

Original Article

Mangiferin suppresses CIA by suppressing the expression of TNF- α , IL-6, IL-1 β , and RANKL through inhibiting the activation of NF- κ B and ERK1/2

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Abstract: Rheumatoid arthritis is a systemic autoimmune disease characterized by chronic inflammation of synovial joints, ultimately leading to a progressive and irreversible joint destruction. Activation of nuclear factor-kappa B (NF- κ B) promotes production of proinflammatory cytokines in various inflammatory diseases including rheumatoid arthritis. Mangiferin, 1,3,6,7-tetrahydroxyxanthone-C2- β -D-glucoside (C-glucosyl xanthone), is a naturally occurring polyphenol. Our previous results showed that mangiferin suppressed NF- κ B activation. However, it is unclear, whether mangiferin can prevent rheumatoid arthritis through suppression of NF- κ B activation and expression of various cytokines, such as tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6), which play a critical role in the pathogenesis of rheumatoid arthritis. In the present study, we found that mangiferin suppressed the progression and incidence of CIA in DBA1/J mice. In CIA mice, mangiferin inhibited the mRNA expression of cytokine genes in thymus and spleen of CIA mice and led to decreased serum levels of IL-1 β , IL-6, TNF- α , and receptor activator NF- κ B ligand (RANKL) via inhibition of NF- κ B and activation of extracellular signal-regulated kinase 1/2 (ERK1/2). In addition, mangiferin markedly inhibited not only developing but also clinically evident CIA. These findings suggest that mangiferin has potential clinical applications for the treatment of rheumatoid arthritis.

Keywords: Mangiferin, rheumatoid arthritis, NF- κ B, ERK1/2, CIA

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by synovial inflammation and hyperplasia that leads to progressive cartilage and bone destruction [1]. Although the precise etiology and underlying pathophysiology of RA are still unclear, cumulative evidence suggests that genetic susceptibility along with environmental factors may trigger the sustained inflammatory process. Collagen-induced arthritis (CIA) is a well-established experimental model of RA and can be induced in susceptible strains of mice by immunizing them with native heterologous type II collagen (CII) [2]. Unlike other experimental arthritis models, the CIA model resembles human RA

more closely in terms of the clinical, histological, and immunological features and the genetic linkage [3].

Nuclear factor-kappa B (NF- κ B) is a dimeric transcription factor formed by the homodimerization or heterodimerization of Rel family proteins [1, 4]. To date, five Rel proteins have been identified, which includes RelA (p65), RelB, c-Rel, (having transactivation domains), and p50 and p52, (expressed as the precursor proteins of p105 [NF- κ B1] and p100 [NF- κ B2], respectively). These precursors require post-translational processing and do not contain transactivation domains. It has been clearly demonstrated that NF- κ B is highly activated and involved in the pathogenesis of RA and ani-

Mangiferin suppresses collagen-induced arthritis

mal models of experimental arthritis [5-7]. It is well established that NF- κ B is involved in the regulation of multiple pro-inflammatory mechanisms. Activation of NF- κ B is necessary and sufficient for the transcriptional activation of TNF- α , IL-1 β , IL-6, receptor activator of NF- κ B ligand (RANKL), and cyclooxygenase 2 (COX-2) [8-10], all of which are required for the initiation, amplification, and maintenance of chronic inflammations, including that seen in RA. Especially, TNF- α is critical in the pathogenesis of RA [9]. In an inflammatory disorder, TNF- α is believed to play the role of early and crucial cytokine, which triggers various positive and negative feedback loops leading to exacerbated inflammation [11]. Therefore, inhibition of NF- κ B and TNF- α expression is believed to inhibit RA.

Mangiferin, 1,3,6,7-tetrahydroxyxanthone-C2- β -D-glucoside (C-glucosyl xanthone), is a naturally occurring polyphenol. It is widely distributed in plants of Thymelaeaceae family (e.g., *Phaleria cumingii*), and is found abundantly in the leaves (Ongaël). Our recent report demonstrates that mangiferin suppresses NF- κ B activation in acute myeloid leukemia (AML) cells. [12] However, the activity of mangiferin in RA was not elucidated. In this study, we investigated the effect of mangiferin in CIA in DBA/1 mice.

Materials and methods

Materials

Mangiferin (1,3,6,7-hydroxyxanthone-C2- β -D-glucoside) was purchased by Sigma (St. Louis, MO, USA); it was dissolved in dimethyl sulfoxide. The dissolved reagent was diluted in phosphate-buffered saline (PBS; pH 7.4), filtered through 0.45 μ m syringe filters (IWAKI GLASS, Tokyo, Japan), and used for various assays as described below.

Mice

Male DBA/1 mice (age, 8 weeks) were purchased from Shimizu Laboratory Animals (Kyoto, Japan). The mice were maintained in a pathogen-free environment at 25°C under controlled light conditions (12-h light/12-h dark) and allowed free access to water and food pellets. All animal studies were performed in accordance with the Recommendations for

Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. The ethical procedures followed met the requirements of the UKCCCR guidelines.

Induction and assessment of arthritis

The DBA/1 mice received an intradermal injection of 200 μ g of bovine CII (Wako, Osaka, Japan) in complete Freund's adjuvant (CFA) (Wako) (Day 0). On Day 21, collagen (200 μ g in PBS) was administered subcutaneously (s.c.) in the tail. The mice were monitored daily for signs of arthritis, and severity scores were assigned as follows: 0 = normal, 1 = erythema, 2 = erythema plus swelling, 3 = extension/loss of function, and the total score = sum of the scores of 4 limbs. Disease onset was characterized by erythema and/or paw swelling observed between Days 21 and 34 or 42. No difference was observed in terms of the degree of response between the early and late responders.

