The Role of Proinflammatory Cytokines in Joint Tissues During Development of Arthritis

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Abstract

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by chronic inflammation of joints, leading to synovial hyperplasia, infiltration of leukocytes and progressive destruction of cartilage and bone. Proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1 and IL-6, contribute to the development of RA by induction of various inflammatory mediators and recruitment, differentiation and proliferation of inflammatory cells. However, the role of proinflammatory cytokines in joint tissues during development of RA is poorly understood. To elucidate this, I focused on how the expression and function of the proinflammatory cytokines in joint tissues are regulated in mouse collagen-induced arthritis (CIA).

Collagen-induced arthritis is known to be accelerated by lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria. LPS-accelerated CIA (LPS-CIA) may have characteristics of some pathogenetic mechanisms similar with RA because systemic infections cause acute exacerbations of RA. To uncover the pathogenetic mechanisms of LPS-CIA, I analyzed the sequence and timing of the production of inflammatory mediators and anti-type II collagen (CII) antibodies. LPS-CIA is accompanied by acute onset and histopathological changes that are characterized in RA. LPS-CIA mice showed marked increases in expression levels of mRNA of inflammatory mediators, such as TNFα, in their arthritic paws and of serum anti-CII antibody concentration after LPS injection. Moreover, anti-TNFα neutralizing antibodies inhibited the development of LPS-CIA and a single injection of recombinant mouse TNFα induced increases in anti-CII antibody concentrations, suggesting TNFα may contribute to the development of arthritis by both initiation of inflammation and production of autoantibodies. These data suggest that exacerbation of CIA by LPS is associated with rapid production of proinflammatory cytokines and anti-CII antibodies.

Most proinflammatory cytokines associated with RA are regulated by the nuclear factor-κB (NF-κB). NF-κB is activated by a variety of inflammatory stimuli, such as TNFα and LPS, mediated by IκB kinase beta (IKKβ). To elucidate the role of NF-κB in regulation and function of proinflammatory cytokines in joint tissues, I investigated the mechanisms of the anti-arthritic effect of a novel IKKβ inhibitor Compound D. Compound D selectively inhibited IKKβ kinase activity and blocked the NF-κB signaling pathway. In addition, Compound D inhibited NF-κB-driven production of proinflammatory cytokines *in vitro* and *in vivo*. Furthermore, Compound D distributed to the arthritic paws compared to healthy paws, where it downregulated proinflammatory cytokines. Moreover, Compound D completely inhibited arthritis progression even when treatment occurred after disease onset. These data suggest that NF-κB-dependent proinflammatory cytokines in local joint tissues play an important role in the development of CIA.

In conclusion, I demonstrated that the NF-κB-dependent production of proinflammatory cytokines in local joint tissues amplify inflammation and develop arthritis.

Abbreviations

AP-1, activator protein-1

BSA, bovine serum albumin

CRE, cAMP response element

CIA, collagen-induced arthritis

CII, type II collagen

E. coli, Escherichia coli

EDTA, ethylenediaminetetraacetic acid

FBS, fetal bovine serum

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IC_{50}, 50% inhibitory concentration

IκB, inhibitor of nuclear factor-κB

IKK, IκB kinase

IL, interleukin

ISRE, interferon-stimulated response element

LPS, lipopolysaccharide

LPS-CIA, lipopolysaccharide-accelerated collagen-induced arthritis

MIP-2, macrophage inflammatory protein-2

MMP, matrix metalloproteinase

ND, not detected

NS, not significant

NFAT, nuclear factor of activated T cells

NF-κB, nuclear factor-κB

PBS, phosphate buffered saline

PCR, polymerase chain reaction

RA, rheumatoid arthritis

RANKL, receptor activator of nuclear factor-κB ligand

SD, standard deviation

SE, standard error

SRE, serum response element

TLR, Toll-like receptor

TNFα, tumor necrosis factor alpha

General Introduction

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that affects approximately 1% of the population worldwide. The disease is primarily manifested as joint inflammation characterized by synovitis. Histopathological features in the joints are synovial hyperplasia, infiltration of leukocytes and progressive destruction of cartilage and bone (Firestein, 2003). The joint inflammation results in pain, swelling and stiffness, which lead to loss of function of the joints.

