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Betulinic acid inhibits the migration and invasion of fibroblast-like synoviocytes from patients with rheumatoid arthritis

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ABSTRACT

The aggressive phenotype displayed by fibroblast-like synoviocytes (FLSs) contributes to cartilage and bone destruction in rheumatoid arthritis (RA). Betulinic acid has been demonstrated to have a positive therapeutic effect on tumor, inflammation and immune disorder, however, the effects of betulinic acid on RA FLSs have not been verified. Therefore, in the present study, we observed the effect of betulinic acid on the migration and invasion of RA FLSs and explored its underlying signal mechanisms. Our results showed that betulinic acid treatment suppressed the migration, invasion and reorganization of the actin cytoskeleton of RA FLSs. In addition, we found that the mRNA expression of IL-1β, IL-6, IL-8 and IL-17A were markedly down-regulated by treatment with betulinic acid in TNF-α-induced RA FLSs. To gain insight into the molecular mechanisms, we evaluated the effect of betulinic acid on NF-κB activation in RA FLSs. The results indicated that betulinic acid treatment reduced the TNF-α-induced activation of NF-κB signal pathway and the NF-κB nuclear accumulation. We also observed that treatment with betulinic acid attenuated synovial inflammation and joint destruction in mice with CIA. Taken together, these results suggest that betulinic acid inhibits the migration and invasion of RA FLSs by blocking NF-κB signal pathway activation.

1. Introduction

RA is an immune-mediated joint disease characterized by chronic inflammation, synovial hyperplasia and progressive destruction of bone and cartilage [1]. Increasing evidence indicates that fibroblast-like synoviocytes (FLSs) in the synovial intimal lining plays an essential role in the development of synovial inflammation and joint erosion [2]. Stable activated RA FLSs exhibits tumor-like properties and abnormal biological behaviors, such as anchorage-independent growth, aggressive migration and invasion and overproduction of inflammatory cytokines [3,4]. Therefore, modulating the migration and invasion of RA FLSs may be a novel therapeutic strategy to prevent the destructive progress of RA.

Betulinic acid, a pentacyclic triterpene, is commonly separated from many plants such as *Ziziphus mauritiana*, *Tryphyllum peltaum*, *Tetracera boliviana* and *Ancistrocladus heyneaus* [5,6]. Previous studies have suggested that betulinic acid could inhibit the migration of various cells, such as human medulloblastoma cells, lung carcinoma cells and stellate cells [7,8]. Betulinic acid also inhibited lipopolysaccharide (LPS)-stimulated pro-inflammatory mediator and cytokine production via inhibition of nuclear factor kappa B (NF-κB) binding activity in RAW 264.7 macrophages [9,10]. Interestingly, it had been reported to show anti-inflammatory activity in the carrageenan and serotonin paw edema tests [11] and exhibited synergistic effect on toll-like receptor-4 mediated anti-atherogenic mechanism in type II collagen induced arthritis (CIA) [12], which provided the evidence supporting the potential role

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of betulinic acid in RA. However, to date, the effects of betulinic acid on the migration and invasion in RA FLSs are still unknown. Therefore, in the present study, we evaluated the effect of betulinic acid on regulating the migration and invasion of RA FLSs and its underlying mechanisms. Our findings indicated that betulinic acid may possess therapeutic potential for RA.

2. Materials and methods

2.1. Antibodies and reagents

Betulinic acid, anti-β-actin antibody and type I collagenase were purchased from Sigma Chemicals (St. Louis, MO, USA). Foetal bovine serum (FBS), antibiotics, Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F12), phosphate-buffered saline (PBS) were obtained from Invitrogen (Carlsbad, CA, USA). Anti-NF-κB, anti-phosphor-NF-κB, anti-IκBa, anti-phosphor-IκBa, anti-IKK, anti-phosphor-IKK antibodies were obtained from Cell Signal Technology (Beverly, MA, USA). Tumor necrosis factor (TNF)-α was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Preparation and culture of RA FLSs

For preparing FLS, synovial tissue specimens (STs) were obtained from eight patients who were diagnosed with RA [13] (6 women and 2 men, aged 40–66 years) and had undergone synovectomy or joint replacement. The study was approved by the Medical Ethical Committee of the First Affiliated Hospital of Jinan University, and was performed based on the recommendations of the Declaration of Helsinki. All patients provided written informed consent before participating in the study.

Based on the reported method [14], STs were cut into small pieces and digested with 1 mg/ml type I collagenase in DMEM/F12 medium at 37 °C and 5% CO2 for 2 h to isolate synoviocytes. The synoviocytes were cultured in DMEM/F12 with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C and 5% CO2. The cells were trypsinized and passaged. RA FLSs obtained from the 3th to 5th passages were used for all experiments.

2.3. Cell viability assays

RA FLSs were pre-incubated for 48 h with betulinic acid at various concentrations (0, 2.5, 5, 10, 20 μM) in a 96-well plate. After removed the culture supernatants, the adherent cells were incubated with a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt (1 mg/ml in PBS) for 30 min at 37 °C. The dark blue crystals of formazan were dissolved in acidified isopropanol, and formed formazan was quantified at a test wavelength of 570 nm using a microplate reader.

2.4. Wounding migration

RA FLSs were plated to confluence on 35 mm culture dishes and wounded with pipette tips and treated with betulinic acid in different concentrations (0, 2.5, 5, 10 μM) for 48 h. The cells that had migrated beyond a reference line were quantified by counting.

2.5. Migration and invasion assay in vitro

Chemotaxis assay of RA FLSs was performed using the Boyden chamber method in 24-well plate with a filter of 6.5 mm diameter and 8.0 mm pore size (Corning, USA). The cells were preprocessed with betulinic acid in different concentrations (0, 2.5, 5, 10 μM) for 24 h and then seeded in a Boyden chamber. The lower wells were place with DMEM/F12 containing 10% FBS, while the upper chambers placed serum-free DMEM/F12. The chamber was incubated for 24 h at 37 °C in 5% CO2. The filters were fixed in methanol for 15 min and stained with 0.1% crystal violet for 15 min. After removed the nonmigrating cells from the upper surface of the filter, an optical microscope was used to quantify the chemotaxis by counting the stained cells that migrated to the lower side of the filter. The stained cells were calculated in 8 random fields to obtain the mean number for each assay. In vitro invasion assay, similar procedures were performed using inserts coated with a Matrigel basement membrane matrix (BD Biosciences, USA).