Treatment protocols

For the prophylactic protocol, DBA/1 mice were administered daily orally with increasing doses of mangiferin (50, 100, and 400 mg/kg; each groups, n = 10) from Days 21 to 34. For the therapeutic approach, DBA/1 mice were treated with mangiferin (100 and 400 mg/kg; each groups, n = 10) orally for a total of 14 days 1 day after CIA became clinically detectable (Day 27). Vehicle mice (n = 10) received PBS alone in both experiments.

Determination of serum cytokine levels

Determination of RANKL levels were measured using Quantikine RANKL enzyme immunoassay kits (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. Determination of IL-1 β , IL-6, and TNF- α levels by multiplex cytokine detection assays using Luminex technology (Cytokine Profiler from Millipore, Upstate).

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using RNAiso (Takara Biomedical; Siga, Japan). One microgram of

Mangiferin suppresses collagen-induced arthritis

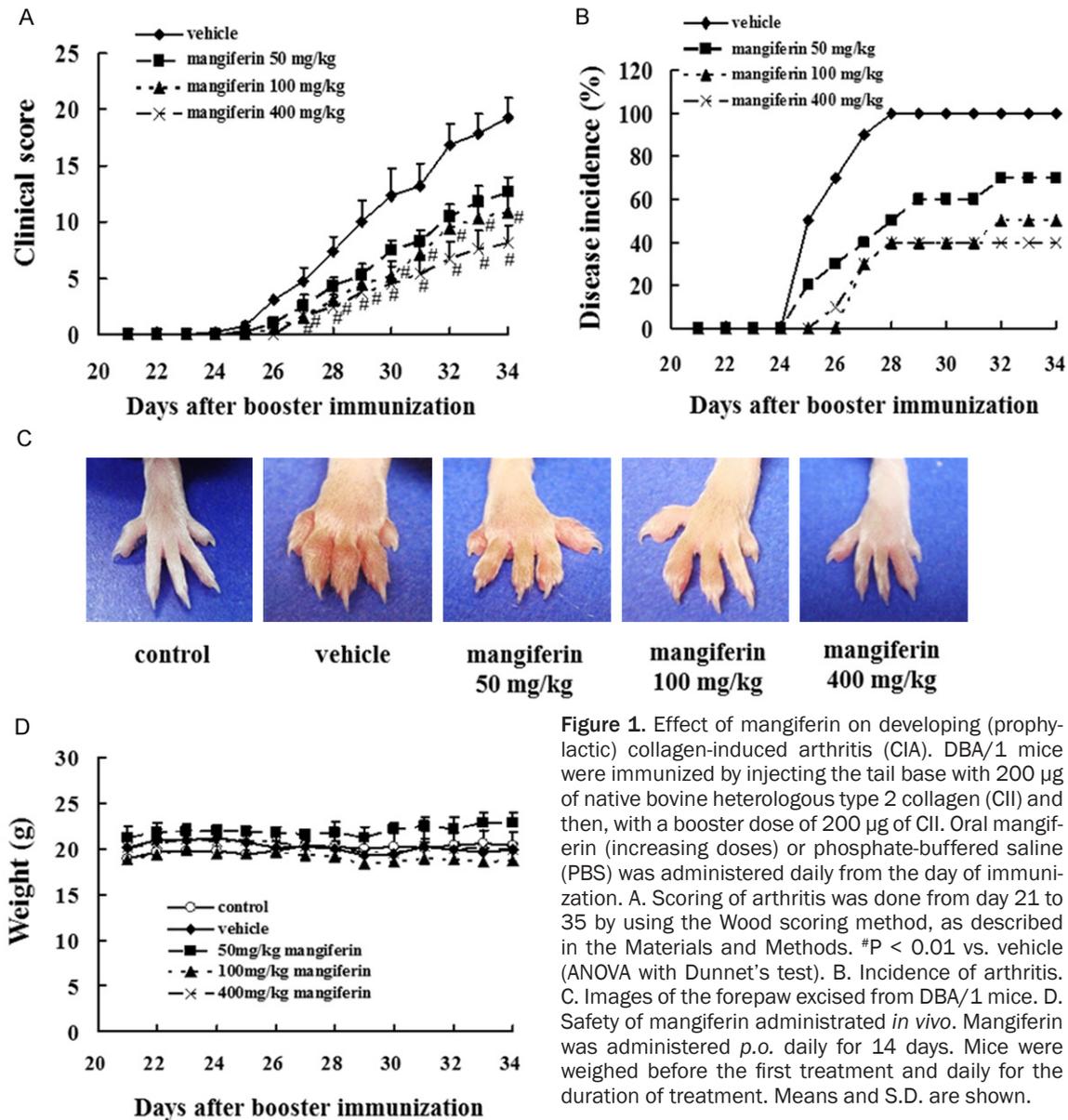


Figure 1. Effect of mangiferin on developing (prophylactic) collagen-induced arthritis (CIA). DBA/1 mice were immunized by injecting the tail base with 200 μ g of native bovine heterologous type 2 collagen (CII) and then, with a booster dose of 200 μ g of CII. Oral mangiferin (increasing doses) or phosphate-buffered saline (PBS) was administered daily from the day of immunization. **A.** Scoring of arthritis was done from day 21 to 35 by using the Wood scoring method, as described in the Materials and Methods. # $P < 0.01$ vs. vehicle (ANOVA with Dunnet's test). **B.** Incidence of arthritis. **C.** Images of the forepaw excised from DBA/1 mice. **D.** Safety of mangiferin administrated *in vivo*. Mangiferin was administered *p.o.* daily for 14 days. Mice were weighed before the first treatment and daily for the duration of treatment. Means and S.D. are shown.