Development of RA is thought to occur in a complex process associated with autoimmunity and inflammation. RA is understood to be an autoimmune disease initiated by autoantibodies. Rheumatoid factor is the classic autoantibody in RA (Scott et al., 2010). The autoantibodies activate complement systems by formation of immune complexes in combination with self-antigens and induce recruitment of inflammatory cells into the synovium (Firestein, 2003; Nandakumar and Holmdahl, 2006). Synovitis in RA patients is characterized by an infiltrate of inflammatory cells, including macrophages and $CD4^+$ T cells. Macrophages are activated partly by immune complexes binding to Fcγ receptors and complement receptors on their surface. In addition, macrophages present arthritogenic antigen to $CD4^+$ T cells owing to contacts between T cell receptors and major histocompatibility complex molecules, leading to enhanced T cell reactivity. B cells are activated by stimulation from T cells followed by further production of autoantibodies leading to immune complex formation (Firestein, 2003). In the inflammatory joint tissues, the immune complexes and invasive inflammatory cells induce proliferation of synovial cells, which results in synovial hyperplasia and subsequent pannus formation. Synovial cells in the region secrete various mediators, which contribute to cartilage matrix degradation and bone erosion (McInnes and Schett, 2011; Redlich and Smolen, 2012).

Inflammatory mediators, such as cytokines, chemokines and proteases, play an

important role in inducing inflammation and tissue destruction (Godessart and Kunkel, 2001; Goldring, 2002; Firestein, 2003; Brown et al., 2008; Goldring and Marcu, 2009). These inflammatory mediators are detectable in high concentrations in synovial fluid or serum of RA patients (Eastgate et al., 1988; Houssiau et al., 1988; Tetta et al., 1990; Troughton et al., 1996; Yoshihara et al., 2000; Ziolkowska et al., 2000; Kraan et al., 2001; Tchetverikov et al., 2004). In particular, proinflammatory cytokines including tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6 and IL-17 contribute to the development of RA (Firestein, 2003; Brown et al., 2008). These cytokines induce recruitment, differentiation, and proliferation of inflammatory cells and amplify inflammation by further production of various inflammatory mediators (Choy and Panayi, 2001; Firestein, 2003; Brown et al., 2008). Therefore, proinflammatory cytokines are believed to be promising targets for RA therapeutics. Supporting this idea, some biologics targeting proinflammatory cytokines have improved the effectiveness of RA therapy (Taylor and Feldmann, 2009). Thus, the proinflammatory cytokines may be a key factor in the development of arthritis.

As described above, various factors, such as proinflammatory cytokines, are involved in the development of arthritis. Based on the pathology, several therapeutics targeting immune response and inflammatory processes were developed and expanded treatment options; however, some patients with RA remain unresponsive to the therapy. Furthermore, some patients have acute onset and exacerbations of the disease. So far, the pathogenic mechanisms underlying these cases are poorly understood. Thus, RA pathology is complex and has not yet been fully elucidated. In particular, the regulation of the expression and function of the proinflammatory cytokines in the joint tissues, which are the primary target tissue of RA, is not clarified.

In my present study, in order to elucidate the role of proinflammatory cytokines in

joint tissues during development of arthritis, I analyzed the mechanism of lipopolysaccharide (LPS)-induced exacerbation of arthritis and the role of nuclear factor-κB (NF-κB) in regulation of expression of proinflammatory cytokines in joint tissues using the mouse collagen-induced arthritis (CIA) model.

Chapter I

"Mechanism of LPS-induced exacerbation of arthritis"

Introduction

It is well known that some patients with RA have acute onset and exacerbations of their disease. Microbial infections are thought to be one of the triggers for onset and exacerbation of autoimmune diseases, including RA (Carty et al., 2003; Leirisalo-Repo, 2005; Getts and Miller, 2010). A number of studies suggest a possible role for LPS, a major component of the outer membrane of gram-negative bacteria, in RA. The serum and synovial fluids of RA patients contain higher concentrations of antibodies against *Escherichia coli* (*E. coli*) or LPS-binding protein than do those of healthy subjects (Heumann et al., 1995; Aoki et al., 1996).

CIA is a common animal model with similarities to RA in both cause and pathology. After immunization of DBA/1J mice with type II collagen (CII) in complete Freund's adjuvant, synovial hyperplasia, infiltration of inflammatory cells and destruction of cartilage and bone occur in the joint tissue of both CIA mice and humans with RA (Williams, 2007). In addition, an autoantibody, namely an anti-CII antibody, triggers CIA (Cho et al., 2007). Injection of a cocktail with monoclonal anti-CII antibodies induces arthritis in mice (Holmdahl et al., 1990; Terato et al., 1992). Furthermore, proinflammatory cytokines play important roles in the pathogenetic process of CIA. Antibodies against TNFα, IL-1β and IL-6 are effective against CIA (Williams et al., 1992; Takagi et al., 1998; Williams et al., 2000). It is well known that CIA is accelerated by LPS (Caccese et al., 1992; Terato et al., 1995; Terato et al., 1996; Takahashi et al., 1999). Caccese et al. have demonstrated that LPS induces acute onset of inflammation after LPS injection in CII-immunized mice, in contrast with unmodified CIA, which develops slowly (Caccese et al., 1992). Moreover, an administration of LPS reactivates paw inflammation in CIA mice (Yoshino et al., 1999; Yoshino and Ohsawa,