2.6. Confocal laser scanning fluorescence microscopy

RA FLSs were incubated on sterile glass coverslips to approximately 60% confluent. The cells were starved and pre-treated with 1% (v/v) dimethyl sulfoxide (DMSO) or betulinic acid (10 μM) for 24 h. After that, the cells were fixed with 4% paraformaldehyde for 15 min and permeated with 0.1% TritonX-100 in PBS for 5 min. The cells were incubated with AlexaFluor-546 phalloidin (Invitrogen, USA) for 20 min at room temperature to stain filamentous actin (F-actin) and then with 4′-6-diamidono-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, USA) for 3 min at room temperature to stain nuclear. For detection of NF-κB, the cells were incubated with anti-NF-κB antibody for 1 h at room temperature and then stain nuclear. The coverslips were mounted on glass slides with antifade mounting media and observed using confocal fluorescence microscopy.

2.7. Actin cytoskeleton scoring system

Based on the reported method [15], the distribution and characteristics of the cytoskeletal F-actin were evaluated (0 = no filaments, 1 = fine filament at the center of the cell, 2 = at least two thick filaments at the center of the cell, 3 = 90% thick filaments). One investigator assessed all slides which could avoid group assignment. Besides, a minimum of 10 cells was studied in each group.

2.8. RNA isolation and quantitative polymerase chain reaction

Total RNA of RA FLSs were isolated using Trizol reagent (Invitrogen, USA) and reverse-transcribed with the Takara PrimeScript RT reagent kit according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Bio-Rad CFX96 system. The expressions of interleukin (IL)-1β, IL-6, IL-8 and IL-17A were analysed using the SYBR Green PCR Master Mix (Toyobo, Japan). The detector was programmed with the following PCR conditions: 40 cycles of 5 s denaturation at 95 °C and 34 s amplification at 60 °C. The RT-PCR primers used are listed in Supplementary Table S1. All reactions were normalized to housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and were run in triplicate.

2.9. Western blot analysis

A total of 5×10^5 RA FLSs were seeded and incubated to 70% confluent, then the cells were made quiescent in DMEM/F12 medium containing 0.5% FBS for 24 h and treated with various agents. After that, the cells were rinsed twice with ice-cold PBS and added 0.5 ml of ice-cold lysis buffer (CST, USA) for 20 min on ice, and then scraped with cell scraper and centrifuged. Protein concentrations were measured using the Bicinchoninic Acid Protein Assay Kit (Pierce, USA). Protein of each groups were balanced to identical amounts and solubilized in Laemmli buffer, boiled for 5 min, separated by SDS-PAGE, and then electrotransferred to nitrocellulose membranes. The membranes were probed with primary antibodies in Tris buffered saline and then treated with TBS-Tween 20 containing 5% non-fat milk at 4 °C overnight. The membranes were incubated with the secondary antibodies at room temperature for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence (NJ, USA). Each blot was a representative of at least three similar independent experiments.

2.10. Treatment of mice with CIA

The animal experiment was approved by the Ethics Committee of Jinan University and the animals were treated in accordance with the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. Male DBA/1 mice were used to induce CIA model by intradermal injection of 100 mg of bovine type II collagen emulsified at a 1:1 ratio (vol/vol) in Freund's complete adjuvant on day 0, and boosted using bovine type II collagen emulsified at a 1:1 ratio (vol/vol) in incomplete Freund's adjuvant on day 21. CIA mice were divided randomly into two groups ($n = 8$ each), which were treated daily by intraperitoneal injection of either DMSO (as control) or betulinic acid (20 mg/kg/day) for 21 days, beginning on the day of arthritis onset. The clinical score for each paw was evaluated every other days in a blinded fashion, and based on the reported method [16]: 0, no change; 1, significant swelling and redness of one digit; 2, mild swelling and erythema of the limb or swelling of more than two digits; 3, marked swelling and erythema of the limb; 4, maximal swelling and redness of the limb and later, ankylosis. The total score was recorded as the sum of the scores in the four limbs. After 21 days of treatment, the hind limbs were removed and fixed in 4% paraformaldehyde, decalcified in 8% formic acid and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin (H&E).

2.11. Statistical analysis

The data are expressed as mean ± standard error of the mean (S.E.M.), and analysed using SPSS 19.0 software. The significance of the differences was evaluated using Student's *t*-test, one-way analysis of variance (ANOVA) and post hoc LSD tests. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of betulinic acid on the viability of RA FLSs

To evaluate the toxic effect of betulinic acid on RA FLSs, we pre-treated the cells with betulinic acid at various concentrations (0, 2.5, 5, 10, 20 μM) for 48 h, and then used the MTT assay to assess the potential cytotoxicity. As shown in Fig. 1, betulinic acid did not affect cell viability of RA FLSs at concentrations of 0–10 μM.

3.2. Effect of betulinic acid on the migration and invasion of RA FLSs

To determine whether betulinic acid effected cell migration of RA FLSs, we examined chemotaxis migration of the cells using transwell Boyden chamber. As shown in Fig. 2A, betulinic acid treatment inhibited the migratory capacity of RA FLSs. Furthermore, we used a monolayer wound scratch assay to confirm the effect of betulinic acid on RA FLSs migration. We found that betulinic acid-treated cells displayed a significant reduction of cell migration, consistent with the previous result (Fig. 2B).

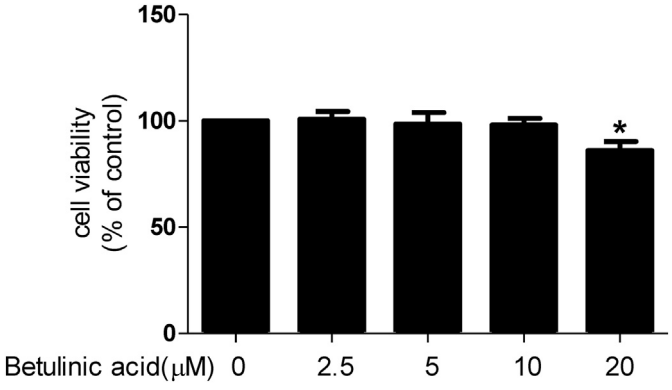


Fig. 1. The effect of betulinic acid on the viability of RA FLSs. Cell viability was measured with an MTT assay in RA FLSs after 48 h of treatment with betulinic acid. The results are shown as the mean ± SEM from three independent experiments. *$P < 0.05$, betulinic acid-treated vs DMSO-treated cells.

