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CT20126, a novel immunosuppressant, prevents collagen-induced arthritis through the down-regulation of inflammatory gene expression by inhibiting NF-κB activation

Seon-Jin Lee [a,1], *Woo-Dong Nam* [b,1], *Hee-Jun Na* [a], *Young-Lai Cho* [a], *Kwon-Soo Ha* [a], *Jong-Yun Hwang* [c], *Hansoo Lee* [d], *Soon-Ok Kim* [e], *Young-Guen Kwon* [f], *Young-Myeong Kim* [a,*]

[a] *Department of Molecular and Cellular Biochemistry and Vascular System Research Center, School of Medicine, Kangwon National University, Chunchon, Kangwon-Do 200-701, Republic of Korea*

[b] *Department of Orthopaethic Surgery, Kangwon National University Hospital, Chunchon, Kangwon-Do 200-701, Republic of Korea*

[c] *Department of Obstetrics & Gynecology, Kangwon National University Hospital, Chunchon, Kangwon-Do 200-701, Republic of Korea*

[d] *Division of Life Sciences, College of Natural Sciences, Kangwon National University, Chunchon, Kangwon-do 200-701, Republic of Korea*

[e] *Team of Biotech and Drug Development, Chem-Tech Research Incorporation, 10th Floor of Kowoon Institute, Suwon University, Hwaseoung, Kyunggi-Do 445-743, Republic of Korea*

[f] *Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Republic of Korea*

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ABSTRACT

The colchicine-derived CT20126 compound has recently been shown to exert an immune regulatory effect and prolong the survival of allograft skins. In this study, we explored the anti-inflammatory and anti-arthritic effects of CT20126 *in vivo* and *in vitro* as well as investigated its underlying action mechanism. CT20126 suppressed the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2, tumor necrosis factor-alpha, and interleukin-1beta as well as the production of nitric oxide and prostaglandin E_2 in lipopolysaccharide (LPS)-treated macrophages as well as LPS-administered mice. This drug also inhibited the production of nitric oxide, prostaglandin E_2, and the chemokines, RANTES, GROα, and ENA-78, in cytokine-stimulated human synoviocytes. CT20126 suppressed NF-κB activation and iNOS promoter activity, which correlated with its inhibitory effect on phosphorylation-dependent IκB kinase activation, IκB phosphorylation and degradation, and NF-κB nuclear translocation, in LPS-stimulated macrophages. This compound also inhibited LPS-induced NF-κB-inducing kinase (NIK) and Akt phosphorylation, which are upstream of NF-κB activation. Furthermore, CT20126 significantly decreased the incidence and severity of arthritis as well as inhibited the expression of inflammatory cytokines, chemokines, iNOS, and cyclooxygenase-2 in the paws of collagen-induced arthritic mice. These findings indicate that CT20126 exerts an anti-inflammatory effect through NF-κB-responsive inflammatory gene expression by inhibiting the NIK- and Akt-dependent canonical NF-κB pathway and can be used as a therapeutic agent for rheumatoid arthritis related to chronic inflammation.

* *Corresponding author*. Tel.: +82 33 2508831; fax: +82 33 2443286.
E-mail address: ymkim@kangwon.ac.kr (Y.-M. Kim).

[1] These authors contributed equally to this work.

Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; NIK, NF-κB-inducing kinase; IKK, IκB kinase; RA, rheumatoid arthritis; NSAIDs, non-steroidal anti-inflammatory drugs; CIA, collagen-induced arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; PBS, phosphate-buffered saline; CM, cytokine mixture.

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1. Introduction

Nuclear factor κB (NF-κB) plays a central role in the regulation of many immune and inflammatory processes involved in the pathogenesis of autoimmune and chronic inflammatory diseases including rheumatoid arthritis (RA) [1]. Examples of genes dependent on the activation of NF-κB include cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL-1β), and IL-6 as well as chemokines such as RANTES, ENA-78, and GROα [2,3]. The pro-inflammatory cytokines such as TNF-α and IL-1β induce the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), resulting in the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), respectively. Therefore, functional inhibition of biological activities of these inflammatory cytokines, chemokines, and enzymes by neutralizing antibodies, receptor antagonists or specific inhibitors can be an effective therapy for inflammatory arthritis [4,5].

NF-κB generally exists in a heterodimeric inactive form in the cytosol bound to distinct inhibitory IκB subunits. NF-κB is activated as a consequence of phosphorylation, ubiquitination, and subsequent proteolytic degradation of IκB through activation of the IκB kinase (IKK) [6]. The liberated NF-κB translocates into nuclei and binds to κB motif in the promoters of target genes, leading to the induction of many inflammation-associated genes. It has been clearly demonstrated that NF-κB is highly activated and involved in the pathogenesis of many inflammatory diseases including sepsis and RA [1,7], indicating that the regulation of NF-κB activation represents an opportunity for the development of novel therapeutics for inflammatory diseases. Many anti-inflammatory drugs, such as glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), and other immunosuppressants, which act as inhibitors of the NF-κB pathway and suppress the expression of various inflammatory genes, have been used for the treatment of inflammatory diseases including RA [8].

We previously demonstrated that the colchicine derivative CT20126 was a potent suppressant of NO, TNF-α, and IL-1β production in the macrophage cell line RAW264.7 stimulated with lipopolysaccharide (LPS) and in the sponge matrix allograft model [9]. These observations suggest that CT20126 can ameliorate chronic inflammatory diseases such as RA, probably by the suppression of NF-κB-dependent inflammatory gene expression. We here investigated the effect of CT20126 on the expression of NF-κB-dependent inflammatory cytokines, chemokines, and enzymes *in vitro* and *in vivo* as well as examined its underlying molecular mechanism. Furthermore, its pharmacological effect on the development of arthritis was examined in a mouse collagen-induced arthritis (CIA) model. Our results showed that CT20126 suppressed the expression and production of inflammatory cytokines (TNF-α and IL-1β), chemokines (RANTES, GROα, and ENA-78), and enzymes (iNOS and COX-2) through the suppression of the NF-κB signaling pathway as well as significantly attenuated the arthritic incidence and severity in a CIA model. It indicates that CT20126 may be a beneficial agent for treating chronic inflammatory arthritis.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Life Technology Inc. (Rockville, MD). Poly (dI-dC) and NF-κB-specific oligonucleotide were obtained from Promega (Madison, WI). Antibodies for IKKα (sc-7183), IKKβ (sc-34673), IKKα (sc-7183AC), IRAK1 (sc-5288), p-IκBα (sc-7977), IκBα (sc-1643), p65 (sc-372-G), NIK, iNOS (sc-7271), COX-2 (sc-1745), TNF-α (sc-1351), IL-1β (sc-1251), p-IKKα (Thr23, sc-21660), p-NIK (sc-12957-R), MyD88 (sc-8196AC), TLR4 (sc-16240), p-IKKαβ (Ser176/Ser177, sc21661S), and p-JNK (sc-12882) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Akt (#9272), p-Akt (#9271), and p-IKKαβ (Ser180/Ser181, #2681S) were obtained from Cell Signaling Technology (Beverly, MA). ELISA kits for PGE_2, TNF-α, IL-1β, RANTES, GROα, and ENA-78 as well as a TNF-α antibody (AF-410-NA) for immunohistochemistry were obtained from R&D Systems (Minneapolis, MN). Stock solution of CT20126 (10 mM, Chem Tech Research Incorporation, Whasung, Korea) was prepared in dimethyl sulfoxide/ethanol/polyethylenglycol 400/distilled water (v/v/v/v, 1/1/3/6). Other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