2000). LPS-accelerated CIA (LPS-CIA) is more useful than CIA alone for screening of antirheumatic drugs because it enables not only shortening the duration of experiments but also more accurate evaluation of incidence and severity (Caccese et al., 1992). On the other hand, these findings suggest that LPS plays a role in the exacerbation of RA and the characteristics of some pathogenetic mechanisms are similar in LPS-CIA and RA. Therefore, LPS-CIA is a valuable disease model for investigating the acute phase of RA. However, few studies have analyzed the pathogenetic mechanisms of LPS-CIA. In particular, there are no detailed analyses focusing on the period before the onset of arthritis.

The purpose of this chapter is to uncover the mechanism of LPS-induced exacerbation of arthritis. In order to achieve the purpose, I analyzed the pathology from the perspective of the sequence and timing of the production of inflammatory mediators and anti-CII antibodies and related this to histopathological findings.

Materials and Methods

Animals

Female DBA/1JNCrj mice were purchased from Charles River (Tokyo, Japan). All mice were used at the age of 7-10 weeks. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Induction and assessment of arthritis in mice

The mice were immunized by intradermal injections at the base of the tail with an emulsion containing 150 μg of bovine CII (Collagen Gijutsu-Kenshukai, Tokyo, Japan) in Freund's complete adjuvant containing *Mycobacterium butyricum* (Difco Laboratories, Detroit, USA). After 17-21 days, the mice were injected subcutaneously with 0.2 mg/kg of LPS of *E. coli* O111:B4 (Difco Laboratories) or intravenously with 2, 20 and 200 ng/kg of recombinant mouse TNFα (Genzyme, Boston, USA) in place of LPS. Anti-TNFα neutralizing antibodies and control IgG were purified from the ascites of mice injected with hybridoma (MP6-XT22 and Y13-259, respectively). The antibodies (25 mg/kg) were intraperitoneally administered once daily from the day before to 6 days after LPS injection. The severity of arthritis of each paw was scored periodically on a scale of 0-3 according to the following criteria: 0, normal; 0.5, swelling of one digit; 1, swelling of more than two digits or redness of paw; 2, swelling of part of paw; and 3, swelling of entire paw.

Histopathology

The hind paws of the mice were severed between the knee and ankle, fixed in phosphate

buffered saline (PBS) containing 10% (v/v) formaldehyde, decalcified in 10% (w/v) ethylenediamine tetraacetic acid (EDTA) and embedded in paraffin. The paws were sliced horizontally to the footpad, sectioned, and stained with hematoxylin and eosin. Synovium and bone/cartilage tissues of their tarsal joints were evaluated by light microscopy. Infiltration of leukocytes, proliferation of synovial cells and osteoclast formation were scored on a scale of 0-3 depending on the number of cells in the synovium, ranging from normal to spreading in most areas. Edema was scored on a scale of 0-3 depending on accumulation of excess liquid in the synovium or articular cavities, ranging from normal to appearance in most areas. Destruction of bone and cartilage tissues was scored on a scale of 0-3 ranging from no damage to complete loss of the articular cartilage or bone structure. The scoring was performed according to the following criteria: 0, no abnormality detected; 0.5, very slight; 1, mild; 2, moderate; and 3, severe or marked.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the hind paws of the mice that had been homogenized in ISOGEN (Nippon Gene, Tokyo, Japan) using Polytron and reverse transcribed to cDNA using SuperScript III First-Strand Synthesis Super Mix (Life Technologies, Carlsbad, USA). A polymerase chain reaction (PCR) was performed using a 7500 real-time PCR system (Life Technologies) with TaqMan Gene Expression Assays (Life Technologies) and Premix Ex-Taq (Takara Bio, Otsu, Japan) according to the manufacturers' instructions. The mRNA expression levels were normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of anti-CII antibody concentration

The concentrations of anti-CII IgG in mouse serum were measured using ELISA. Briefly, a

96-well plate coated with 0.1 μg/well of bovine CII was incubated overnight at 4°C, then washed with PBS containing 0.05% (v/v) Tween 20 followed by blocking with PBS containing 1% (w/v) bovine serum albumin (BSA), 5% (w/v) sucrose, and 0.05% (w/v) sodium azide for 1 h at room temperature. After washing, the plates were incubated with the serum for 2 h at room temperature. The plates were then washed and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase for 2 h at room temperature. After washing, the plates were incubated with tetramethyl benzidine substrate for 20 min and the optical density of the wells at 450 nm was measured. The concentrations of anti-CII IgG were quantitated using a standard curve derived from anti-human CII IgG (Daiichi Fine Chemical, Takaoka, Japan).

