μg·mL−1 streptomycin at 37 °C, 5% CO2. The FLS between passages 3 and 8 were used in the subsequent experiments.

**Fig. 1  The Chemical structure of madecassoside**

MTT assay

The cell viability was determined using the MTT method [20]. The FLS (4 × 10^4 cells/well) were plated in 96-well plates. After a 24-h incubation with Madec (10, 30, and 50 μmol·L−1) and IL-1β (Abcam, Cambridge, UK) (10 nmol·mL−1), MTT (Sigma–Aldrich, Saint Louis, MS, USA) (5 mg·mL−1) was added and incubated for another 4 h. The absorbance at 570 nm represents the number of viable cells with a Multiskan Spectrum Microplate Reader (Thermo Fisher Scientific, Waltham, USA).

In vitro scratch-wound-closure assay

In vitro wound-healing assay was carried out as previously described [21]. The FLS were seeded into 24-well plates at a density of 10^5 cells/mL. When the cells reached 90% confluence, they were incubated with 10 μg·mL−1 of mitomycin C (Keygen Biotech. Co., Ltd., Nanjing, China) for 2 h before scratching with a 200-μL pipette tip. The wounded culture was rinsed twice with PBS to get rid of the floating cells, and treated with Madec (10 and 30 μmol·L−1) and IL-1β (10 ng·mL−1) for 24 h and observed and photographed under a microscope.

Transmembrane cell invasion assay

Transmembrane invasion analysis was performed in 24-well inserts pre-coated with Matrigel (Keygen Biotech. Co., Ltd., Nanjing, China) [22]. After incubation with Madec (10 and 30 μmol·L−1) and IL-1β (10 ng·mL−1) for 24 h in 75-cm² flasks, the FLS were split by 0.25% trypsin and collected. The treated FLS were seeded at the upper chamber in serum-free DMEM. 5% FCS-DMEM in the bottom chamber were regarded as chemo-attractant. After a 24-h culture, a cotton swab was used to remove the matrigel and left cells from polycarbonate filters. The cells that had migrated to the lower surface of the inserts were fixed with methanol, sub-
MMP-13 were established as previously reported [23]. The membranes were blocked in non-fat milk in Tris-buffered saline-Tween 20 (TBST) for 1 h at 37 °C. The membranes were developed by ECL (Boster, Wuhan, China). The dual luciferase activity was assayed with a Luminometer (Bender MedSystems, Vienna, Austria) according to the manufacturer’s protocol (Keygen Biotech. Co., Ltd., Nanjing, China). All the results are expressed as percentage of normalized firefly luciferase activity against Renilla luciferase activity.

Real time PCR

Total RNA was extracted from FLS with a RNA isolation kit (Keygen Biotech. Co., Ltd., Nanjing, China). RNA was reverse transcribed into cDNA using a Reverse Transcription kit (R&D Systems, Minneapolis, MN, USA) [24]. qPCR was performed on a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). All the reactions were performed using Lightcycler®FastStart DNA Master SYBR Green I. The primers are listed in Table 1. All the reactions were performed on a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The results were quantified using the 2−ΔΔCt method. GAPDH mRNA was used as an internal control.

Table 1 | Sequences of primers used in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>MMP-2</td>
<td>ACCTACACCAAGAACCCTCCG</td>
<td>TTGGTTCTCCAGCTTCCG</td>
</tr>
<tr>
<td>MMP-3</td>
<td>ATCCCATGGAACGCGCTTTTC</td>
<td>CATTGGGTCAAACTCTCATGTG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>TCCCTGGAGACCTGAGAAC</td>
<td>CGGCAAGTCTTCCGAGATTT</td>
</tr>
<tr>
<td>MMP-13</td>
<td>CGCCAGAAGAATCTGCTTAAAA</td>
<td>CCAAATTTAGGGAGAGATGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCTCTCTGAATTCAACAGCGAC</td>
<td>CCTGTGTTGCTGTAAGCAATTC</td>
</tr>
</tbody>
</table>