Because the invasion ability of RA FLSs played a crucial role in regulating the joint destruction of RA, to explore the effect of betulinic acid in the invasive behavior of RA FLSs in vitro, we pre-treated the cells with betulinic acid at various concentrations (0, 2.5, 5, 10 μM) and then measured with a thin layer of reconstituted extracellular matrix (Matrigel). As shown in Fig. 2C, betulinic acid treatment caused a mardly suppression of invasiveness of RA FLSs.

3.3. Effect of betulinic acid on cytoskeletal reorganization of RA FLSs

Dynamic reorganization of the actin cytoskeleton is important for optimal cell migration [17], to confirm the role of betulinic acid in regulating cytoskeletal reorganization in RA FLSs, we pre-treated the cells with betulinic acid (10 μM) or 1% (v/v) DMSO and then used immunofluorescence staining to visualize cytoskeletal reorganization. As shown in Fig. 3A–B, treatment with betulinic acid decreased the formation of actin stress fibers and actin cytoskeleton score compared with those treated using DMSO.

3.4. Effect of betulinic acid on TNF-α-induced production of interleukin in RA FLSs

To understand whether betulinic acid effected inflammatory cytokines of RA FLSs, we examined the effect of betulinic acid on TNF-α-induced production of interleukin in RA FLSs. As shown in Fig. 4A–D, the mRNA expression of IL-1β, IL-6, IL-8 and IL-17A were decreased by treatment with betulinic acid in TNF-α-induced RA FLSs.

3.5. Effect of betulinic acid on NF-κB activation by TNF-α-induced RA FLSs

As NF-κB pathway is considered a key signal factors in regulating synovial inflammation and joint destruction, we explored the effect of betulinic acid on NF-κB activation in TNF-α-induced RA FLSs. We found a significant decrease in phosphorylated NF-κB, IκBα and IKK following treatment with betulinic acid in TNF-α-stimulated RA FLSs (Fig. 5A). As shown in Fig. 5B, we also performed immunofluorescence staining to showed the translocation of NF-κB into the nucleus of RA FLSs with the treatment of TNF-α and betulinic acid. We observed a reduction in NF-κB nuclear accumulation in RA FLSs treated with betulinic acid, compared with that in cells treated with TNF-α alone. We also observed an inhibitory effect of NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) on the formation of actin stress fibers and actin cytoskeleton score of RA FLSs (Fig. 5C).

3.6. Attenuation of betulinic acid in the severity of arthritis in mice with CIA

The vivo effect of betulinic acid on synovial inflammation and joint destruction of RA was explored in mice with CIA, a classic animal model of RA. As shown in Fig. 6A–C, compared with that of animals injected with DMSO (as control), intraperitoneal injection of betulinic acid (20 mg/kg/day) suppressed the increase in the clinical score and paw volume. Furthermore, we also used H&E staining to illustrate the joint pathology in mice. Compared with the DMSO-treated group, betulinic acid-treated group mice showed a significant reduction in synovitis, synovial hyperplasia, and invasion into calcified cartilage and bone (Fig. 6D).

4. Discussion

In the present study, we demonstrated that betulinic acid

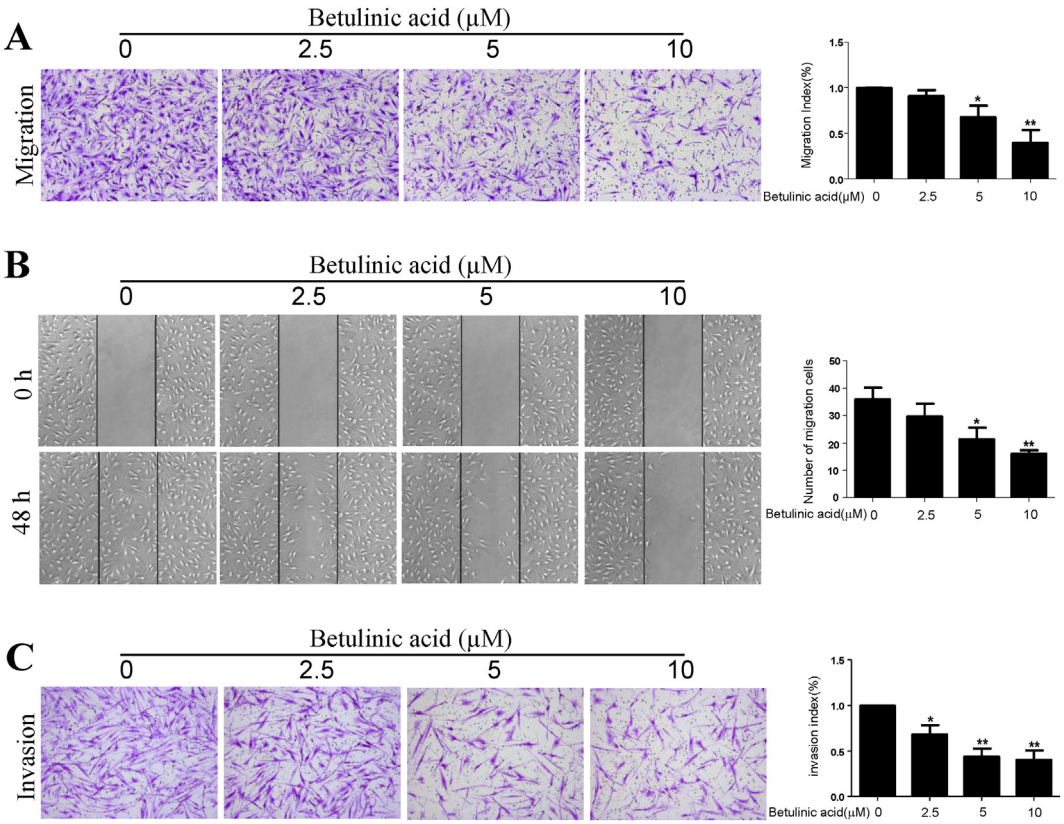


Fig. 2. The effect of betulinic acid on RA FLSs migration and invasion. (A) Effect of betulinic acid on RA FLSs migration was tested using a Boyden chamber, and chemotaxis was quantified by counting the migration index (100×). (B) Effect of betulinic acid on the wound migration of RA FLSs. The cells that migrated beyond the reference line were counted and photographed (50×). (C) Effect of betulinic acid on RA FLSs invasion was explored using a Matrigel basement membrane matrix chamber, and the invading cells were quantified by counting the invasion index (100×). All values represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. DMSO (as control).