Statistical analysis

The data are expressed as mean ± standard error (SE). Statistical significance was determined by a parametric Dunnett's test or Student's *t*-test for expression level of mRNA and anti-CII IgG concentrations, by a nonparametric Dunnett's test for histopathological scores and by a Wilcoxon's rank sum test for arthritis scores. To elucidate the correlation between the expression level of mRNA and the arthritis score, a Spearman's rank order correlation test was used.

Results

LPS induces arthritis in CII-immunized mice

CII-immunized DBA/1JNCrj mice were subcutaneously injected with LPS. Paw swelling was observed from Day 2; it reached a peak on Day 7, and then declined slowly until Day 17 (**Figure 1A**; page 75, 76). The prevalence of swelling remained stable and high until Day 17 (**Figure 1B**; page 75, 76). In contrast, the CII-immunized mice injected with saline did not show significant arthritis in terms of both severity and prevalence until Day 17 (**Figure 1A, B**; page 75, 76). These findings show that LPS induces arthritis in CII-immunized mice.

Next, the histopathological features of synovium and bone/cartilage tissues in the tarsal joints of LPS-CIA mice were assessed. The inflammatory features were not observed in the normal mice (**Figure 1D, G**; page 75, 76). From the onset of the arthritis on Day 1, characteristic features, especially infiltration of leukocytes and edema, were observed (**Figure 1C**; page 75, 76). The leukocytes were mainly neutrophils. On Day 3, a number of neutrophils infiltrated the synovium (**Figure 1E**; page 75, 76). In addition, edema reached maximal levels (**Figure 1C, H**; page 75, 76). Furthermore, proliferation of synovial cells, destruction of bone/cartilage and osteoclast formation appeared (**Figure 1C, E, H**; page 75, 76). The proliferating synovial cells were both macrophage-like and fibroblast-like. On Day 7, the proliferation of synovial cells and destruction of bone/cartilage progressed (**Figure 1C, F, I**; page 75, 76). Along with development of bone destruction, an increase in the number of osteoclasts around the area of destruction was observed. The infiltrated neutrophils reached sustained level and remained locally. On Day 19, proliferation of synovial cells and tissue destruction remained mild to moderate, although most histopathological features, such as infiltration of leukocytes and edema, resolved almost completely (**Figure 1C**; page 75, 76).

These findings indicate that joints of LPS-CIA mice have similar histopathological features to those of humans with RA.

Gene expression of inflammatory mediators increases in LPS-CIA paws

To evaluate the association of inflammatory mediators such as cytokines, chemokines and proteases with the development of LPS-CIA, gene expression level of TNFα, IL-1β, IL-6, IL-17A, receptor activator of NF-κB ligand (RANKL), macrophage inflammatory protein-2 (MIP-2: the functional IL-8 homolog in mice), matrix metalloproteinase (MMP)-3 and MMP-9 in the mouse paws were measured. The expression level of TNFα increased at 6 h after LPS injection (**Figure 2A**; page 77, 78). TNFα expression returned to basal levels on Day 3 (after the onset of arthritis) and increased again on Day 6 in parallel with the development of arthritis (**Figure 2A**; page 77, 78). Furthermore, on Days 0, 3 and 6, the expression level of TNFα was positively correlated with the arthritis scores (**Figure 2B**; page 77, 78). Moreover, the gene expression levels of IL-1β, IL-6, IL-17A, RANKL, MIP-2, MMP-3 and MMP-9 also increased and their increase correlated with the development of arthritis (**Table 1, 2**; page 69, 70). These data suggest that these inflammatory mediators are related to the development of arthritis in this model.

Next, I focused on the period before the onset of arthritis and analyzed short-term changes in gene expression up to 24 h after LPS injection. Because LPS directly induces the expression of many inflammatory mediators including TNFα, IL-1β, IL-6 and MIP-2 through NF-κB, gene expression of inhibitor of nuclear factor-κB alpha (IκBα), which represents the NF-κB target gene, was measured. The mRNA level of IκBα in the paws increased by 2 h after LPS injection, and then decreased to the basal level at 6 h, which was maintained until 24 h (**Figure 2C**; page 77, 78). These data indicate that LPS directly activated NF-κB in the

paws around 2 h after LPS injection. The expression of TNFα also increased by 2 h after LPS injection and continued to increase, at least until 24 h (**Figure 2D**; page 77, 78). IL-1β and MIP-2 also increased by 2 h and were induced again 8 h after LPS injection (**Figure 2D, E**; page 77, 78). The expression of IL-6 also increased by 2 h and was induced again 24 h after LPS injection (**Figure 2D**; page 77, 78). In contrast, the expression of MMP-3 and MMP-9 did not change until 8 h, and the expression level of MMP-3 increased by 24 h (**Figure 2E**; page 77, 78). These data demonstrate that inflammatory mediators, in particular TNFα, IL-1β and MIP-2, are upregulated rapidly and continuously after direct activation of NF-κB and before the onset of LPS-CIA.