Western blotting assays

Whole lysate samples were loaded and separated by SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes [25]. The membranes were blocked in non-fat milk in Tris-buffered saline-Tween 20 (TBST) for 1 h at 37 °C and rinsed with TBST. The membranes were probed with primary antibodies (GAPDH, MMP-13, and Lamin A antibodies obtained from Cell Signaling Technology (Danvers, MA, USA); p-p65, IκBα, p-IκBα, and Iκk kinase antibodies from Abcam (Cambridge, UK) at 4 °C overnight, and then with horseradish peroxidase secondary antibodies (Sigma–Aldrich, Saint Louis, MS, USA) for 2 h at 37 °C. The membranes were developed by ECL (Boster, Wuhan, China). Membranes were visualized with chemo luminescence reagents. Image pro plus (IPP) software for densitometry analysis is applied for the quantification of protein expressions.

MMP-13 activity

The MMP-13 enzymatic activity was quantified by a fluorescent intensity assay as previously reported [20]. Briefly, the isolated proteins were incubated with the fluorescent MMP-13 peptide probe (PerkinElmer, Groningen, Netherlands) in the reaction buffer at 37 °C for 120 min. Fluorescent intensity, an indicator of enzymatic activity, was determined at excitation 560 nm and emission 590 nm, respectively. The activity levels were calculated using a control solution containing no protein, and normalized by the content of total proteins in the samples.

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out with a Gel Shift Assay System kit according to the manufacturer’s instructions (ThermoFisher Scientific, Waltham, MA, USA) [27]. Nuclear protein was extracted. A “cold probe” was used, which lacked 5′-biotin labeling. Nuclear protein (10 μg) was incubated with biotin-labeled probes at 4 °C in binding buffer containing poly (dl : dc), 50 mmol·L−1 NaCl, 0.5 mmol·L−1 EDTA, 10 mmol·L−1 Tris-HCl, 1 mmol·L−1 MgCl2, 4% (V/V) glycerol, and 0.5 mmol·L−1 DTT. The Protein-DNA binding reaction was run on a 7% acrylamide gel and analyzed.

Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde at room temperature for 10 min [29]. Then the cells were permeabilized in 0.25% Triton X-100 in PBST, followed by blocking with 1% BSA and 5% goat serum in PBST for 2 h, and then incubated with the rabbit polyclonal p65 antibody (Abcam, Cambridge, UK) (diluted 1 : 200) at 4 °C overnight. Afterwards, the sample was washed with PBST thrice and incubated with secondary donkey anti-rabbit antibody conjugated to FITC. Finally, the chamber slides were mounted with Vectashield® anti-fade solution containing DAPI (Sigma–Aldrich, Saint Louis, MS, USA) (Vector Laboratories Inc.). Fluorescence images were captured under a fluorescence microscope (Zeiss Axio Observer A1, Germany).

Statistics analysis

Statistical analysis was carried out with GraphPad Prism software 5.01 (GraphPad Software Inc., San Diego, CA, USA). All the results are expressed as means ± SD. One-way ANOVA was used to compare the differences among multiple groups. P < 0.05 was regarded as statistically significant.

Results

Effects of Madec on AIA severity in rats

Change in body weight in the AIA rats is regarded as a good marker of the anti-inflammatory and immuno-suppressive effects of the test drugs. During the initial 7 days after adjuvant injection, the AIA rats’ body weights increased initially but decreased gradually afterwards. Body weight loss in the Madec-treated group was greatly alleviated. However, DEX failed to reverse body weight loss. Polyarthritis index


– 332 –
(PI) scores were assessed according to a scale of 0–4. Madec (25 mg·kg\(^{-1}\)) and DEX (0.5 mg·kg\(^{-1}\)) treatments noticeably reduced PI scores. Swelling of paws was also examined to compare the inflammation status among different groups. From the variation tendency of swelling in the model group, we proposed that secondary arthritis onset began on Day 10. Both Madec and DEX decreased the paw volumes evidently. Histopathology results displayed that the AIA rat joints showed aggressive inflammatory cell infiltration, synovial hyperplasia, pannus, and cartilage destruction. Madec displayed an obvious therapeutic effect in reversing these histological lesions. The above results are shown in Fig. 2.