purified total RNA was used for the real-time PCR analysis with the PrimeScript RT reagent kit (Takara Biomedical). cDNA was subjected to quantitative real-time PCR by using SYBR Premix Ex *Taq* (Takara Biomedical) and the Thermal Cycler Dice Real Time system (Takara Biomedical) in a 96-well plate according to the manufacturer's instructions. The PCR conditions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IL-1 β , IL-6, TNF- α , and RANKL were 94°C for 2 min; followed by 40 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 0.5 min. The following primers were used: IL-1 β , 5'-TGA AAT GCC ACC TTT TGA CAG-3' (5'-primer) and 5'-CCA CAG CCA CAA TGA GTG

ATA C-3' (3'-primer); IL-6, 5'-CTG CAA GAG ACT TCC ATC CAG-3' (5'-primer) and 5'-AGT GGT ATA GAC AGG TCT GTT GG-3' (3'-primer); TNF- α , 5'-GGC AGG TCT ACT TTG GAG TCA T-3' (5'-primer) and 5'-CAG AGT AAA GGG GTC AGA GTG G-3' (3'-primer); RANKL, 5'-GGT CGG GCA ATT CTG ATT T-3' (5'-primer) and 5'-GGG GAA TTA CAA AGT GCA CCA G-3' (3'-primer); GAPDH, 5'-ACT TTG TCA AGC TCA TTT-3' (5'-primer) and 5'-TGC AGC GAA CTT TAT TG-3' (3'-primer). As an internal control for each sample, the GAPDH gene was used for standardization. Cycle threshold (Ct) values were established, and the relative difference in expression from GAPDH expression was determined according to the $2^{-\Delta\Delta Ct}$