2.2. Cell culture

Peritoneal macrophages were harvested from female BALB/c mice (6–8 weeks old, Orient, Korea) at day 5 following intraperitoneal (i.p.) injection with 1 ml of sterile 4% thioglycollate broth (Difco Laboratories, Detroit, MI). RAW264.7 cells were obtained from the American Type Culture Collection. Human fibroblast-like synoviocytes were provided by Dr. Wan-Uk Kim (Catholic University, Seoul, Korea). All cells were cultured in DMEM containing 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum in a humidified incubator with 5% CO_2/95% air at 37 °C. Macrophages were treated with LPS (100 ng/ml) in the presence or absence of CT20126, and human fibroblast-like synoviocytes were stimulated with a cytokine mixture (CM; 10 units/ml IL-1β, 100 units/ml IFN-γ, and 25 ng/ml TNF-α) in the presence or absence of CT20126.

2.3. Animal treatment

Mice (BALB/c and DBA/1, 6–8 weeks old, Orient, Sungnam, Korea) were maintained at the specific pathogen-free housing facility of the School of Medicine, University of Kangwon National University (Chunchon, Korea). All procedures performed on these animals were in accordance with the guidelines of the University Animal Care and Use Committee. Bovine type II collagen (Chondrex, Seattle, USA), 2 mg/ml in 0.05 M acetic acid, was emulsified in an equal volume of Freund's complete adjuvant (Sigma Chemical Co.). Arthritis was induced by injecting DBA/1 mice intradermally at the base of the tail with 100 μl of this emulsion on days 0 and 21, as described previously [10]. At day 15 after primary immunization, mice were i.p. injected with CT20126 (1 mg/kg) or saline daily. The incidence and severity of arthritis were assessed by

daily physical examination. Clinical arthritis scores of four paws were evaluated using a scale of 0–3 for each paw as previously described with minor modifications, with 0 indicating normal and 1–3 indicating minimal, moderate, and severe erythema and swelling. Each limb was graded, resulting in a maximal clinical severity score of 12 per animal. The time of onset was expressed as the mean time when paw swelling score was over 3 per mouse. For some experiments, BALB/c mice were i.p. injected with CT20126 (1 mg/kg) and 4 h later i.p. injected with LPS (2 mg/kg). After 12 h of LPS injection, blood samples were collected by cardiac puncture, and serum was prepared by centrifugation at 12,000 × *g* for 30 min. All experimental groups consisted of 8–10 mice per group.

2.4. Histological and immunohistochemical analysis

At day 30 or 42 after primary immunization, paws were fixed overnight in 10% formalin, decalcified in 30% citrate-buffered formic acid for 3 days at 4 °C, dehydrated in a graded series of ethanol and xylene, and then embedded in paraffin. Thin sections (7-μm thick) were stained with H&E, and histopathologic scoring was performed under a light microscope by blinded observers. The degrees of inflammation, synovial hyperplasia, pannus formation, and bone erosion in the joints were examined by three independent observers using a standard scoring protocol [9], where the severity was scored on a scale from 0 to 3; score 0, absent; score 1, weak; score 2, moderate; score 3, severe. The expression level of TNF-α in the joints was identified by immunohistochemical staining of the paraffin section with an antibody against TNF-α. Sections were fixed in xylene for 10 min and incubated with methanol plus 3% H_2O_2 for 15 min at room temperature to inactivate endogenous peroxidase. The sections were incubated with an antibody against TNF-α for 1 h at room temperature. After washing three times with phosphate-buffered saline (PBS), the sections were incubated with an anti-rabbit IgG secondary antibody for 20 min. Sections were treated with 3,3′-diaminobenzidine (Sigma, St. Louis, MO) for 5 min. The chromogenic reaction was stopped by immersion in water. The sections were counterstained in hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted in DPX (BDH, Poole, UK).

2.5. Measurements of NO, PGE_2, cytokines, and chemokines

NO production was determined by measuring the amount of nitrite in the culture media and nitrite plus nitrate (NOx) in serum using Griess reagents and a nitrate reductase-based colorimetric assay kit [10]. The levels of PGE_2, cytokines, and chemokines were determined using ELISA kits purchased from R&D Systems (Minneapolis, MN). Tissue lysates were prepared by homogenizing the joint tissues in HEPES buffer (10 mM HEPES, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin) and then subjected to centrifugation (12,000 × *g*) at 4 °C for 10 min.

2.6. Western blot analysis

Western blot analysis was performed as previously described [10]. For measuring phosphorylation of IκB, IKK, Akt, and NIK, RAW264.7 cells were treated with LPS in the presence or absence of 10 μM CT20126 for 10–15 min. Cells were harvested, washed twice with ice-cold PBS, suspended in 10 mM Tris–HCl (pH 7.4), and lysed by three cycles of freezing and thawing. Cell extracts were obtained by centrifugation at 12,000 × *g* at 4 °C for 20 min. Cytosolic proteins (30 μg) were electrophoretically resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat-dried milk and hybridized with primary antibodies. After washing four times, the membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies. The membranes were incubated for 2 min with ECL solution and exposed to X-ray film.

2.7. IKK activity assay

Cells treated with LPS for 10 min were harvested and washed with PBS, and the pellets were resuspended in 80 μl of immunoprecipitation lysis buffer and stored on ice for 20 min before centrifugation at 14,000 × *g* at 4 °C for 20 min as described previously [11]. IKKαβ complex was immunoprecipitated by incubation for 2 h at 4 °C with polyclonal IKKα antibody (Santa Cruz, CA) bound to agarose. The immunoprecipitates were washed twice with immunoprecipitation buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM DTT, and 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 1 μg GST-IκBα fusion protein as substrate and 0.5 μCi [γ-^{32}P] ATP. Reaction mixtures were incubated for 30 min at 30 °C, and phosphorylated IκBα was determined by SDS-PAGE, followed by autoradiography.