suppressed the migration and invasion and inflammatory cytokine production of RA FLSs. We also observed that betulinic acid blocked the TNF-α-mediated activation of NF-κB signal pathways. Moreover, treatment with betulinic acid attenuated synovial inflammation and joint destruction in mice with CIA. Taken together, our results suggest that betulinic acid has potential as a novel therapeutic agent for RA might be associated with its inhibitory effect on the aggressive behavior of RA FLSs by blocking the TNF-α-mediated activation of NF-κB signal pathways.

Betulinic acid is a naturally occurring triterpenoid widely distributed throughout the plant kingdom. Several studies have shown that it exhibits a wide range of biological activities, including anti-tumor, anti-inflammatory, anti-osteoarthritic and immunomodulatory effects [18–20]. These observations suggest benefits of betulinic acid for the treatment of inflammatory and immune disorders. However, whether betulinic acid has the potential for the migration and invasion in RA FLSs is unknown.

The migration of stable activated FLSs to cartilage and bone is an essential process for joint destruction in RA. If RA FLSs arrive at the joint surface, they can destroy cartilage and activate osteoclasts, leading to enhance bone erosion and destruction [21,22]. Thus, regulation of activated FLSs migration and invasion may be a new beneficial strategy for preventing the destructive progress in RA [4]. Therefore, we evaluated the effect of betulinic acid on the migration and invasion of FLSs from RA patients. We determined that betulinic acid inhibited the migration and invasion of RA FLSs. AS cell cytoskeleton rearrangement plays an essential role in regulating the direction of cell migration and invasion [15], we determined the effect of betulinic acid on cytoskeletal reorganization in RA FLSs. We demonstrated that betulinic acid prevented F-actin remodeling in RA FLSs. Moreover, our data also demonstrated the amelioration of betulinic acid treatment in synovial inflammation and joint destruction of mice with CIA, which further support the hypothesis that betulinic acid may have potential as an agent to treat RA.

Increasing evidence suggests that inflammatory cytokines, such as interleukin, implicated in the pathogenesis of RA and anticytokine therapy has become a common clinical treatment for RA [23]. Previous study has showed that betulinic acid suppressed LPS-induced IL-1β and IL-6 expression in RAW 264.7 macrophages [9]. Besides, it has been reported that betulinic acid showed an inhibiting activity of topoisomerase [24–26], which inhibitor has anti-inflammatory actions [27] and has an effect of ameliorating the development of RA [28,29]. To measure the effect of betulinic acid on interleukin expression of RA FLSs, we used real-time PCR to test the expression of interleukin. We found that the mRNA expression of IL-1β, IL-6, IL-8 and IL-17A were markedly down-regulated by treatment with betulinic acid in TNF-α-induced RA FLSs, suggesting that inhibition of interleukin production may be one of the mechanisms for betulinic acid to regulate aggressive behavior of RA FLSs.

NF-κB is a transcription factor that participates in modulating the expression of inflammatory cytokines [30]. It was considered a key signal molecule in the control of synovial inflammation and joint destruction [31,32]. In this study, we observed that betulinic acid treatment reduced the TNF-α-induced activation of NF-κB signal pathway, including NF-κB, IκBα and IKK pathways in RA FLSs. We also confirmed that betulinic acid inhibited the NF-κB nuclear accumulation induced by TNF-α. Consistent with our data, previous studies have shown that betulinic acid inhibited activation of NF-κB signal pathway in human umbilical vein endothelial cells [33], RAW 264.7 macrophages [9] and kidney mesangial cells [34]. Previous works in our laboratory revealed

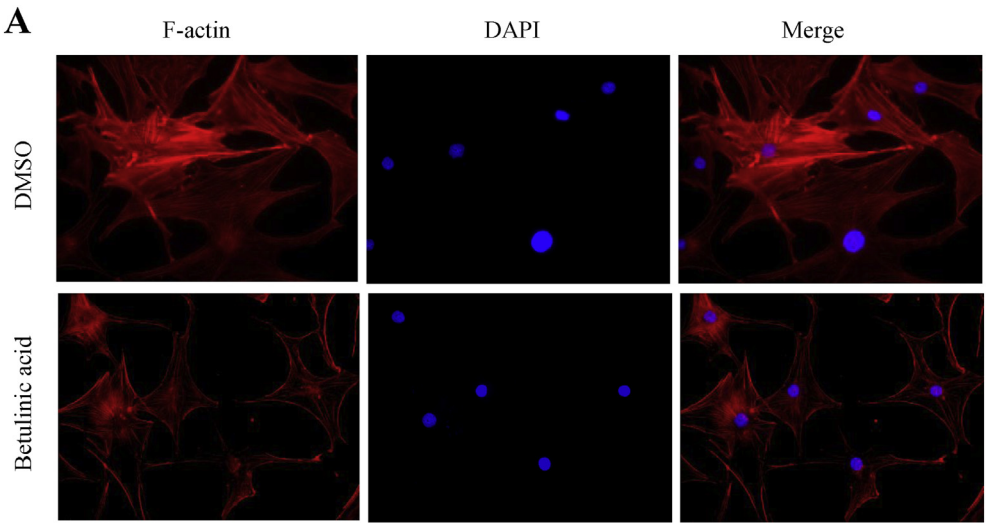


Fig. 3. The effect of betulinic acid on cytoskeletal reorganization in RA FLSs. (A) RA FLSs were fixed and stained with fluorescent phalloidin and DAPI to visible F-actin (red) and nuclei (blue), after that imaged using fluorescence microscopy (200×). Representative images from three independent experiments are shown. (B) Immunofluorescence morphologic of RA FLSs was scored for actin cytoskeleton characteristics. All values represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$ vs. DMSO (as control). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