LPS induces rapid production of anti-CII antibodies in CII-immunized mice

To examine whether LPS induces production of specific autoantibodies, anti-CII IgG concentrations in the mice serum were measured. Anti-CII IgG concentrations in CII-immunized mice were increased by Day 6 after LPS injection although the difference in the concentrations between the saline and LPS injection was not statistically significant (P = 0.0896, **Figure 3A**; page 79). Furthermore, analysis of short-term changes (up to 24 h after LPS injection) showed that anti-CII IgG concentrations increased from 2 h after LPS injection (**Figure 3B**; page 79). These data suggest that LPS induces rapid and continuous production of anti-CII antibodies in CII-immunized mice.

TNFα is involved in development of LPS-CIA

Because TNFα was expressed rapidly and continuously after LPS injection, the involvement of TNFα in the development of LPS-CIA was examined. When TNFα was injected into CII-immunized mice in place of LPS, severe arthritis did not occur in contrast to LPS-injected

mice (**Figure 4A**; page 80). However, in TNFα-injected mice, serum anti-CII IgG concentrations increased in a dose-dependent manner (**Figure 4B**; page 80). These data indicate that TNFα can induce production of anti-CII antibodies, but is not sufficient on its own to induce arthritis. Finally, the contribution of TNFα to LPS-CIA development was investigated using anti-TNFα neutralizing antibodies. Administration of anti-TNFα antibodies once daily from the day before to 6 days after LPS injection significantly inhibited development of LPS-CIA (**Figure 4C**; page 80). These data indicate that TNFα contributes to the development of LPS-CIA and participates in the process of anti-CII antibody induction.

Discussion

Autoimmune diseases sometimes have acute onsets and periods of exacerbation, the causes for which have not yet been clarified. Because a number of studies have shown that LPS may play a role in the exacerbation of autoimmune diseases including RA, LPS-CIA is a valuable disease model for investigation of the acute phase of RA (Yoshino et al., 1999; Yoshino and Ohsawa, 2000). In this study, I elucidated details of the pathogenesis of LPS-CIA from the perspective of the sequence and timing of production of inflammatory mediators and anti-CII antibodies and related this to histopathological findings.

Inflammatory mediators such as cytokines, chemokines and proteases are involved in the development of RA through induction of inflammation and destruction of tissues. Upregulation of inflammatory mediators has been observed in both RA and CIA (Eastgate et al., 1988; Houssiau et al., 1988; Tetta et al., 1990; Troughton et al., 1996; Thornton et al., 1999; Yoshihara et al., 2000; Ziolkowska et al., 2000; Kraan et al., 2001; Tchetverikov et al., 2004). Previous studies have reported increased expression of mRNA of IL-1β and MMPs in arthritic paws of animals with LPS-CIA (Joosten et al., 1996; Raychaudhuri et al., 2003). In the present study, I clearly demonstrated that LPS-CIA mice have marked increases in the gene expression level of inflammatory mediators, including IL-1β, MMP-3 and MMP-9 in their arthritic paws (**Table 1**; page 69). The mRNA levels in them increased in parallel with the development of arthritis and correlated positively with arthritis severity (**Table 2**; page 70). Importantly, TNFα, IL-1β and MIP-2 were rapidly and continuously expressed after direct activation of NF-κB and before the onset of arthritis. In particular, the expression level of TNFα was greater before the onset of arthritis than after its onset. In contrast, I did not observe these responses for MMP-3 and MMP-9, which are overexpressed as a result of

aspects of joint inflammation such as the proliferation of synovial cells and infiltration of inflammatory cells (Yoshihara et al., 2000). These findings suggest that upregulation of TNFα, IL-1β and MIP-2 is involved in the mechanisms of induction of LPS-CIA. Furthermore, it is well known that IL-17 is involved in human RA and arthritis in animal models (Miossec and Kolls, 2012). IL-17A was upregulated in parallel with the development of arthritis in the paws of LPS-CIA mice (**Table 1**; page 69); suggesting IL-17A plays important roles in the pathogenesis of LPS-CIA.