Mangiferin suppresses collagen-induced arthritis

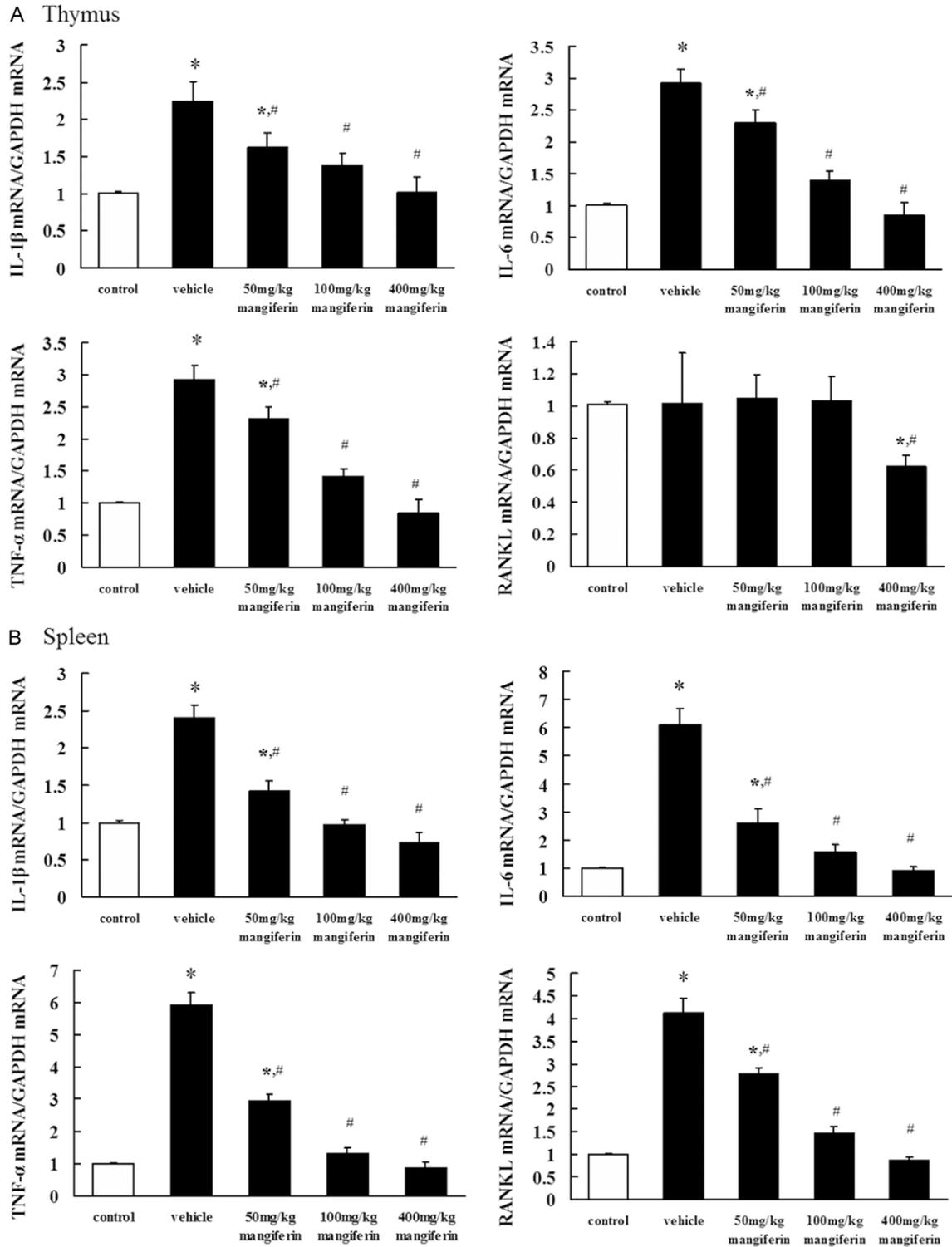


Figure 2. Effect of mangiferin treatment on cytokine gene expression in CIA mice. Total RNA was extracted from thymus (A) and spleen (B), and the interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and receptor activator NF- κ B ligand (RANKL) mRNA levels were determined by real-time PCR. Samples were obtained on day 35. The results are expressed as the test:control ratio after correction of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. The results are representative of 10 independent experiments. *P < 0.01 vs. control, #P < 0.01 vs. vehicle, (ANOVA with Dunnett's test).

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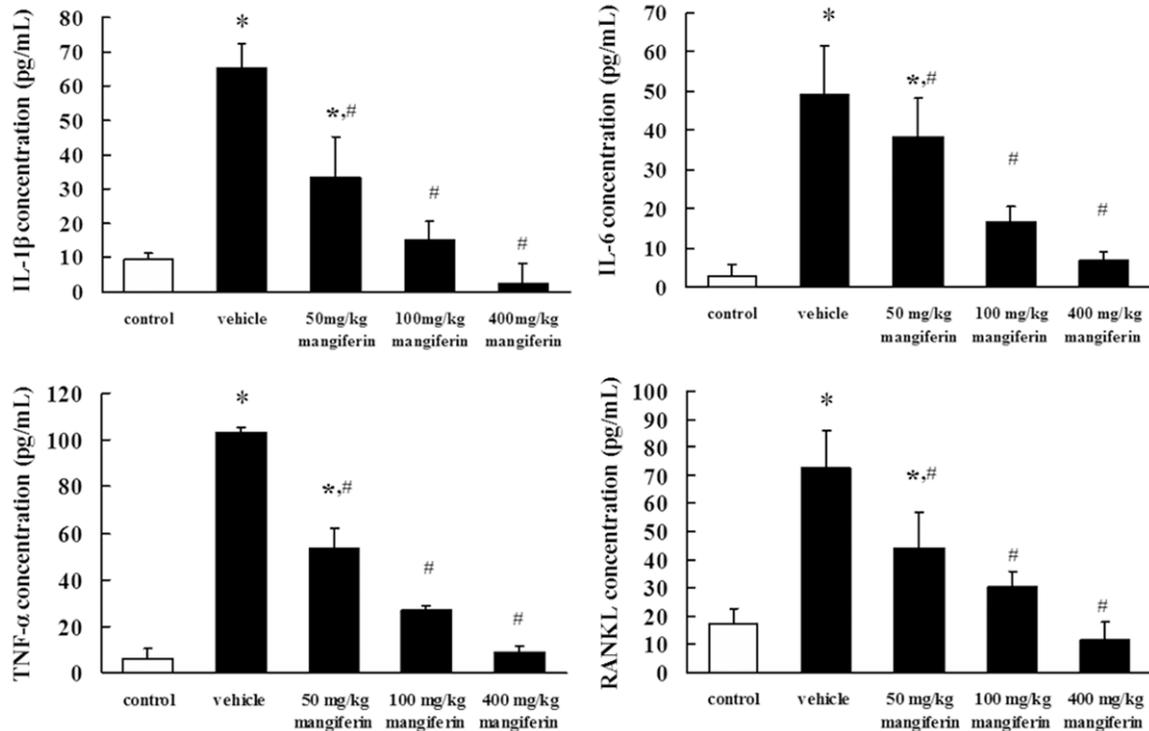


Figure 3. Inhibitory effect of mangiferin on serum interleukin and other markers in CIA mice. Serum titers of IL-1 β , IL-6, TNF- α , and RANKL were measured by Luminex technology or ELISA assay in mice treated with various doses of mangiferin ($n = 10$) or phosphate-buffered saline (PBS, $n = 10$) and normal control mice ($n = 10$). Serum samples were obtained on day 35. Each serum sample was diluted with PBS. The results are representative of 10 independent experiments. * $P < 0.01$ vs. control, # $P < 0.01$ vs. vehicle, (ANOVA with Dunnett's test).

method of analysis and compared to the expression in vehicles.

Western blotting

Thymuses and spleens treated under various conditions were lysed with a lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP-40, 1 μ g/ml leupeptin, 1 μ g/ml antipain, and 1 mM PMSF), and the protein concentrations of the resulting cell lysates were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). A 40- μ g protein aliquot of each extract was fractionated by electrophoresis in a SDS-PAGE and transferred to a PVDF membrane (Amersham, Arlington Heights, IL, USA). The membranes were blocked with a solution containing 3% skim milk, and then incubated overnight at 4°C with each of the following antibodies: anti-phospho- extracellular signal-regulated kinase (ERK) 1/2 antibody, anti-ERK1/2 antibody, anti-phospho-Akt antibody, anti-Akt antibody, and phospho-NF- κ B p65, NF- κ B p65 antibody (Cell Signaling Technology, Beverly, MA, USA).