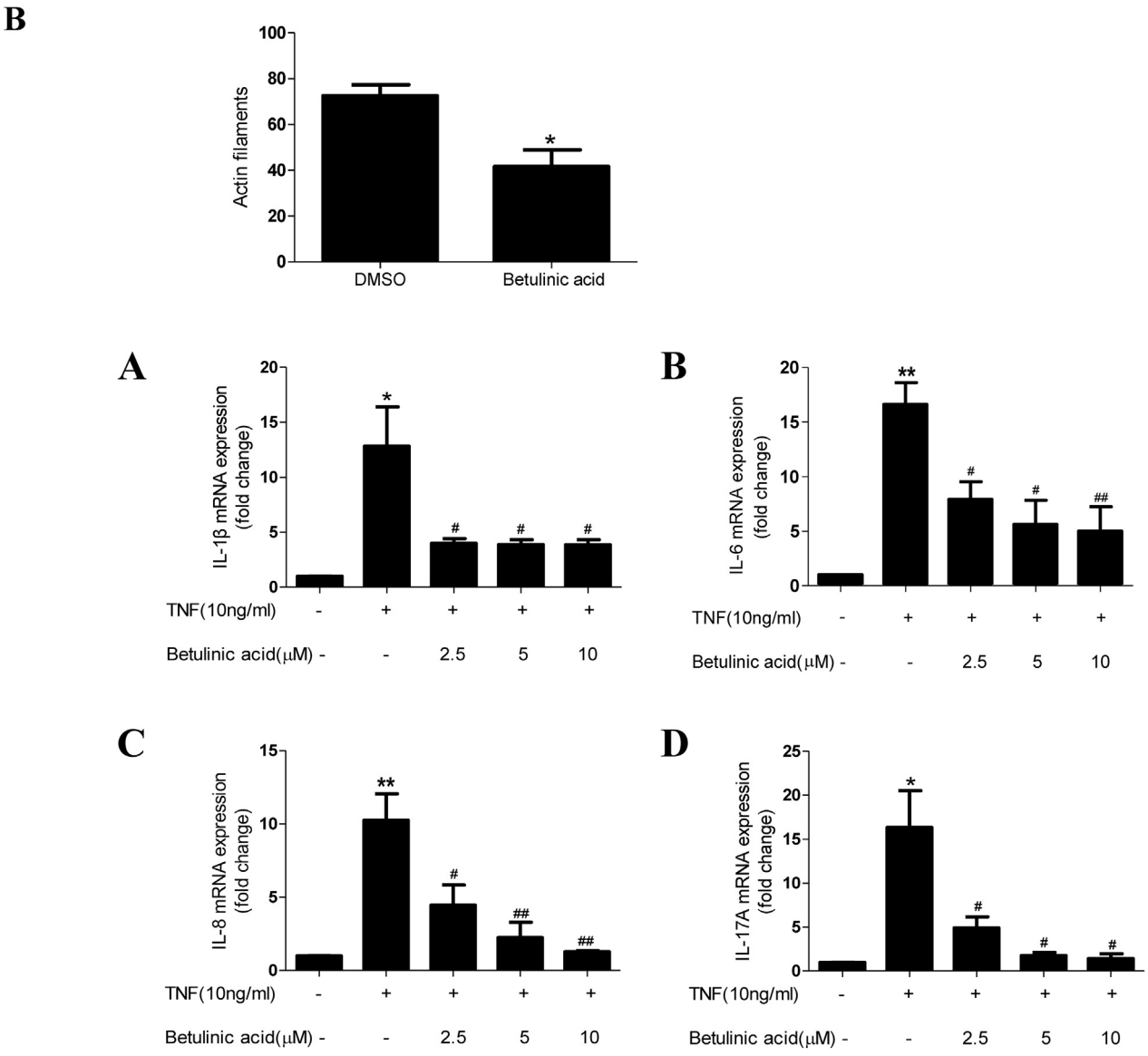
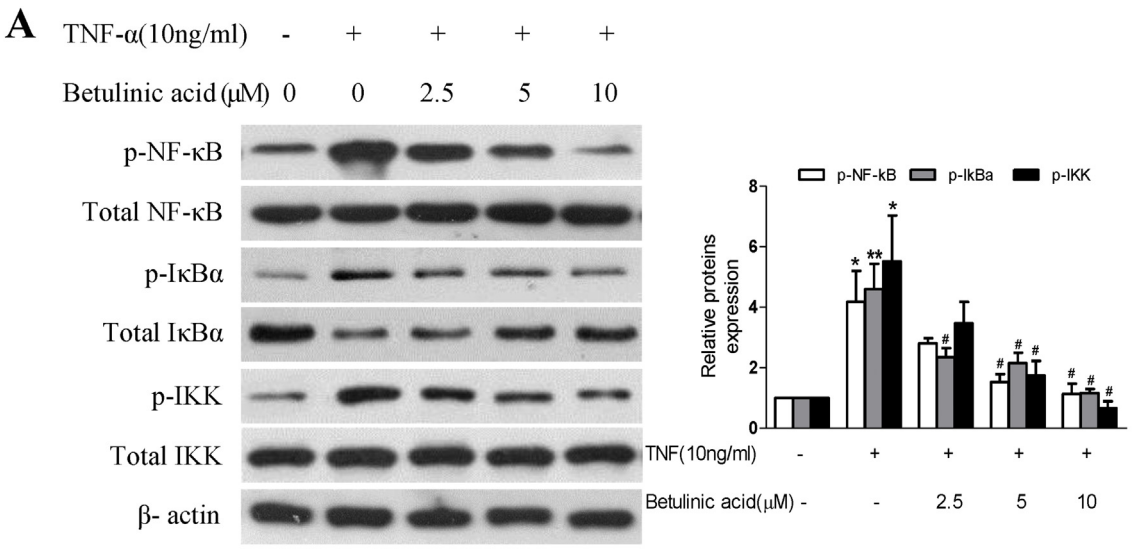
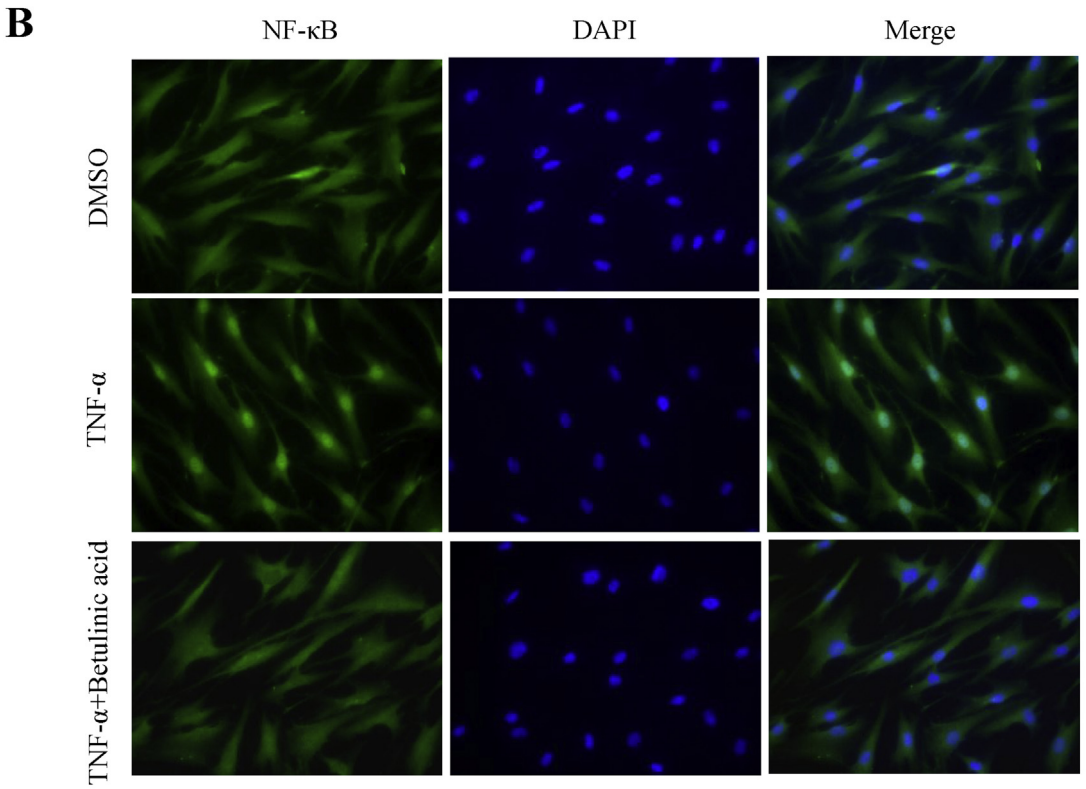