I confirmed that arthritic paws of LPS-CIA mice are characterized by similar histopathological features as human RA; these include bone and cartilage destruction, synovial change and infiltration of inflammatory cells. Moreover, expression of inflammatory mediators is likely to correlate with histopathological findings. There is consensus that synovial hyperplasia caused by a marked increase in macrophage-like and fibroblast-like synovial cells is a hallmark of RA (Firestein, 2003). I observed proliferation of both macrophage-like and fibroblast-like synovial cells in parallel with the development of LPS-CIA, which demonstrates that LPS-CIA has this pathological hallmark in common with RA. In addition, I observed the increase in synovial cells from the early stages of arthritis. The increase in MMP-3 observed 24 h after LPS injection might reflect the proliferation of synovial cells, which generally takes 1 day. Notably, infiltration of neutrophils accompanied by edema occurred first; this coincided with the onset of arthritis, suggesting that neutrophil infiltration is the main cause of the inflammation in LPS-CIA. Recent studies have highlighted the possible contribution of neutrophils in the early phases of RA physiopathology. Although it is well known that mononuclear cells such as T cells, B cells and macrophages infiltrate the synovium in RA, neutrophils migrate to the synovial fluid before mononuclear cells do (Cascao et al., 2010; Wright et al., 2010; Dominical et al., 2011). Previous studies

have reported accumulation or participation of neutrophils in commonly-used arthritis models including CIA (Thornton et al., 1999; Wipke and Allen, 2001; Tanaka et al., 2006). In particular, the histopathology of LPS-CIA is similar to that of anti-CII antibodies and LPS-induced arthritis, in which neutrophils, but not T and B cells, play crucial roles in the development of arthritis (Tanaka et al., 2006). Thus, neutrophil infiltration may cause the acute inflammation in LPS-CIA; this suggests that LPS-CIA has pathogenetic mechanisms in common with the early phase of RA. Upregulation of MIP-2 may play a part in recruitment of neutrophils to inflammatory sites. Likewise, TNFα and IL-1β may result in edema formation by initiating acute inflammation with enhanced vascular permeability. The increase in osteoclasts associated with bone and cartilage destruction is consistent with increased expression of RANKL (**Table 1**; page 69). Similarly, overexpression of MMP-3 and MMP-9 may cause cartilage destruction.

The rapid and continuous increase in serum anti-CII antibody concentrations may, in part, contribute to the development of LPS-CIA. CII is the major constituent protein of the cartilage of joints and autoimmunity to CII occurs in RA patients (Nandakumar and Holmdahl, 2006). The rapidity of increase in production of serum anti-CII antibodies induced by LPS may be mediated *via* a direct effect on B cells. LPS injection results in production of polyclonal antibodies *via* stimulation of B cells (Dziarski, 1982a; Dziarski, 1982b). Anti-CII antibodies have the potential to induce arthritis, as evidenced by induction of articular inflammation by serum transfer or collagen antibody cocktail injection (Holmdahl et al., 1990; Terato et al., 1992). Complex formation between anti-CII antibodies and CII in cartilage activates the complement systems and initiates recruitment of neutrophils and macrophages, which are activated by Fcγ receptor ligation and secrete inflammatory mediators (Cho et al., 2007). In the present study, I detected a second induction in gene expression of IL-1β and

MIP-2 2-8 h after LPS injection, this interval of time being just after the increase in serum anti-CII IgG. Additionally, a previous study has shown that LPS injection of mice without CII immunization does not induce arthritis (Caccese et al., 1992; Takahashi et al., 1999). Thus, production of anti-CII antibodies and the consequent induction of continuous expression of inflammatory mediators may be necessary for acute onset of LPS-CIA. Furthermore, T cells might be involved in LPS-CIA *via* modulation of pathogenic B cells, which produce anti-CII antibodies.

The expression pattern of TNFα mRNA did not decrease after LPS injection and before the onset of arthritis, implying its important role in the development of LPS-CIA. Previous studies have shown that neutralizing antibodies against TNFα and soluble TNFα receptor fusion protein significantly ameliorate CIA (Piguet et al., 1992; Williams et al., 1992; Wooley et al., 1993). Additionally, anti-TNFα antibodies are effective when treated after the onset of arthritis induced by a booster injection of CII and subsequent LPS injection (Joosten et al., 1996); however, whether the antibodies are effective when treated before the onset of LPS-CIA has yet to be clarified. In the present study, I have confirmed the effectiveness of anti-TNFα antibodies treated before the onset of LPS-CIA, validating that TNFα is an important molecule in the pathogenesis of LPS-CIA. A single intra-articular injection of TNFα accelerates the onset of arthritis in rats immunized with CII (Cooper et al., 1992). In addition, comparable to LPS, an intraperitoneal administration of recombinant IL-1β can induce arthritis in CII-immunized mice (Caccese et al., 1992). However, TNFα did not induce severe arthritis when it was injected intravenously, suggesting that systemic exposure of TNFα is insufficient to induce arthritis. Interestingly, this experiment showed TNFα has the potential to induce production of anti-CII antibodies. This finding suggests that TNFα contributes to the development of LPS-CIA through participation in the production process of