Subsequently, the membranes were incubated for 1 h at room temperature with anti-rabbit IgG sheep antibody coupled to horseradish peroxidase (Amersham). Reactive proteins were visualized using a chemiluminescence kit (Amersham) according to the manufacturer's instructions.

Statistical analysis

All experimental results are expressed as mean (SD) of several independent experiments. Multiple comparisons of data were carried out by analysis of variance (ANOVA) with Dunnett's test. P values of less than 5% were regarded as significant.

Results

Mangiferin suppresses CIA

Daily oral administration of mangiferin from day 21 to 34 was found to suppress the incidence and severity of CIA in a dose-dependent manner followed by s.c. CII challenge (**Figure 1A**

Mangiferin suppresses collagen-induced arthritis

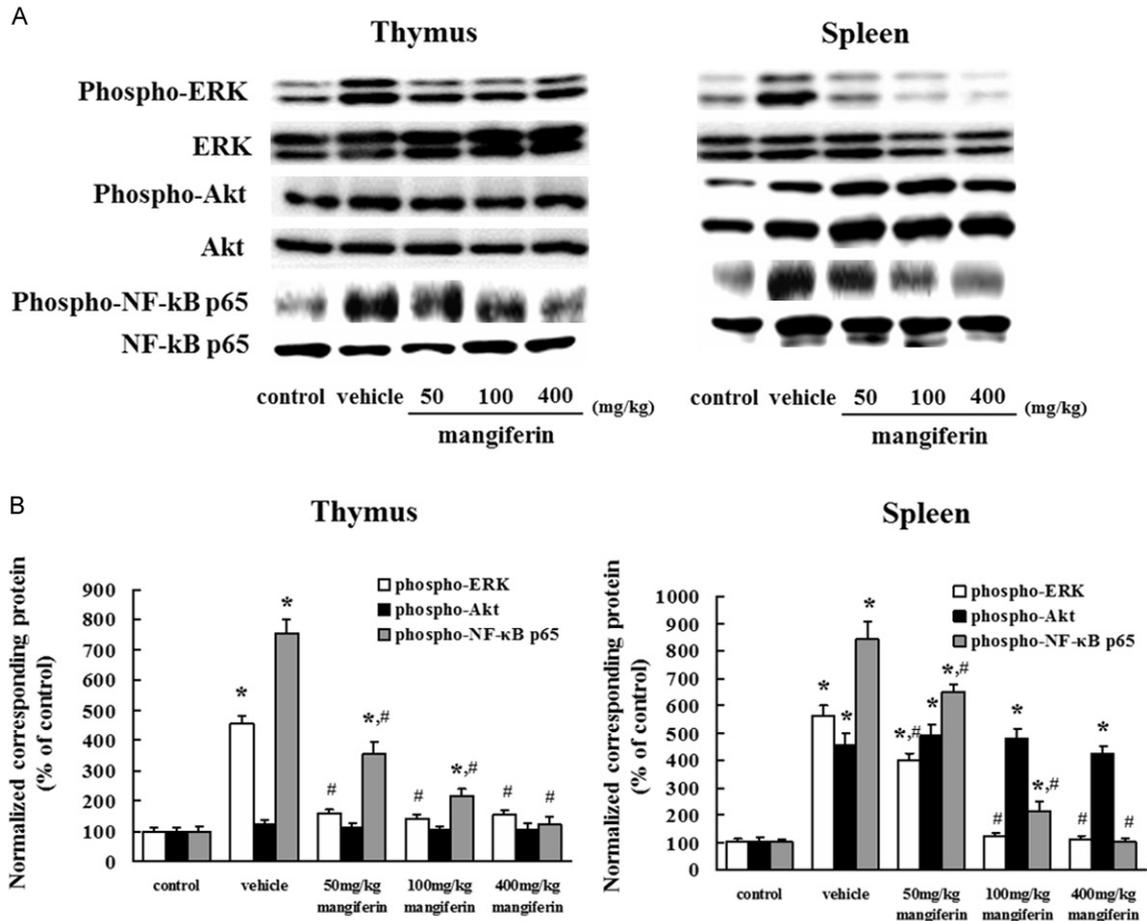


Figure 4. Mangiferin suppresses NF- κ B and ERK1/2 in thymus and spleen of CIA mice. A. Thymus and spleen lysate samples were obtained on day 35. Thymus and spleen lysates were generated and immunoblotted with antibodies against phospho-ERK1/2, phospho-Akt, phospho-NF- κ B p65, ERK1/2, Akt, and NF- κ B p65. B. Quantification of the amount of phospho-ERK1/2, phospho-Akt, or phospho-NF- κ B p65, normalized to the amount of ERK, Akt, or NF- κ B, respectively. The results are representative of 10 independent experiments. * $P < 0.01$ vs. control, # $P < 0.01$ vs. vehicle, (ANOVA with Dunnett's test).

and 1B). Figure 1C shows a representative photograph of a typical forepaw. Mice administered with mangiferin did not exhibit any weight loss (Figure 1D).

Mangiferin inhibits IL-1 β , IL-6, TNF- α , and RANKL expressions in CIA mice

Because inflammatory cytokines secreted by T cells and B cells are thought to play a critical role in the pathogenesis of RA, we monitored the mRNA expression of the pro-inflammatory cytokines IL-1 β , IL-6, TNF- α , and RANKL in the thymus and spleen of CIA mice by using real time PCR. The mRNA expression levels of IL-1 β , IL-6, TNF- α , and RANKL increased in the mice challenged with CII, which was significantly reversed by treatment with 50, 100, and 400

mg/kg mangiferin (Figure 2). Moreover, suppression of these cytokine mRNA expressions in the groups treated with 100 and 400 mg/kg mangiferin was comparable to the expression levels exhibited by control (no treatment of CII group).