Fig. 4. The effect of betulinic acid on interleukin expression in RA FLSs. RA FLSs were pretreated with DMSO, as control, or various concentrations of betulinic acid for 24 h and then stimulated with or without TNF-α (10 ng/ml) for 12 h. The effect of betulinic acid on IL-1β (A), IL-6 (B), IL-8 (C) and IL-17A (D) mRNA expression was determined by quantitative real-time PCR. Data were normalized to GAPDH. All values represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. DMSO (as control), $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. treatment with TNF-α.





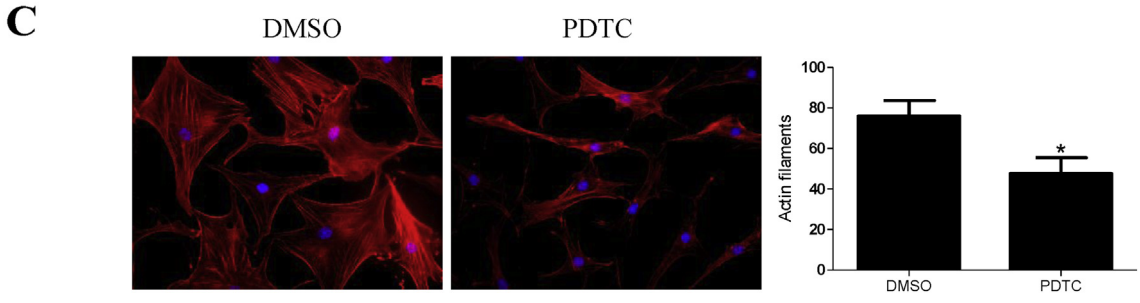


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Fig. 5. The effect of betulinic acid on the TNF-α-induced activation of NF-κB pathway in RA FLSs. (A) RA FLSs were treated with betulinic acid or DMSO for 24 h, followed by stimulation with or without TNF-α for 10 min. The Western blot analysis was performed to evaluate the expression of NF-κB, IκBα and IKK and phosphorylated NF-κB, IκBα and IKK. The protein expression was quantitated using densitometry analysis (right panel) from three independent experiments. (B) Effect of betulinic acid on TNF-α-induced nuclear translocation of NF-κB (green stain) with nuclei stained with DAPI (blue stain) (200×). (C) Effect of NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) on the formation of actin stress fibers (200×) and actin cytoskeleton score of RA FLSs. All values represent the mean ± SEM from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. DMSO (as control), $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. treatment with TNF-α. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