anti-CII antibodies. On the other hand, because TNFα alone is not sufficient to induce arthritis, the findings raise the possibility that involvement of other proinflammatory cytokines, such as IL-1β and IL-6, could be necessary for the development of LPS-CIA.

The development and severity of RA are linked to genetic and environmental factors. Microbial infections, which may be a potent source of LPS, may be one of the triggers for autoimmune diseases, including RA (Carty et al., 2003; Leirisalo-Repo, 2005; Getts and Miller, 2010). It is recognized that systemic infections cause acute exacerbations of RA. One of the multiple possible mechanisms by which infections may trigger autoimmunity is the adjuvant effects of pathogens mediated by Toll-like receptors (TLRs) and other pattern-recognition receptors on antigen-presenting cells; this would lead to production of inflammatory mediators which in turn would result in tissue damage (Getts and Miller, 2010). A number of studies suggest a possible role for LPS or its cellular receptor, TLR4, in RA. The serum and synovial fluids of RA patients contain higher concentrations of antibodies against *E. coli* or LPS-binding protein than do those of healthy subjects (Heumann et al., 1995; Aoki et al., 1996). In animal models, the spontaneous arthritis of IL-1 receptor antagonist knockout mice is markedly suppressed by crossing with TLR4-deficient mice (van den Berg et al., 2007). Moreover, blocking of TLR4 by receptor antagonists suppresses experimental arthritis in mice (van den Berg et al., 2007; O'Neill et al., 2009). I confirmed that, after LPS injection, LPS-CIA mice had increased inflammatory mediators in their paws, similar to that associated with infection. Taken together with these findings, LPS-CIA may have pathogenetic mechanisms in common with infection-associated RA.

In summary, I have demonstrated that LPS induces arthritis in CII-immunized mice and that the onset of the arthritis is preceded by rapid and continuous production of inflammatory mediators and anti-CII antibodies. This finding suggests that production of

these humoral factors induces the development of LPS-CIA. In particular, TNFα may contribute to the development of LPS-CIA *via* both initiation of inflammation and production of anti-CII antibodies. It is possible that the rapid and continuous production of inflammatory mediators and autoantibodies is the mechanism underlying the exacerbation of arthritis.

Chapter II

“Role of NF-κB in regulation of expression of proinflammatory cytokines in joint tissues”

Introduction

Most proinflammatory cytokines associated with RA are regulated by NF-κB. NF-κB plays an important role in immune response, inflammation, cell differentiation, proliferation and survival (Baeuerle and Henkel, 1994; Hayden and Ghosh, 2004; Perkins, 2007). Dysregulation of NF-κB is observed in various diseases, such as cancer and inflammatory diseases including RA (Tak and Firestein, 2001; Senftleben and Karin, 2002; Viatour et al., 2005; Brown et al., 2008). In resting cells, NF-κB exists as an inactive form through association with IκB in the cytoplasm. In the canonical pathway, a variety of stimuli such as TNFα and LPS induces phosphorylation and subsequent polyubiquitination of IκB, leading to degradation through the 26S proteasome pathway. NF-κB released from IκB translocates into the nucleus and initiates the transcription of target genes, including TNFα, IL-1 and IL-6 (DiDonato et al., 1996; Karin, 1999; Guha and Mackman, 2001; Makarov, 2001; Tak and Firestein, 2001).

The phosphorylation of IκB is catalyzed by the IκB kinase (IKK) complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modulator (Karin, 1999; Ghosh and Karin, 2002). Ample evidence indicates that IKKβ, but not IKKα, is required for NF-κB activation in response to inflammatory stimuli (Li et al., 1999; Ghosh and Karin, 2002), suggesting that IKKβ is a key factor in the production of proinflammatory cytokines in inflammatory conditions. Therefore, further understanding of the mechanisms of NF-κB regulation by IKKβ is of great interest for RA therapeutics. Several small molecule inhibitors of IKKβ have been identified, and the efficacy of each inhibitor has been clearly demonstrated in arthritis models (McIntyre et al., 2003; Podolin et al., 2005; Schopf et al., 2006; Mbalaviele et al., 2009); however, studies using diverse chemical classes