**Effects of Madec on the viability, migration, and invasion of FLS**

The cytotoxic effect of Madec was examined by the MTT assay. The FLS were cultured in DMEM with IL-1\(\beta\) (10 ng·mL\(^{-1}\)) with or without Madec for 24 h. The results showed that Madec (10 and 30 \(\mu\)mol·L\(^{-1}\)) exerted no obvious cytotoxicity on FLS, as shown in MTT assay and apoptosis analysis (Figs. 3A and 3B). The two concentrations were applied in the following experiments. Whether Madec could inhibit FLS invasion was analyzed by using a Transwell chamber. Madec (10 and 30 \(\mu\)mol·L\(^{-1}\)) showed a dose-dependent inhibitory effect on FLS invasion mediated by IL-1\(\beta\) in vitro. Similar inhibitory effect of Madec on FLS migration was observed in the scratch-wound healing assay.

**Effects of Madec on IL-1\(\beta\)-induced MMP-2, MMP-3, MMP-9 and MMP-13 mRNA**

MMP family plays a critical role in degrading collagen present in the extracellular matrix. The FLS were treated with Madec (10 and 30 \(\mu\)mol·L\(^{-1}\)) and IL-1\(\beta\) (10 ng·mL\(^{-1}\)) for 24 h. The effects of Madec on IL-1\(\beta\)-induced MMP-2, MMP-3, MMP-9, and MMP-13 mRNA levels were measured by real-time PCR. Madec selectively repressed both MMP-13 mRNA level and activity (Fig. 4). Additionally, to confirm the essential role of MMP-13 in the invasive property of FLS, siRNA MMP-13 was used. After siRNA transfection and IL-1\(\beta\) incubation, an obvious decrease in the number of cells passing through the basal membrane was observed in the MMP-13 siRNA-treated group. These data suggest that MMP-13 is greatly involved in the invasion induced by IL-1\(\beta\).

**Effects of Madec on the binding activity of NF-\(\kappa\)B on the MMP-13 promoter**

Both mRNA and activity of MMP-13 were inhibited by Madec. Involvement of NF-\(\kappa\)B in regulation of MMP13 mRNA in chondrocytes in response to surviving stress has been reported [29]. To examine whether the transcription factor NF-\(\kappa\)B modulates MMP-13 gene expression in FLS, the wild-type MMP-13 promoter or a promoter with mutations in the NF-\(\kappa\)B responsive element site were transfected into the FLS. Madec reduced IL-1\(\beta\)-driven elevation to the basal level in the transcription activity of the reporter with the wild-type promoter, but didn’t alter transcription activity with NF-\(\kappa\)B mutation, signifying that NF-\(\kappa\)B was probably the target of Madec. EMSA method was used to evaluate the manipulating effect of Madec on the binding activity of NF-\(\kappa\)B to MMP-13 promoter. As expected, IL-1\(\beta\) stimulated NF-\(\kappa\)B binding to MMP-13 promoter, which was greatly abrogated after exposure to Madec (Fig. 5B).
Fig. 3  Madec suppressed the invasion and migration of FLS. Cells were stimulated with IL-1β (10 ng·mL⁻¹) for 24 h with or without Madec (10 and 30 μmol·L⁻¹). (A) Cell viability by MTT assay. (B) Cell apoptosis. (C) Invasion was analyzed by using a Transwell chamber (magnification, 100 ×). (D) Scratch-wound healing assays (magnification, 100 ×). The data are expressed as mean ± SD.

Fig. 4  Madec suppressed mRNA and protein expressions of MMP-13. The cells were stimulated with IL-1β (10 ng·mL⁻¹) for 24 h with or without Madec (10 and 30 μmol·L⁻¹). (A) mRNA expressions of MMP-2, MMP-3, MMP-9 and MMP-13 by real-time PCR. Relative gene expression was normalized to GAPDH and compared with the control. (B) MMP-13 enzymatic activity was quantified by fluorescent intensity after incubation with a purified peptide probe. (C) Protein expression of MMP-13 was analyzed by Western blotting. (D) siRNA was applied to explore of the essential effect of MMP-13 in FLS invasion induced by IL-1β. After MMP-13 siRNA transfection, and followed with IL-1β stimulation, FLS invasion was evaluated 24 h later (magnification, 100 ×). The data are expressed as mean ± SD, n = 6. *P < 0.05 vs untreated cells. **P < 0.05 vs IL-1β-treated group.
Fig. 5  Madec suppressed the transcriptional activity of MMP-13 via repression of IL-1β induced-NF-κB activity. (A) The FLS were transfected with pGL2-MMP-13WT or pGL2-MMP-13 mutant-NF-κB. Transfected cells were treated with Madec and/or IL-1β (10 ng/mL) for 24 h. The relative luciferase activity (RLA) was normalized to Renilla luciferase activity and is expressed as fold of the control. (B) Madec inhibits the binding activity of NF-κB in the MMP-13 promoter. The DNA binding activity was evaluated by EMSA using a probe containing NF-κB motif in the MMP-13 promoter. The data are expressed as means ± SD of three independent experiments. ##P < 0.01 vs normal, *P < 0.05 vs IL-1β-treated group