2.8. NF-κB activation assays

NF-κB activation was determined by electromobility shift assay, nuclear translocation of the p65 subunit of NF-κB, and an iNOS promoter activity assay. The electromobility shift assay was performed as described previously [10]. Nuclear extracts (10 μg of protein), prepared from cells stimulated with LPS (100 ng/ml) in the presence or absence of CT20126 for 1 h, were incubated with a ^{32}P-labeled double stranded NF-κB-specific probe (~40,000 cpm, 5′-AGTTGAGGGGACTTTCCCAGGC-3′) in the presence or absence of a 100-fold excess of cold probe or antibody for NF-κB p65 subunit for 20 min at room temperature. Samples were resolved on native 5% polyacrylamide gel, and the gel was dried and subjected to autoradiography. Nuclear translocation of the p65 subunit of NF-κB was examined by an immunocytochemical method as described previously [11]. Briefly, cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. After washing with PBS, the slides were blocked with 3% bovine serum albumin for 1 h and incubated with goat polyclonal anti-p65 antibody for 2 h at 4 °C. The cells were washed and incubated with anti-goat IgG-FITC (1:200, Sigma) and propidium iodide (10 μg/ml, Sigma) for 1 h. After staining, the cells were mounted with mounting medium and observed by a confocal microscopy. iNOS promoter activity was measured as described in a previous method [11]. Briefly, cells were transfected with 1 μg of a murine iNOS promoter-luciferase plasmid by using Lipofectamine (Invitrogen). Cells were

stimulated with LPS in the presence or absence of CT20126 for 12 h. Cellular luciferase activity was measured after lysis.

2.9. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured macrophages and animal tissues using a Trizol reagent kit (Life Technology Inc., Rockville, MD), as described in a previous method [10]. Real-time RT-PCR analysis was carried out on the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan® One Step PCR Master Mix Reagents Kit (polynucleotide: 4309169) as recommended by the manufacturer. Primers for iNOS (cat. # Mm004408-485_ml), COX-2 (cat. # Mm00478374_ml), TNF-α (cat. # Mm00443258_ml), IL-1β (cat. # Mm0043228_ml), and GAPDH (cat. # Mm99999915_gl) were purchased from Applied Biosystems' TaqMan Gene Expression Assay. The cycling condition was programmed for the initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Quantitation of gene expression was determined using comparative threshold multiplex PCR in the same rack. GAPDH mRNA was used as endogenous control.

2.10. Co-immunoprecipitation assay

Cells treated with LPS in the presence or absence of CT20126 for 10 min were resuspended in 0.5 ml of NP-40 lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, and a protease inhibitor cocktail and stored on ice for 20 min. Immune complexes were precipitated by incubation for 4 h at 4 °C with polyclonal MyD88 antibody, which had been pre-coupled with agarose. The immune complexes were washed three times with PBS containing protease inhibitors, separated

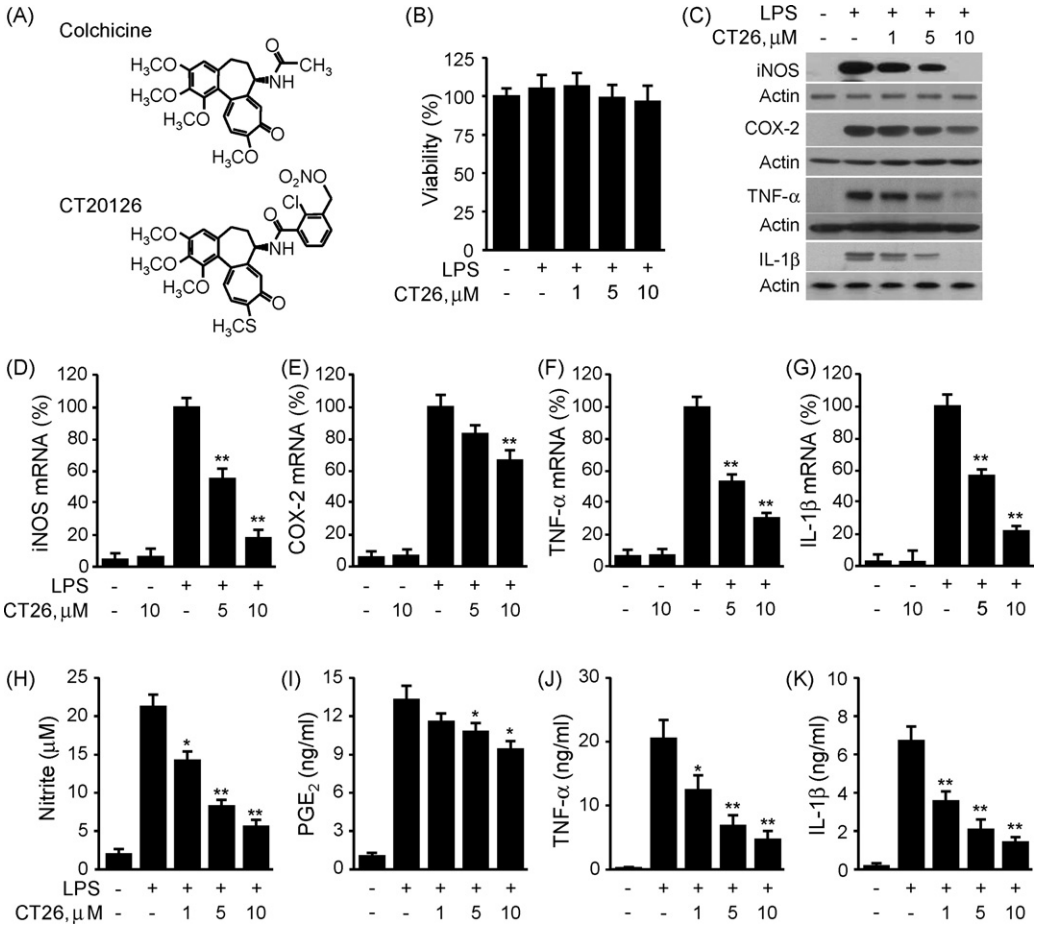

Fig. 1 – Effect of CT20126 on the expression of inflammation-related genes in mouse primary macrophages. (A) Chemical structures of colchicine and CT20126. (B) Treatment of mouse peritoneal macrophages with LPS (100 ng/ml) in the presence or absence of 0–10 μM CT20126 (CT26) for 12 h, and cell viability was determined by crystal violet staining. Total RNA and protein were obtained from peritoneal macrophages stimulated with LPS in the presence or absence of CT20126 for 8 h and 12 h using a Trizol reagent kit and three cycles of freezing and thawing, respectively. (C) The protein levels of inflammatory cytokines and enzymes were determined by Western blot analyses. The levels of iNOS (D), COX-2 (E), TNF-α (F), and IL-1β (G) mRNAs were measured by real-time RT-PCR, with the use of gene-specific TaqMan primers and a universal PCR master mixture. GAPDH was used as an internal control. The levels of nitrite (H), PGE_2 (I), TNF-α (J), and IL-1β (K) were measured in the culture medium using Griess reagents and ELISA kits following treatment with LPS in the presence or absence of different concentrations of CT20126 for 18 h. All graphic data shown are the mean ± S.D. ($n = 4$). $^{**}p < 0.01$ versus LPS alone.

by SDS-PAGE, and then transferred to a nitrocellulose membrane. Interaction between TLR4, MyD88, and IRAK1 was measured by Western blot analysis.

2.11. Statistic analysis

The data are presented as the mean ± standard deviation (S.D.) of at least three separate experiments. The two-tailed Student's *t*-test was used for statistical analysis. ANOVA was used when more than two groups of samples were compared. Statistical significance was established at a p value <0.05. The mean value of arthritis index and histological score in each treatment group is compared with that in the CIA-no treatment group by using the Mann–Whitney *U*-test.