Next, we investigated whether mangiferin can inhibit the secretion of these cytokine levels in CIA mice. Serum was collected and analyzed for IL-1 β , IL-6, TNF- α , and RANKL levels by Luminex technology or ELISA assay. Treatment with mangiferin suppressed the serum expression levels of IL-1 β , IL-6, TNF- α , and RANKL in a dose-dependent manner (Figure 3). These observations suggest that mangiferin suppressed CIA via inhibition of IL-1 β , IL-6, TNF- α , and RANKL expressions.

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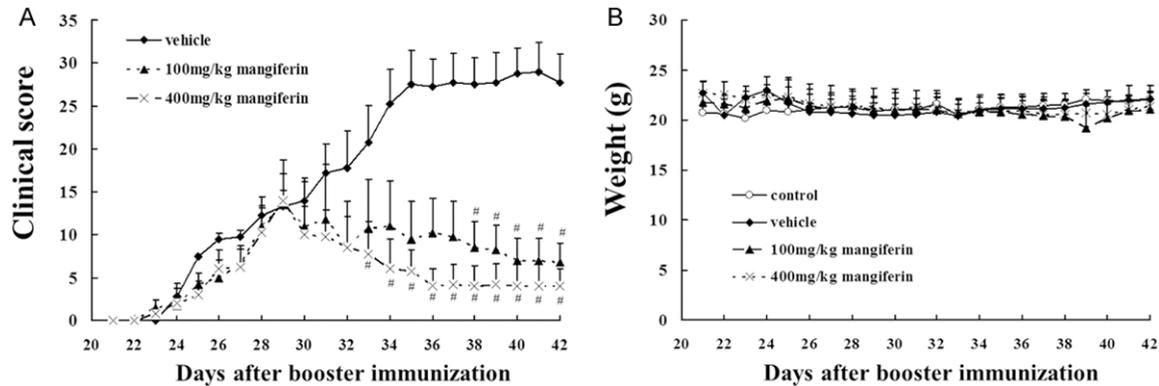


Figure 5. Effect of mangiferin on clinically evident CIA. A. Mice were treated 1 day post-onset of clinically evident articular disease for a total of 14 days either with mangiferin 100 (n = 10) and 400 mg/kg (n = 10), or PBS (n = 10). #P < 0.01 vs. vehicle (ANOVA with Dunnett's test). B. Safety of mangiferin administered *in vivo*. Mice were weighed before collagen challenge and daily for the duration of treatment. Means and S.D. are shown.

Mangiferin inhibited the NF- κ B and ERK1/2 activation in thymus and spleen of CIA mice

Signal transduction factors were studied, to determine the mechanism by which mangiferin suppressed CIA. The thymus and spleen of CIA mice showed activated ERK1/2 and NF- κ B. In spleen, activation of Akt increased in CIA mice. Mangiferin suppressed ERK1/2 and NF- κ B activation in a dose-dependent manner in thymus and spleen, while Akt activation remained unaffected in spleen (Figure 4). These results indicate that the inhibitory effect of mangiferin on CIA is exerted via inhibition of NF- κ B and ERK1/2 activation.

Inhibitory effect of mangiferin after onset of CIA

It was important to determine whether similar effects could be obtained in mice after onset of CIA. Therefore, mangiferin was administered to DBA/1 mice after CIA became clinically detectable (day 27; therapeutic protocol). Significant reduction of arthritis progression was apparent within 7 days of treatment compared with the vehicle-treated controls (Figure 5A). Disease was suppressed until at least 14 days after cessation of treatment (data not shown). In addition, mice treated with mangiferin did not exhibit any weight loss (Figure 5B). Commensurate with clinical measurements, mRNA expressions of IL-1 β , IL-6, TNF- α , and RANKL in thymus and spleen, and serum IL-1 β , IL-6, TNF- α , and RANKL levels were also significantly suppressed (Figures 6 and 7). Together, these data clearly indicate that mangiferin potently sup-

pressed both developing and after onset phases of inflammatory CIA.

Discussion

In the present study, we demonstrated that mangiferin suppresses progression and incidence of CIA by inhibiting NF- κ B and ERK1/2 activation. A previous report indicated that dehydroxymethylepoxyquinomicin (DHMEQ), an NF- κ B inhibitor, suppressed CIA [13]. An IKK inhibitor, ML120B, has been reportedly shown to inhibit antibody-induced arthritis in a murine model [14]. Fasudil inhibited arthritis via suppression of NF- κ B activation in rat adjuvant-induced arthritis model [15]. It is well reported that NF- κ B activation regulates survival and activation of T- and B-lymphocytes at their sites of development in thymus and spleen, progress development of RA [16]. FR180204, an ERK1/2 inhibitor, ameliorated the clinical arthritis and body weight loss occurring in the CIA mice [17]. In addition, PD184352, selective mitogen extracellular kinase kinase 1/2 (MEK1/2) inhibitor, inhibited paw edema and clinical arthritis scores, and levels of phosphorylated ERK1/2 in synovium were higher in RA patients than in normal individuals, suggesting that ERK1/2 activation plays an important role in RA [18]. Taken together, these findings suggest that decreased phosphorylation of ERK and NF- κ B could play an important role in the inhibition of CIA by mangiferin.