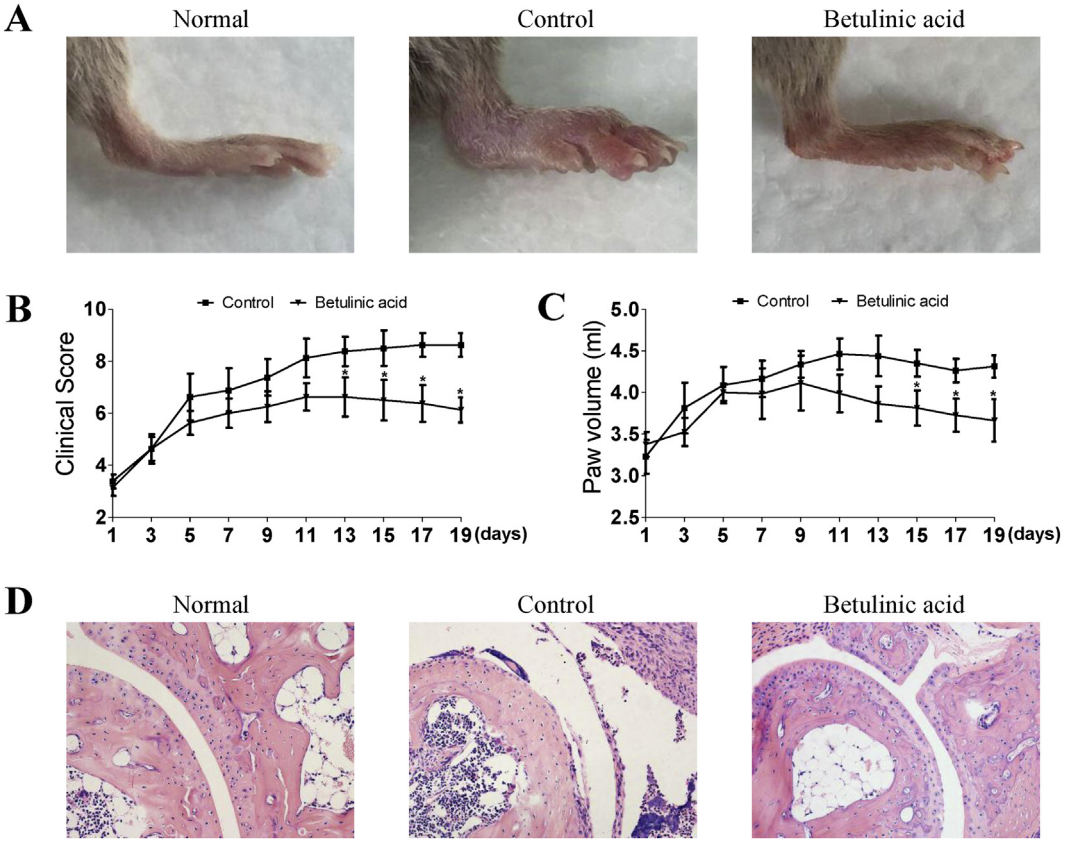

Fig. 6. The effects of betulinic acid on the severity of the arthritis of mice with CIA. The DBA/1 mice were immunized with bovine type II collagen on day 0 and boosted on day 21. The CIA mice were treated with once-daily intraperitoneal injections of DMSO (as control) or betulinic acid (20 mg/kg/day) for 21 days from the day of arthritis onset. (A–C) Effects of betulinic acid on the joint morphology (100×) (A), clinical scores (B) and paw swelling (change in volume) (C) in the CIA mice. (D) Histologic appearance in the joints of normal control and CIA mice treated with DMSO or betulinic acid. H&E staining was used to observe synovial infiltration, cartilage erosion and bone loss (100×). All values represent the mean ± SEM from eight independent experiments. $^{*}P < 0.05$ vs. DMSO-treated CIA mice.

that NF-κB inhibitor PDTC has an inhibitory effect on the migration and invasion in RA FLSs [16]. In the present study, we also found that PDTC suppressed the formation of actin stress fibers of RA FLSs. These results suggest that the NF-κB signal pathway might mediate the action of betulinic acid in the function of RA FLSs.

In summary, our findings demonstrated that betulinic acid inhibits the migration, invasion, actin cytoskeletal reorganization and interleukin expression of RA FLSs by inhibiting the NF-κB signal pathway. Our findings suggest that betulinic acid may be an effective therapeutic agent for RA. However, there are no data with normal FLS in our finding and further studies should be performed to explore the safety and mechanism of betulinic acid for RA.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.042.

Competing interests

No conflict of interest has been declared by authors.

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References

[1] I.B. McInnes, G. Schett, The pathogenesis of rheumatoid arthritis, N. Engl. J. Med. 365 (2011) 2205–2219.
[2] L.C. Huber, O. Distler, I. Tarner, R.E. Gay, S. Gay, T. Pap, Synovial fibroblasts: key players in rheumatoid arthritis, Rheumatology (Oxford) 45 (2006) 669–675.
[3] B. Bartok, G.S. Firestein, Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis, Immunol. Rev. 233 (2010) 233–255.
[4] A. Mor, S.B. Abramson, M.H. Pillinger, The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction, Clin. Immunol. 115 (2005) 118–128.
[5] P. Yogeeswari, D. Sriram, Betulinic acid and its derivatives: a review on their biological properties, Curr. Med. Chem. 12 (2005) 657–666.