3. Results

3.1. CT20126 inhibits NO, TNF-α, and IL-1β production, but not COX-2 production in peritoneal macrophages

We have recently showed that the colchicine-derived CT20126 potently inhibited inflammation-associated gene expression [9] in the immortalized macrophage cell line RAW264.7. We further examined the anti-inflammatory effects of CT20126 in primary mouse peritoneal macrophages stimulated with LPS. Fig. 1A shows the chemical structures of colchicine and its derivative CT20126 used in this study. Treatment of mouse peritoneal macrophages with various concentrations of CT20126 for 12 h did not alter cell viability as measured by crystal violet staining (Fig. 1B). CT20126 treatment significantly suppressed LPS-induced increases in protein and mRNA levels of iNOS, COX-2, TNF-α, and IL-1β at the transcriptional step in a dose-dependent manner, with a nearly maximum inhibitory effect at 10 μM (Fig. 1C–G). Furthermore, CT20126 treatment inhibited the secretion of NO, PGE_2, TNF-α, and IL-1β into the culture media of peritoneal macrophages stimulated with LPS (Fig. 1H–K). These results indicate that CT20126 inhibits the expression of iNOS, COX-2, TNF-α, and IL-1β at the transcriptional step in LPS-stimulated primary mouse macrophages.

3.2. CT20126 suppresses inflammatory mediator production in vivo

To examine the *in vivo* anti-inflammatory effect of CT20126, a model of LPS-induced production for inflammatory cytokines and mediators in mice was used. CT20126 was i.p. administered to mice 4 h prior to challenge with LPS and 12 h later the plasma levels of immune mediators and cytokines were

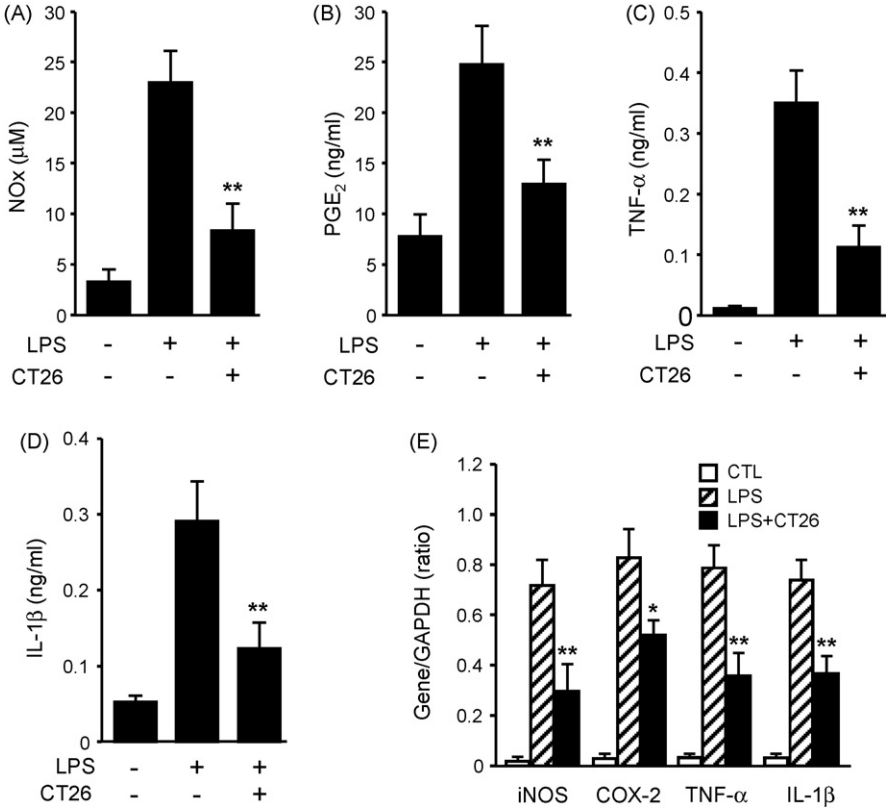

Fig. 2 – CT20126 decreases the production of inflammatory mediators *in vivo*. Mice were injected i.p. with LPS (2 mg/kg) following pretreatment with CT20126 (CT26, 1 mg/kg). After 12 h of LPS treatment, whole blood was collected by cardiac puncture and serum was obtained by centrifugation at 12,000 × *g* for 10 min. (A) Serum levels of nitrite plus nitrate (NOx) were measured using a nitrate assay kit. The levels of PGE_2 (B), TNF-α (C), and IL-1β (D) were determined by ELISA kits. (E) The mRNA levels of iNOS, COX-2, TNF-α, and IL-1β were assessed in livers isolated from mice treated with saline, LPS, and LPS + CT20126 for 6 h by real-time RT-PCR. All data shown are the mean ± S.D. ($n \geq 8$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus LPS alone.

measured. LPS-administered mice were found to have increased serum levels of NOx and PGE_2 compared with saline-treated mice, and this increase was inhibited by intraperitoneal administration of CT20126 (Fig. 2A and B). In addition, CT20126 treatment significantly suppressed LPS-induced increases in TNF-α and IL-1β *in vivo* (Fig. 2C and D). Furthermore, the mRNA levels of these inflammatory genes were significantly increased in the livers from LPS-administered mice compared with those of control mice, and these increases were effectively suppressed by administration of CT20126 (Fig. 2E). These results indicate that CT20126 possesses anti-inflammatory potential by inhibiting the *in vivo* production of these inflammatory mediators under pathologically inflammatory conditions.

3.3. *CT20126 inhibits NO, PGE_2, and chemokine production in human synoviocytes*

It has been shown that RA synoviocytes increase the expression of NF-κB-dependent inflammation-associated genes, such as iNOS, COX-2, and chemokines, resulting in cartilage destruction, proliferation and invasion of these cells into adjacent tissues, and inflammatory angiogenesis [10,12,13]. We examined whether CT20126 regulates iNOS and COX-2 expression in human fibroblast-like synoviocytes following stimulation with CM. Treatment of human fibroblast-like synoviocytes with 0–10 μM CT20126 in the presence of CM for 30 h did not show significant cytotoxicity as determined by crystal violet staining (Fig. 3A). CM treatment significantly increased iNOS and COX-2 expression as well as NO and PGE_2 production, and these increases were inhibited in a dose-dependent manner by pretreatment with CT20126 (Fig. 3B–D). Furthermore, stimulation with CM resulted in significant increases in the production of the chemokines, RANTES, GROα, and ENA-78, in the culture media as compared with the untreated control, and these increases were inhibited in a dose-dependent manner by co-treatment with CT20126 (Fig. 3E–G).