Effects of Madec on NF-κB activation and translocation in FLS induced by IL-1β

The FLS were pre-incubated with Madec (10 and 30 μmol·L⁻¹) for 24 h and then exposed to IL-1β for 15 min. NF-κB p65 translocation was determined by Western blotting in cytosolic and nuclear fractions, respectively. IL-1β promoted the increment of NF-κB p65 translocation to the nucleus, which was greatly reversed by Madec treatment. Visualization of cells in immunofluorescence staining showed reduced expression of NF-κB in the nucleus after Madec treatment. IL-1β stimulation resulted in an evident phosphorylation of both Iκκα and IκκBα in the RA FLS. However, Madec (10 and 30 μmol·L⁻¹) treatment for 24 h only faintly decreased Iκκα phosphorylation stimulated by IL-1β for 15 min and exhibited little or negligible effect on IκκBα phosphorylation and degradation (Fig. 6).

Discussion

RA is an autoimmune disease characterized by chronic synovium inflammation. The orchestrated interactions between inflammatory cytokines (e.g., IL-17, IL-1β, and TNF-α) and regulatory cytokines (e.g., IL-10, IL-4, and TGF-β) play vital roles in the initiation, development, and progression of RA [30]. Hence, to mimic the in vivo pathophysiology environment, IL-1β stimulation was used in the present study to induce FLS invasion and to detect the anti-arthritis mechanism of Madec. First, we established the rat arthritis model by injecting mycobacterium butyricum in complete adjuvant intrademally. Histological analysis showed that the synovial cells in ankle joints of AIA rats were eroded, accompanied with the inflammatory cells infiltration (mainly mononuclear macrophages and neutrophils), chondrocyte degeneration, joint cavity stenosis, as well as vascular and fibroblastic hyperplasia. Oral administration of Madec and DEX for 13 consecutive days reduced the damage in the articular surface of the cartilage and bone tissue and attenuated hyperemia in the synovial tissue. Although previous reports also demonstrated that Madec was effective in alleviating arthritis symptoms in collagen II-induced arthritis mice, the molecule mechanisms by which Madec repressed FLS invasion in response to IL-1β needs further investigation.

The primary application for Centella asiatica herbs are promoting wound healing in Asian countries. In recent study, the anti-arthritis effect of Centella asiatica extract and Madec has drawn wide attention. Thus far, no clinical trials of Madec have been reported for anti-arthritis therapy. Most of the researches are pre-clinical and carried out in rodent animal arthritis model. As one of the most abundant triterpenoid constituents in Centella asiatica and regarded as the major bioactive compound for arthritis treatment, the concentration of Madec
was 3.10 ± 4.58 mg·mL⁻¹ in Centella extract [31]. Madec exhibited remarkable anti-arthritis activities in rodent animals with oral effective dose ranging from 20 to 30 mg·kg⁻¹ [32]. However, Cmax only reached 0.31 μmol·L⁻¹ after a single oral administration (100 mg·kg⁻¹) in normal rats [33-34]. In vitro studies showed that Madec at 50 μmol·L⁻¹ could inhibit lipopolysaccharide-stimulated activation of macrophages. In our study, Madec at 30 μmol·L⁻¹ could attenuate the migration of fibroblast-like synoviocytes. We could see that the effective concentration in vitro was far higher than Cmax. The discrepancy between poor pharmacokinetic property and remarkable bioactivity can also be observed from effective ingredients. We suppose that these ingredients work not only by absorption into blood, but also in an intestine-dependent manner.