[6] E. Pisha, H. Chai, I.S. Lee, T.E. Chagwedera, N.R. Farnsworth, G.A. Cordell, C.W. Beecher, H.H. Fong, A.D. Kinghorn, D.M. Brown, A. Et, Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis, Nat. Med. 1 (1995) 1046–1051.
[7] W. Rzeski, A. Stepulak, M. Szymanski, M. Sifringer, J. Kaczor, K. Wejksza, B. Zdzisinska, M. Kandefer-Szerszen, Betulinic acid decreases expression of bcl-2 and cyclin D1, inhibits proliferation, migration and induces apoptosis in cancer cells, Naunyn Schmiedeberg's Arch. Pharmacol. 374 (2006) 11–20.
[8] A. Szuster-Ciesielska, K. Plewka, M. Kandefer-Szerszen, Betulin, betulinic acid and butein are inhibitors of acetaldehyde-induced activation of liver stellate cells, Pharmacol. Rep. 63 (2011) 1109–1123.
[9] K.S. Kim, D.S. Lee, D.C. Kim, C.S. Yoon, W. Ko, H. Oh, Y.C. Kim, Anti-inflammatory effects and mechanisms of action of coussaric and betulinic acids isolated from *Diospyros kaki* in lipopolysaccharide-stimulated RAW 264.7 macrophages, Molecules 21 (2016).
[10] Y. Yun, S. Han, E. Park, D. Yim, S. Lee, C.K. Lee, K. Cho, K. Kim, Immunomodulatory activity of betulinic acid by producing pro-inflammatory cytokines and activation of macrophages, Arch. Pharm. Res. 26 (2003) 1087–1095.
[11] M.C. Recio, R.M. Giner, S. Manez, J. Gueho, H.R. Julien, K. Hostettmann, J.L. Rios, Investigations on the steroidal anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*, Planta Med. 61 (1995) 9–12.
[12] L.E. Mathew, V. Rajagopal, H. A, Betulinic acid and fluvastatin exhibits synergistic effect on toll-like receptor-4 mediated anti-atherogenic mechanism in type II collagen induced arthritis, Biomed. Pharmacother. 93 (2017) 681–694.
[13] F.C. Arnett, S.M. Edworthy, D.A. Bloch, D.J. McShane, J.F. Fries, N.S. Cooper, L.A. Healey, S.R. Kaplan, M.H. Liang, H.S. Luthra, A. Et, The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis, Arthritis Rheum. 31 (1988) 315–324.
[14] D. Pan, N. Li, Y. Liu, Q. Xu, Q. Liu, Y. You, Z. Wei, Y. Jiang, M. Liu, T. Guo, X. Cai, X. Liu, Q. Wang, M. Liu, X. Lei, M. Zhang, X. Zhao, C. Lin, Kaempferol inhibits the migration and invasion of rheumatoid arthritis fibroblast-like synoviocytes by blocking activation of the MAPK pathway, Int. Immunopharmacol. 55 (2018) 174–182.
[15] M. Verderame, D. Alcorta, M. Egnor, K. Smith, R. Pollack, Cytoskeletal F-actin patterns quantitated with fluorescein isothiocyanate-phalloidin in normal and transformed cells, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 6624–6628.
[16] N. Li, Q. Xu, Q. Liu, D. Pan, Y. Jiang, M. Liu, M. Liu, H. Xu, C. Lin, Leonurine attenuates fibroblast-like synoviocyte-mediated synovial inflammation and joint destruction in rheumatoid arthritis, Rheumatology (Oxford) 56 (2017) 1417–1427.
[17] L.X. Qin, Inflammatory immune responses in tumor microenvironment and metastasis of hepatocellular carcinoma, Cancer Microenviron. 5 (2012) 203–209.
[18] D.A. Eiznhamer, Z.Q. Xu, Betulinic acid: a promising anticancer candidate, IDrugs 7 (2004) 359–373.
[19] W. Jingbo, C. Aimin, W. Qi, L. Xin, L. Huaining, Betulinic acid inhibits IL-1beta-induced inflammation by activating PPAR-gamma in human osteoarthritis chondrocytes, Int. Immunopharmacol. 29 (2015) 687–692.
[20] S.K. Dash, S. Chattopadhyay, S. Tripathy, S.S. Dash, B. Das, D. Mandal, S.K. Mahapatra, B.G. Bag, S. Roy, Self-assembled betulinic acid augments immunomodulatory activity associates with IgG response, Biomed. Pharmacother. 75 (2015) 205–217.
[21] U. Muller-Ladner, T. Pap, R.E. Gay, M. Neidhart, S. Gay, Mechanisms of disease: the molecular and cellular basis of joint destruction in rheumatoid arthritis, Nat. Clin. Pract. Rheumatol. 1 (2005) 102–110.
[22] T. Pap, I. Meinecke, U. Muller-Ladner, S. Gay, Are fibroblasts involved in joint destruction? Ann. Rheum. Dis. 64 (Suppl. 4) (2005) v52–v54.
[23] S. Siebert, A. Tsoukas, J. Robertson, I. McInnes, Cytokines as therapeutic targets in rheumatoid arthritis and other inflammatory diseases, Pharmacol. Rev. 67 (2015) 280–309.
[24] A.R. Chowdhury, S. Mandal, B. Mittra, S. Sharma, S. Mukhopadhyay, H.K. Majumder, Betulinic acid, a potent inhibitor of eukaryotic topoisomerase I: identification of the inhibitory step, the major functional group responsible and development of more potent derivatives, Med. Sci. Monit. 8 (2002) R254–R265.
[25] A. Ganguly, B. Das, A. Roy, N. Sen, S.B. Dasgupta, S. Mukhopadhayay, H.K. Majumder, Betulinic acid, a catalytic inhibitor of topoisomerase I, inhibits reactive oxygen species-mediated apoptotic topoisomerase I-DNA cleavable complex formation in prostate cancer cells but does not affect the process of cell death, Cancer Res. 67 (2007) 11848–11858.
[26] F.M. Bar, M.A. Khanfar, A.Y. Elnagar, H. Liu, A.M. Zaghloul, F.A. Badria, P.W. Sylvester, K.F. Ahmad, K.P. Raisch, S.K. El, Rational design and semisynthesis of betulinic acid analogues as potent topoisomerase inhibitors, J. Nat. Prod. 72 (2009) 1643–1650.
[27] M. Verdrengh, A. Tarkowski, Impact of topoisomerase II inhibition on cytokine and chemokine production, Inflamm. Res. 52 (2003) 148–153.
[28] M. Verdrengh, O. Isaksson, A. Tarkowski, Topoisomerase II inhibitors, irrespective of their chemical composition, ameliorate experimental arthritis, Rheumatology (Oxford) 44 (2005) 183–186.
[29] J.K. Jackson, T. Higo, W.L. Hunter, H.M. Burt, Topoisomerase inhibitors as anti-arthritic agents, Inflamm. Res. 57 (2008) 126–134.
[30] S.E. Sweeney, G.S. Firestein, Signal transduction in rheumatoid arthritis, Curr. Opin. Rheumatol. 16 (2004) 231–237.
[31] A.V. Miagkov, D.V. Kovalenko, C.E. Brown, J.R. Didsbury, J.P. Cogswell, S.A. Stimpson, A.S. Baldwin, S.S. Makarov, NF-kappaB activation provides the potential link between inflammation and hyperplasia in the arthritic joint, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 13859–13864.
[32] P.P. Tak, D.M. Gerlag, K.R. Aupperle, D.A. van de Geest, M. Overbeek, B.L. Bennett, D.L. Boyle, A.M. Manning, G.S. Firestein, Inhibitor of nuclear factor kappaB kinase beta is a key regulator of synovial inflammation, Arthritis Rheum. 44 (2001) 1897–1907.
[33] J.J. Yoon, Y.J. Lee, J.S. Kim, D.G. Kang, H.S. Lee, Protective role of betulinic acid on TNF-alpha-induced cell adhesion molecules in vascular endothelial cells, Biochem. Biophys. Res. Commun. 391 (2010) 96–101.
[34] S. Wang, Z. Yang, F. Xiong, C. Chen, X. Chao, J. Huang, H. Huang, Betulinic acid ameliorates experimental diabetic-induced renal inflammation and fibrosis via inhibiting the activation of NF-kappaB signaling pathway, Mol. Cell. Endocrinol. 434 (2016) 135–143.