3.4. *CT20126 suppresses the signal pathway of NF-κB activation*

NF-κB is an important transcription factor for the induction of various inflammation-associated genes including iNOS, cytokines, and chemokines in response to LPS and TNF-α [1,13]. The murine iNOS promoter contains an NF-κB-binding element at -85 bp, which is a major regulatory factor for iNOS expression and NO production [14]. We examined whether CT20126 suppresses iNOS promoter activity and NF-κB activation. LPS treatment resulted in about a 10-fold increase in iNOS promoter activity, and this increase was suppressed in a dose-dependent manner by the addition of CT20126 (Fig. 4A). This compound also illustrated dose-dependent inhibition of

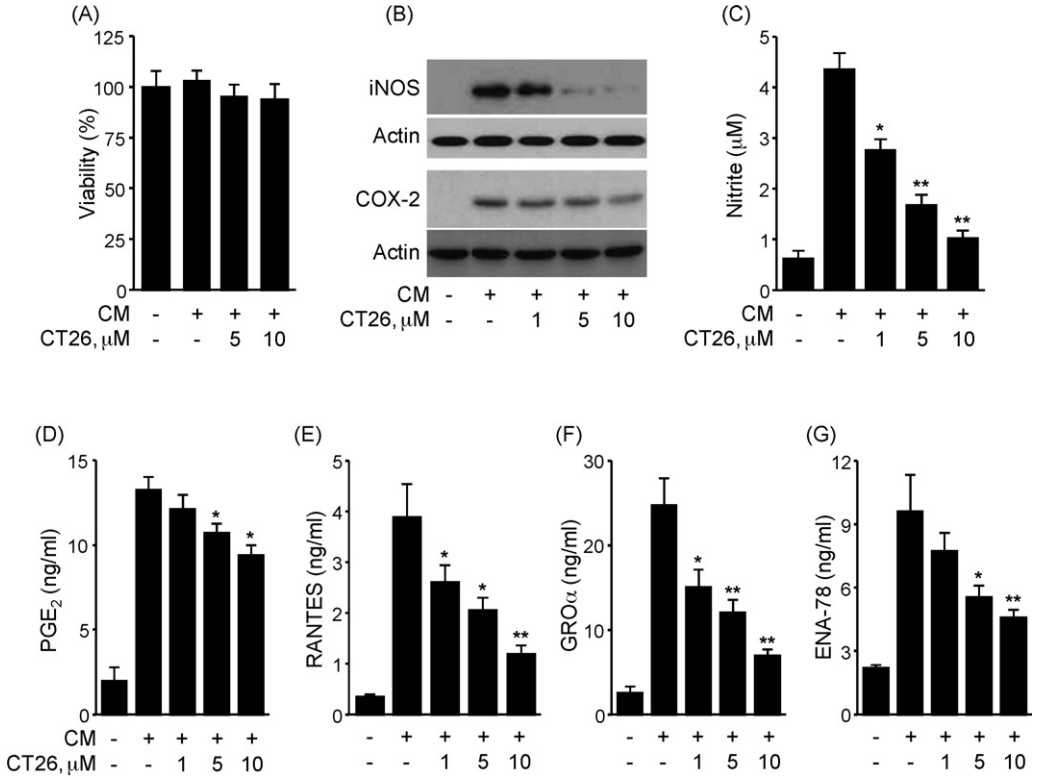

Fig. 3 – CT20126 inhibits the production of NO, PGE_2, and chemokines in CM-stimulated human synoviocytes. Human synoviocytes were stimulated with CM (10 units/ml IL-1β, 100 units/ml IFN-γ, and 25 ng/ml TNF-α) in the presence or absence of various concentrations of CT20126 for 30 h. (A) Cell viability was determined by crystal violet. (B) The protein levels of iNOS and COX-2 were determined in the lysates of synoviocytes by Western blot analyses. The levels of nitrite (C) and PGE_2 (D) were measured in the culture media by Griess reagents and ELISA, respectively. The levels of RANTES (E), GROα (F), and ENA-78 (G) were measured in the culture medium using ELISA kits. All data are presented as the mean ± S.D. ($n = 4$). $^{*}p < 0.05$; $^{**}p < 0.01$ versus CM alone.

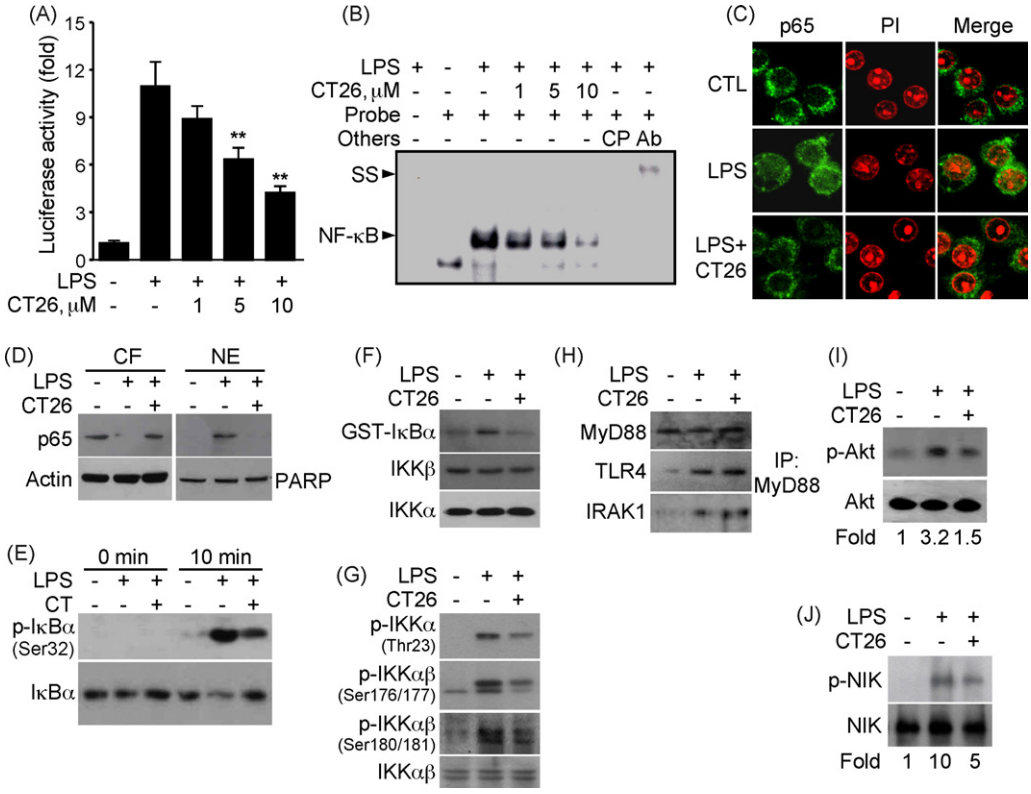

Fig. 4 – CT20126 inhibits NF-κB activation. (A) RAW264.7 cells were transfected with a murine iNOS promoter-luciferase construct by the liposome method. Cells were treated with LPS in the presence or absence of 10 μM or various concentrations of CT20126 for 12 h. Luciferase activity was measured by a luminometer. Data are presented as the mean ± S.D. ($n = 3$). $^{**}p < 0.01$ versus LPS alone. (B) NF-κB activation was determined in the nuclear extracts from RAW264.7 cells treated with or without CT20126 in the presence of LPS for 1 h by electromobility gel shift assay. Specific binding of NF-κB to DNA was determined by competition with a 100-fold excess of cold probe (CP) and supershift (SS) using an antibody for the NF-κB p65 subunit (Ab). (C) Nuclear translocation of NF-κB was determined by immunohistochemistry using an antibody for the NF-κB p65 subunit. (D) Cytosolic fractions (CF) and nuclear extracts (NE) were prepared from the cells treated with LPS in the presence or absence of CT20126 for 30 min. The NF-κB p65 levels were determined by Western blot analysis. (E) IκBα phosphorylation was determined in the whole cell lysates by Western blot analysis. (F) IKK activity was measured as described in the section of Materials and Methods. (G) Phosphorylation of IKKαβ was determined by Western blot using phosphor-specific antibodies for IKKs. (H) The complex of TLR4/MyD88/IRAK1 was immunoprecipitated using a polyclonal MyD88 antibody, and interaction between TLR4, MyD88, and IRAK1 was measured by Western blot analysis. Akt phosphorylation (I) and NIK phosphorylation (J) were determined by Western blot analyses.