Moreover, when comparing the main pharmacokinetic parameters of Madec on the 7th and 14th day after oral administration in normal and collagen-induced arthritic rats, the values of Cmax, AUC₀₋₃ and AUC₀₋∞ were markedly lowered. A possible explanation for this phenomenon is that Madec is more widely distributed in vivo under arthritis situation [35].

IL-1β is a major catabolic factor responsible for cartilage degradation in RA progression. Levels of IL-1β in RA patients’ synovial fluid were higher than that in osteoarthritis synovial fluid [36]. In addition, beneficial anti-IL-1 therapies in vivo prove the important role in arthritis [37]. IL-1β belongs to IL-1 family that consists of three members, namely, IL-1α, IL-1β and IL-1Ra. As an IL-1 receptor antagonist, IL-1Ra is an endogenous inhibitor that blocks IL-1α and IL-1β activity. Unsurprisingly, FLS invasion, MMP-13 mRNA and activity were strengthened by IL-1β induction. Our results from the present study demonstrated that Madec suppressed IL-1β-induced invasion and migration of FLS, decreased the level of MMP-13 mRNA and reduced its activity as well.

MMPs are regarded as the main enzymes responsible for collagens and aggrecan degradation in cartilage. The elevation of MMPs has been found in cartilage and synovial tissues of RA patients [38]. MMPs intervene tissue remodeling including destruction, catabolism and the turnover of ECM [39]. Accordingly, screening drugs with anti-MMP capacity is of great value for arthritis therapy. We found that IL-1β exposure resulted in the increment of MMP-2, MMP-3, MMP-9, and MMP-13 mRNAs. Madec prohibited IL-1β-promoted MMP-13 expressions at the mRNA and protein levels in FLS, with little effect on other MMPs mRNA expression. Similar results were also shown by Song et al. that Madec inhibited keloid fibroblasts migration at least partially via blocking MMP-13 expression [40]. Interestingly, MMP-13 siRNA reduced IL-1β-triggered elevation of FLS invasion. IL-1β can cause the recruitment and oligomerization of TNF-associated factor 6, activate IkkB, and subsequently drive NF-κB pathways, thus intermediating the expressions of genes encoding other cytokines [41]. We showed that IL-1β-driven elevation of
the transcription activity of the reporter with the wild-type promoter was brought down to the basal level by Madec treatment, but didn’t alter the transcription activity of the promoter with NF-xB mutations, indicating that Madec functions probably via targeting NF-xB. And EMSA assay further demonstrated that IL-1β notably stimulated the binding of NF-xB to MMP-13 promoter, which was greatly abrogated after exposure to Madec.

NF-xB activation embraces a sequential cascade, containing Ikk-dependent IκBα phosphorylation, ubiquitination, proteolytic degradation, as well as translocation from cytosol to the nucleus. We paid an attention to the regulatory effects of Madec on NF-xB owing to its importance in inflammatory process. NF-xB is constrained with the IκBα in the cytosol inactively. IL-1 stimulation activates Ikk and phosphorylates IκBα. Then, proteasomes degrade polyubquitinated IκBα. Consistently, noticeable phosphorylation of Iκkα and IκBα was observed in FLS after IL-1β stimulation. Once activated, p65 disconnects from its inhibitory protein IκBα and is translocated into the nucleus, and then binds with the particular position of DNA. Although the role of NF-xB in the regulation of MMP-13 has been well documented [29], Madec’s mechanism of action on these pathways has not been fully understood. In our study, IL-1β elevated p65 phosphorylation and p65 translocation, which was blunted by Madec treatment, although Madec showed little effect on Ikkα phosphorylation. Taken together, these data proved that Madec inhibited MMP-13 by preventing NF-xB translocation and phosphorylation.

Conclusions

Madec significantly inhibited the invasion, migration, and MMP-13 expression of FLS induced by IL-1β, and NF-xB pathway was at least partially involved in this process. These findings provided a reasonable explanation for Madec’s effects on the histological lesions in adjuvant-induced arthritis rats, further suggesting its potential value as a candidate drug for RA therapy.

References