The MEK/ERK pathway and NF- κ B leads to the production of various pro-inflammatory cytokines such as TNF- α , IL-6, IL-1 β , and RANKL

Mangiferin suppresses collagen-induced arthritis

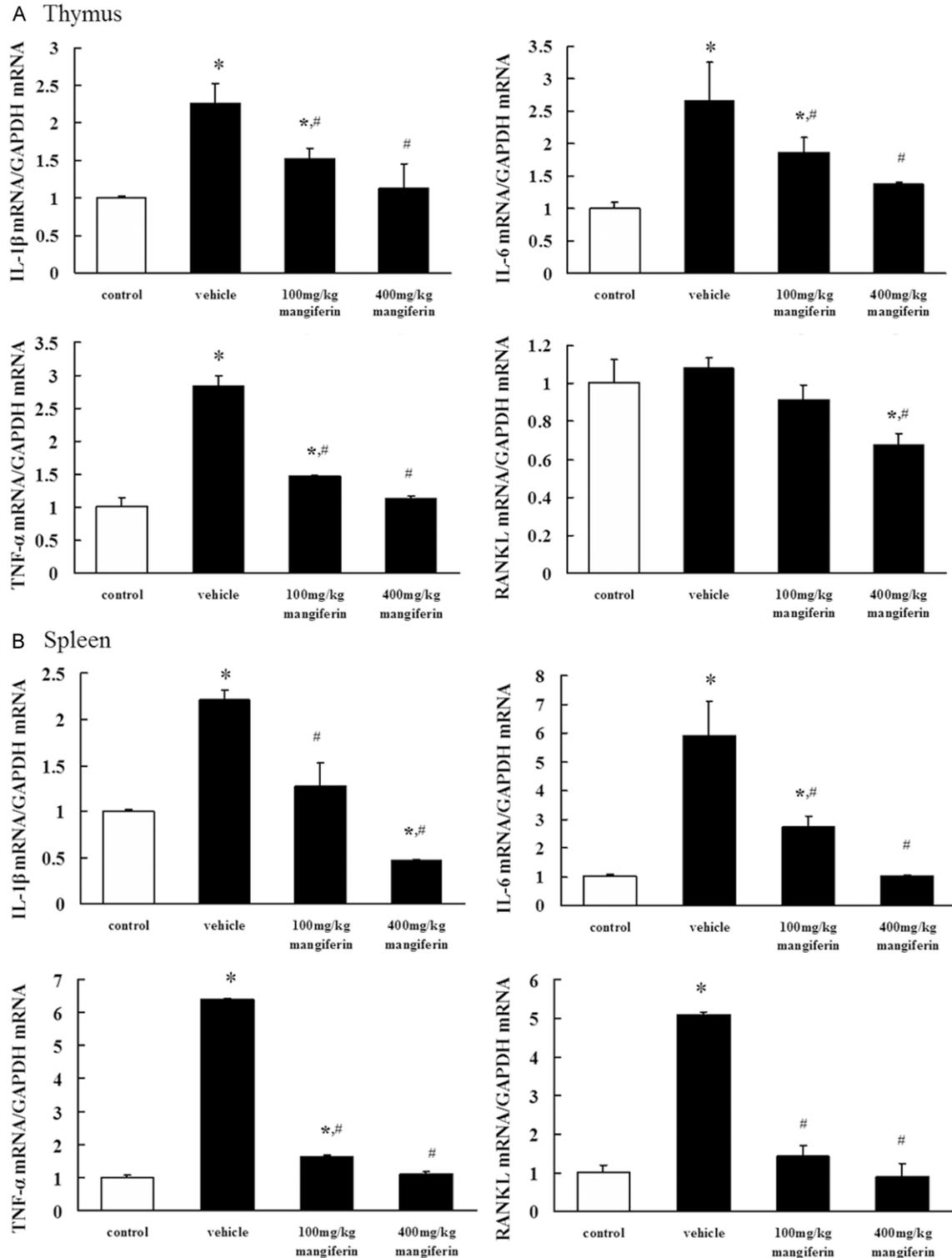


Figure 6. Effect of mangiferin treatment for therapeutic protocol on cytokine gene expression in CIA mice. Mangiferin was administered to DBA/1 mice after CIA became clinically detectable (Day 27). Total RNA was extracted from thymus (A) and spleen (B), and interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and receptor activator NF- κ B ligand (RANKL) mRNA levels were determined by real-time PCR. Samples were obtained on day 42. The results are expressed as the test:control ratio after correction of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. The results are representative of 10 independent experiments. *P < 0.01 vs. control, #P < 0.01 vs. vehicle, (ANOVA with Dunnett's test).