NF-κB-DNA binding activity in the nuclear extract from LPS-stimulated macrophages compared with control cells (Fig. 4B). The specific interaction between DNA and NF-κB was demonstrated by a competitive assay with an excess of cold probe and a supershift analysis of NF-κB using an antibody for the NF-κB p65 subunit. We next examined whether CT20126 regulates the nuclear translocation of NF-κB as well as phosphorylation and proteolytic degradation of IκBα [6,15,16]. Immunocytochemical analysis showed that the cytosolic NF-κB subunit p65 significantly translocated to the nucleus in LPS-treated macrophages compared with control cells, and this event was suppressed by CT20126 treatment (Fig. 4C). Western blot analysis further confirmed that LPS treatment decreased the cytosolic p65 subunit level, resulting in an increase in the nuclear p65 level, while this subunit was mostly present in the cytosol in control cells, and these events were suppressed by CT20126 treatment (Fig. 4D). In addition, CT20126 significantly inhibited the phosphorylation and proteolytic degradation of IκBα in macrophages stimulated with LPS for 10 min compared with control (Fig. 4E). We next tested the effect of CT20126 on IKK activation and activity, which are responsible for IκB phosphorylation. CT20126 significantly suppressed LPS-induced IKKα activity (Fig. 4F) as well as phosphorylation of IKKα (Thr23) and IKKαβ (Ser176/177 and Ser180/Ser181) (Fig. 4G). However, this compound did not affect the apical signalosome formation of TLR4/MyD88/IRAK1 in LPS-stimulated macrophages (Fig. 4H). Recent studies have shown that Akt and NIK are involved in the canonical NF-κB pathway in response to LPS and TNF-α [16–18]. We next examined the effects of CT20126 on Akt and NIK activation. This compound significantly blocked LPS-induced phosphorylation of Akt and NIK (Fig. 4I and J). Theses data suggest that

CT20126 can regulate the IKK-dependent canonical pathway of NF-κB activation.

3.5. CT20126 reduces the incidence and severity of CIA

Since CT20126 inhibited NF-κB-dependent inflammatory gene expression, which is important for the pathogenic process of RA [7,10], we further evaluated the effect of CT20126 on the development of CIA in DBA/1 mice immunized with collagen and monitored for the appearance of clinical signs of arthritis. Mice initially developed an onset of CIA at day 28 after the first immunization of collagen, and the incidence of arthritis rapidly increased and reached 100% by day 34 (Fig. 5A). This development was reduced to 40% by daily administration of CT20126 from day 15 after the first immunization. CT20126 administration significantly decreased arthritic severity compared with control CIA mice (Fig. 5B). In addition, histopathologic analysis was performed on the paws harvested from mice with CIA receiving CT20126 or saline in the preventive and therapeutic experiments. Representative histologic sections of the ankle joints from normal, CIA, and CT20126-treated CIA mice are shown in Fig. 5C. Histopathologic examination revealed marked increases in the scores of inflammation, synovial hyperplasia, pannus formation, and bone erosion in the joints of CIA mice, and these pathological events were significantly reduced in CT20126-treated mice (Fig. 5D).

3.6. CT20126 inhibits the production of inflammatory mediators in CIA mice

We next examined the expression levels of inflammatory enzymes, cytokines, and chemokines in the paws of CIA mice. Consistent with previous reports [10], high levels of iNOS, COX-2, TNF-α, and IL-1β mRNAs were observed in the joint tissues of CIA mice, and administration of CT20126 significantly inhibited the expression levels of these genes (Fig. 6A). CT20126 treatment suppressed the protein levels of TNF-α and IL-1β in the lysates of joint tissues compared with control mice (Fig. 6B and C). The elevated level of PGE_2 in the joint tissues of CIA mice was also reduced by CT20126 treatment (Fig. 6D). Moreover, the elevated level of RANTES production in the joints of CIA mice was significantly suppressed by CT20126 treatment (Fig. 6E). In addition, immunohistochemical analysis showed a similar suppressive effect of CT20126 on TNF-α expression in the joint tissues of CIA mice (Fig. 6F). These results suggest that CT20126 can inhibit the production of NF-κB-dependent inflammatory mediators in rheumatoid arthritis.

4. Discussion

This study was undertaken to elucidate the potential effect and molecular mechanism of CT20126, a new immune

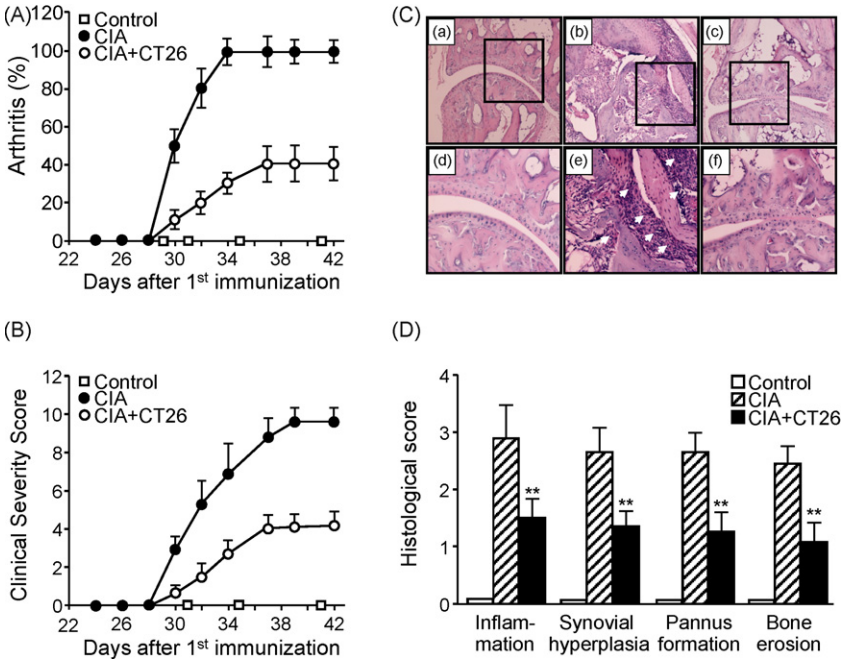

Fig. 5 – CT20126 suppresses collagen-induced arthritis. DBA/1 mice were immunized with type II bovine collagen at the base of the tail on days 0 and 21, as described in the section of Materials and Methods. At day 15 after the first immunization, mice were injected i.p. with saline or 1 mg/kg CT20126 daily. (A) Percentage of mice that developed arthritis and (B) the arthritis index of CIA were determined. Data shown in A and B are presented as the mean ± S.D. from 10 animals per group. (C) Histological features of representative ankle joints harvested from control DBA/1 mice (a) and collagen-induced arthritis mice treated with PBS (b) and CT20126 (c) at day 40 after primary immunization. High-power view of the boxed area (bottom; d, e, and f) showed the infiltration of immune cells. (D) Cellular infiltration, synovial hyperplasia, pannus formation, and bone erosion were determined in the joint ankles of CIA mice treated with or without CT20126. Data are presented as mean ± S.D. from 10 animals per group. $^{**}p < 0.01$ versus CIA.

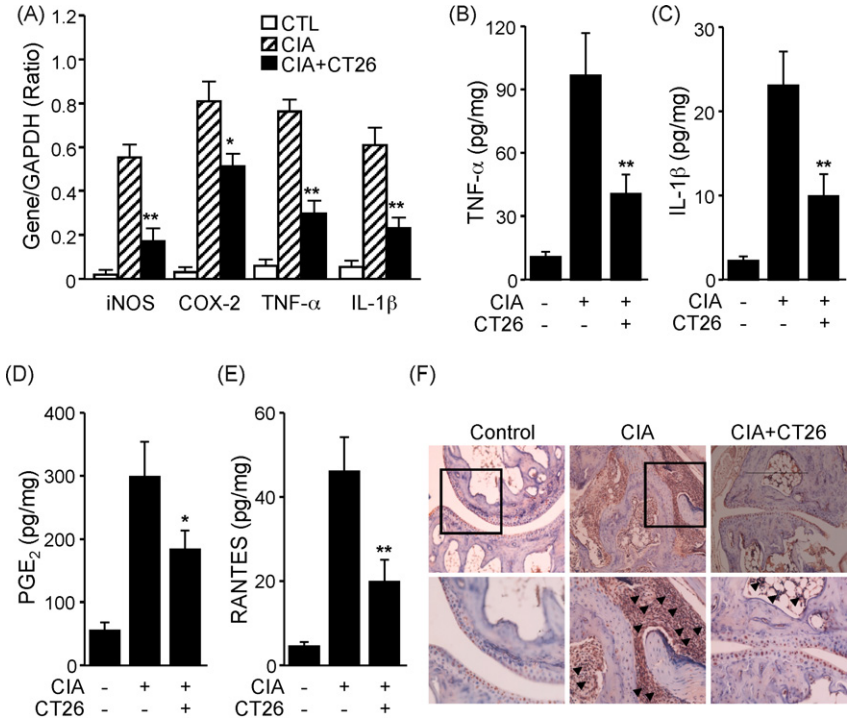

Fig. 6 – CT20126 suppresses inflammatory gene expression and chemokine production in the joints of CIA mice. (A) The mRNA levels of iNOS, COX-2, TNF-α, and IL-1β were assessed in the hind paws from mice at day 30 after the first immunization by real-time RT-PCR. The levels of TNF-α (B), IL-1β (C), PGE_2 (D), and RANTES (E) were determined in the tissue lysates of the joint ankles from normal and CIA mice using ELISA kits. (F) TNF-α expression was determined in the paraffin sections of the joint ankles from normal and CIA mice by immunohistochemistry. Upper pictures are representative histological features of ankle joints harvested from control and CIA mice, and bottom pictures are high-power view of the boxed areas of the upper panel showed the expression level of TNF-α. All graphic data shown are presented as the mean ± S.D. ($n \geq 6$). $^{*}p < 0.05$; $^{**}p < 0.01$ versus CIA.

regulatory drug derived from colchicine, on the production of pro-inflammatory cytokines, chemokines, and mediators *in vitro* and *in vivo* and to determine its anti-arthritic activity in CIA mice, a surrogate model for human RA. We found that CT20126 inhibited the expression iNOS, COX-2, cytokines (TNF-α and IL-1β), and chemokines (RANTES, GROα, and ENA-78), which are NF-κB-regulated inflammation-associated genes, in immune-activated primary macrophages and human synoviocytes *in vitro* as well as *in vivo* animal models. This compound blocked NF-κB activation by inhibiting IKK activation via the suppression of NIK and Akt activation. Furthermore, CT20126 ameliorated the pathogenic development of CIA in DBA/1 mice with the suppression of inflammatory gene expression in the paws of CIA mice. These data indicate that CT20126 possesses a potent anti-arthritic and anti-endotoxemic activity by suppressing NF-κB-dependent inflammatory gene expression.

As therapeutic agents, NSAIDs are clinically common as an inhibitor of NF-κB-dependent expression inflammatory genes such as TNF-α and IL-1β, which are critically involved in the pathogenesis of RA [8,19], but they have been shown to cause side effects in the gastrointestinal tract and other body systems [19]. Recently, TNF-α-neutralizing antibody and IL-1 receptor antagonist have been shown to provide sustained clinical benefits [4,20]. These biological agents also have adverse effects including serious infection [21]. Therefore, new and safe immunosuppressive drugs without side effects are needed to manage inflammatory disorders such as RA. Our previous study showed that CT20126 possessed immune regulatory activity for cytokine production in RAW264.7 cells, which is nearly comparable to that of the well-known anti-inflammatory drug cyclosporine A *in vitro* and *in vivo* [9]. We here also found that CT20126 blocked the expression of NF-κB-dependent pro-inflammatory genes, iNOS, COX-2, TNF-α and IL-1β, in LPS-stimulated primary mouse macrophages and cytokine-activated synoviocytes as well as in the joints of collagen-induced arthritic mice. In addition, CT20126 did not show hepatotoxicity in collagen-induced arthritic mice as judged by measuring serum aspartate aminotransferase activity and liver histology (data not shown). These results indicate that CT20126 can be used for a therapeutic anti-inflammatory drug without liver toxicity.

At inflammatory sites of RA, pro-inflammatory cytokines such as TNF-α and IL-1β are produced by immune-activated macrophages and exert various actions during the process in inflammation through the activation of the NF-κB signal pathway [4,7,19]. In addition, iNOS expression is significantly increased in macrophages, chondrocytes, and synoviocytes in a NF-κB-dependent manner, resulting in the marked elevation of NO in synovial fluid or serum from patients with RA [22]. NO

plays a regulatory role in the activation of metal-dependent proteases (collagenase, stromelysin, and COX) and the production of toxic peroxinitrite [23,24], resulting in cartilage catabolism and chronic synovial inflammation. The suppression of iNOS expression and NO production ameliorated pathogenesis or the development of inflammatory arthritis and decreased arthritis severity [25,26]. There are also many supporting evidences that COX-2 induction and PGE_2 production play an important role in the pathogenesis of RA [27,28]. We here showed that CT20126 inhibited the expression of the NF-κB-dependent genes iNOS, COX-2, TNF-α, and IL-1β in immune-activated macrophages and human synoviocytes as well as in the inflamed joints of CIA mice.