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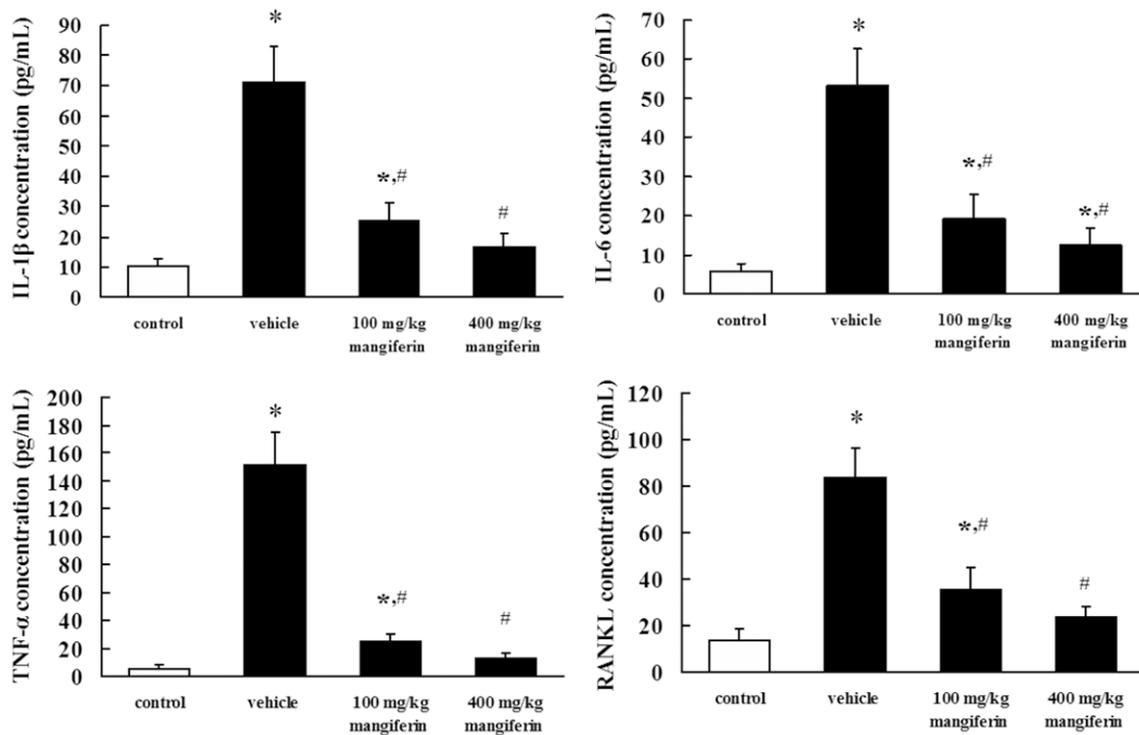


Figure 7. Mangiferin was administered to DBA/1 mice after CIA became clinically detectable (day 27). Serum titers of IL-1 β , IL-6, TNF- α , and RANKL were measured by Luminex technology or ELISA assay in mice treated with various doses of mangiferin ($n = 10$) or PBS ($n = 10$) and normal control mice ($n = 10$). Serum samples were obtained on day 42. Each serum sample was diluted with PBS. The results are representative of 10 independent experiments. * $P < 0.01$ vs. control, # $P < 0.01$ vs. vehicle.

[15, 19-23]. Role of B cells as autoantibody producers from isolated splenocytes in glucose-6-phosphate isomerase-induced arthritis has been previously reported. These autoantibodies can trigger joint inflammation in orchestration with inflammatory cytokines, especially TNF- α [24]. In addition, elevated IL-6 levels have been found in the serum and synovial fluid of RA patients, and positive correlations between IL-6 and RA have been observed [25]. It has been indicated that IL-6/soluble IL-6 receptor complexes and IL-1 β induce RANKL expression in fibroblast-like synoviocytes in RA patients, and induction of IL-6 was accelerated by TNF- α and IL-1 β [26]. Furthermore, T cells and B cells expressed RANKL suggesting that induction of osteoclast differentiation was promoted in synovium [27]. Collectively, these findings suggest that TNF- α , IL-6, IL-1 β , and RANKL expressions play an important role in the progression of RA. In this study, we found that mangiferin suppressed mRNA expressions of TNF- α , IL-6, IL-1 β , and RANKL in the thymus and spleen, and serum levels of these cytokines in CIA mice. A previous study indicated that Raf

kinase (an upstream signal molecule of MEK/ERK pathway) inhibitor protein suppressed TNF- α -induced IL-6 expression via inhibition of MEK/ERK pathway in rheumatoid fibroblast-like synoviocytes [28]. Another report showed that ARRY-162, a MEK1/2 inhibitor, inhibited IL-1 β , IL-6, and TNF- α [29]. NF- κ B inhibition by an IKK inhibitor is known to suppress the expression of IL-6, pannus formation, and cartilage and bone erosion [30]. Bortezomib, a proteasome inhibitor, suppressed the TNF- α , IL-1 β , and IL-6 production via inhibition of NF- κ B activation in activated T cells from RA patients [31]. Taken together, these results suggest that a decrease in the activation of NF- κ B and ERK1/2 plays an important role in the suppression of TNF- α , IL-6, IL-1 β , and RANKL expressions even in our experimental model.

Hence, it can be concluded that mangiferin can suppress the progression of CIA in DBA1/J mice. Importantly, treatment with mangiferin was effective even after the onset of arthritis via suppression of TNF- α , IL-6, IL-1 β , and RANKL expressions. These data indicate that

Mangiferin suppresses collagen-induced arthritis

mangiferin may hold promise for RA therapeutics.

In conclusion, we present preclinical characterization of mangiferin, providing first evidence of effective suppression of CIA. Our findings suggest that CIA is associated with the activation of NF- κ B and ERK1/2 and inhibition of these signal molecules by mangiferin effectively suppresses CIA. Moreover, our study demonstrated the anti-rheumatic effects of mangiferin through the inhibition of TNF- α , IL-6, IL-1 β , and RANKL expressions *in vivo*. Therefore, mangiferin may be therapeutically useful for preventing RA and possibly for other inflammatory and autoimmune diseases.

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Disclosure of conflict of interest

None.

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Mangiferin suppresses collagen-induced arthritis

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