The role of NF-κB in regulating COX-2 expression is ambiguous, although NF-κB activation is importantly involved in COX-2 induction [29], several studies failed to confirm its critical importance [28,30,31]. Indeed, a study to analyze the function of *cis*-acting elements such as, CREB, C/EBPβ, and NF-κB using mutant COX-2 promoters has clearly demonstrated that mutant CREB or C/EBPβ dramatically reduced LPS-induced promoter activity, but a mutation in the NF-κB site inhibited about 30% of COX-2 promoter activity [28,30]. It indicates that NF-κB is much less important in inducing COX-2 expression in LPS-stimulated macrophages compared with the other two elements. Although CT20126 inhibited the NF-κB signal pathway, this compound did not block phosphorylation-dependent activation of JNK (responsible for C/EBPβ activation) and CREB in LPS-stimulated macrophages (data not shown), indicating that this compound specifically inhibits NF-κB activation, but not C/EBPβ and CREB activation. Therefore, CT20126 showed slightly lower inhibition on COX-2 expression than other genes, such as iNOS, TNF-α, and IL-1β, which are critically dependent on NF-κB activation, in both *in vitro* and *in vivo* conditions. These results indicate that CT20126 possesses anti-inflammatory activity with potential specificity for suppressing NF-κB-dependent pro-inflammatory gene expression responsible for the pathogenesis of arthritis in collagen-treated mice.

The pro-inflammatory cytokines TNF-α and IL-1β induce the expression of chemokines such as RANTES, ENA-78, and GROα in synoviocytes and macrophages by activating NF-κB [13], and elevated levels of these chemokines are also detected in RA synovial fluids [32]. Indeed, treatment with receptor antagonists for these chemokines prevented the onset of arthritis as observed by joint swelling and histological evaluation of the joints [33–35]. These observations indicate that the production of chemokines plays an important role in trafficking leukocytes and lymphocytes to the RA synovium and promotes inflammatory processes. A recent study has shown that the potent NF-κB inhibitors, epigallocatechin-3-gallate and pyrrolidine dithiocarbamate, suppressed the production of these chemokines in synoviocytes stimulated with IL-1β [13]. Our data showed that CT20126 inhibited the production of these chemokines in cytokine-stimulated human synoviocytes and joint tissues of CIA mice as well as significantly improved the clinical symptoms of arthritis, similar to previous studies using treatment with neutralizing antibodies or receptor antagonists against GROα and RANTES in animal models [33–35].

Fischer et al demonstrated that although NIK cannot directly phosphorylate IκBα *in vitro* [36], but appears to be a functionally important subunit for the NF-κB pathway, because mutated NIK inhibited NF-κB activation in TNF-α- or LPS-stimulated monocytes more effectively than mutated IKKα or IKKβ [17]. More recent data also demonstrated that NIK is an important factor for the activation of the canonical NF-κB activation pathway using techniques of transient gene transfer and siRNA of NIK [15,16,37,38], indicating that NIK participates in signaling events in the activation of both the canonical and noncanonical pathways. In addition, Akt is involved in NF-κB activation in TNF-α- and LPS-stimulated cells by the phosphorylation of IKKα [15,39], which promotes IKKβ phosphorylation in a reciprocal activation mode, supporting an important role of Akt in canonical NF-κB activation pathway. Therefore, both NIK and Akt have been implicated in the canonical NF-κB pathway by converging on IKK to promote NF-κB activation at two sites: IKKα at threonine 23 (Akt-mediated) and IKKαβ at serines 176/177 (NIK-mediated) [15,16,36–39]. NIK can also directly activate IKKαβ via the phosphorylation at serines 180/181 [40]. Therefore, these events promote the phosphorylation and proteolytic degradation of IκB. Our data showed that CT20126 effectively suppressed phosphorylation-dependent activation of NIK and Akt, phosphorylation of IKKα at threonine 23, and phosphorylation of IKKαβ at serines 176/177 and serines 180/181 in LPS-stimulated macrophages. These results indicate that this compound can regulate the canonical NF-κB activation pathway by inhibiting Akt and NIK activation.

Colchicine has been well known to inhibit microtubule polymerization, which can play a role of movement of cytosolic molecules to the nucleic or cytoplasmic membrane [41,42]. Because CT20216 is a derivative of colchicine, it is possible that this compound inhibits NF-κB activation by perturbing the movement of signaling molecules to signalosomes or the cytoplasmic membrane via the inhibition of microtubule organization. We found that CT20216 did not significantly affect microtubule organization and cell migration compared with colchicine as determined by immunofluorescence microscopy and Boyden chamber assay (data not shown). These different effects may be due to modification of the chemical structure. It suggests that CT20216 can not regulate microtubule-dependent movement of cytosolic molecules to their target sites such as the cytoplasmic membrane, signalosome or nuclei. Indeed, our results showed that this compound did not inhibit apical signalosome formation via recruitment of the cytosolic components, MyD88 and IRAK1, to the cytoplasmic domain of TLR4 in LPS-stimulated macrophages. These data indicate that the inhibitory effect of CT20216 on NF-κB is not associated with the inhibition of microtubule polymerization.

Activated IKK promotes the dissociation of the cytosolic inactive NF-κB-IκB complexes via the serine phosphorylation and degradation of IκB, leading to NF-κB translocation to the nucleus and transcriptional up-regulation of inflammatory genes. Thus, the abnormal, constitutive activation of NF-κB is associated with a number of chronic inflammatory arthritis [19]. These evidences indicate that an inhibitor of NF-κB would be useful in the treatment of inflammatory diseases including RA [8]. In the present study, CT20126 was found to inhibit the NF-κB signal pathway by blocking phosphorylation, ubiquitination, and proteosomal degradation of IκB as well as

subsequent translocation of the cytosolic NF-κB p65 subunit to the nucleus through the suppression of NIK- and Akt-dependent IKK activation. The inhibitory effect of CT20126 on the NF-κB signal pathway could be critically associated with the suppression of inflammatory genes in the synovium of CIA mice, resulting in the amelioration of arthritic clinical symptoms.

Adoptive immune responses of T cells, dendritic cells, and B cells are also important in the pathogenesis of RA by producing cytokines and chemokines. Although the regulatory effect of CT20126 on adaptive inflammatory response was not determined in this CIA animal model, our previous study showed that this compound can suppress Th1-related pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-2, with minimal suppression of Th2-related anti-inflammatory cytokines IL-4 and IL-10 in the sponge matrix allograft model [9]. These evidences suggest that the regulation of an adoptive response by CT20126 can be also involved in its anti-arthritic effect. This possibility is currently under investigation.

In conclusion, this study demonstrated that the new, small anti-inflammatory compound CT20126 inhibits the NF-κB activation pathway by blocking NIK- and Akt-dependent IKK activation and subsequently inhibits the transcriptional expression of inflammatory genes such as iNOS, cytokines, and chemokines, resulting in the amelioration of arthritic incidence and severity in CIA. These results, therefore, suggest that CT20126 may be useful as a potential therapeutic drug for RA.

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