of IKKβ inhibitors with characteristic mechanisms of action are still required to validate the role of NF-κB in the development of RA. In fact, there is no report where IKKβ inhibitors are analyzed from the perspective of the relationship between the tissue distribution of the compounds and the regulation of proinflammatory cytokines in the arthritic joints, which are the primary target tissues of RA. Compound D is a novel IKKβ inhibitor, which shows potent IKKβ inhibitory activity, favorable physical properties, good plasma exposure and efficacy in CIA models (Shimizu et al., 2010; Shimizu et al., 2011a; Shimizu et al., 2011b). However, the molecular mechanisms and joint tissue distribution of the compound have not been fully investigated.

The purpose of this chapter is to clarify the role of NF-κB in regulation and function of proinflammatory cytokines in joint tissues. In order to achieve the purpose, I investigated the mechanisms of anti-arthritic effect of Compound D because the compound exhibited suitable properties for studying the role of NF-κB in the local joint tissues, such as selective inhibition of the NF-κB signaling pathway and preferential distribution in the arthritic paws.

Materials and Methods

Animals

Male BALB/c mice and female DBA/1J mice were purchased from Charles River and Japan SLC (Shizuoka, Japan), respectively. All mice were used at the age of 7-10 weeks. All experimental procedures were performed in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Materials

Compound D was synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). LPS (*E. coli* O111:B4) and Freund's complete adjuvant containing *Mycobacterium butyricum* were purchased from Difco Laboratories. PathDetect *cis*-reporter luciferase plasmids for NF-κB, activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT), serum response element (SRE), cAMP response element (CRE) and interferon-stimulated response element (ISRE), were purchased from Agilent Technologies (Santa Clara, USA). Fugene HD, EDTA-free protease inhibitor cocktail, Pefablock and leupeptin were purchased from Roche Diagnostics (Basel, Switzerland).

Kinase assay

Kinase selectivity was assessed using a commercial kinase profiling service (Carna Biosciences, Kobe, Japan). Kinase activities of IKKβ and IKKα were measured by an IMAP^{TM} assay using ATP at the concentrations around *Km* for each kinase.

Reporter assay

Reporter assay was performed as previously reported (Wen et al., 2006), with some modifications. In brief, 293T cells (American Type Culture Collection) grown in a 10 cm dish in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum (FBS), 50 units/mL of penicillin and 50 μg/mL of streptomycin were transfected with 10 μg of reporter luciferase plasmids using Fugene HD. The following day, the transfected cells were harvested and plated at a density of 2×10^4 cells/well in a poly-D-lysine 96-well plate (BD Biosciences, San Jose, USA). The cells were pretreated with various concentrations of Compound D just before the stimulation as follows: 3 ng/mL of TNFα for NF-κB, 5 ng/mL of phorbol 12-myristate 13-acetate for AP-1 and SRE, 5 ng/mL of phorbol 12-myristate 13-acetate and 1 μg/mL of ionomycin for NFAT, 5 μM forskolin for CRE, 4,000 units/mL of interferon beta for ISRE. After incubation for 6 h (TNFα) or 24 h (the others), the cells were lysed with Bright-Glo (Promega, Madison, USA) and their luciferase activities were measured using ARVO-MX (PerkinElmer, Waltham, USA). For evaluation of cytotoxicity, untransfected 293T cells were plated at a density of 2×10^4 cells/well in a poly-D-lysine 96-well plate. The cells were pretreated with various concentrations of Compound D and incubated for 21 h. The cells were treated with WST-8 (Cell counting kit-8, Dojindo, Kumamoto, Japan) and incubated for 3 h. The absorbance at 450 nm was measured.

IκBα phosphorylation and degradation assays

HeLa cells (American Type Culture Collection) grown in a 24-well plate in RPMI-1640 medium supplemented with 10% (v/v) FBS, 50 units/mL of penicillin and 50 μg/mL of streptomycin were starved with serum-free medium for 1 day. The cells were pretreated with various concentrations of Compound D for 15 min, followed by stimulation with 10 ng/mL of TNFα for 10 min (IκBα phosphorylation) or 15 min (IκBα degradation). After washing with

Hank's balanced salt solutions, the cells were lysed with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4), 150 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 1% (v/v) Triton X-100, and EDTA-free protease inhibitor cocktail by freeze-thawing. The supernatant was obtained by centrifugation (13,000*g* at 4°C for 5 min) and boiled with Laemmli sample buffer. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Blots were blocked with 5% (w/v) BSA in tris(hydroxymethyl)aminomethane-buffered saline with 0.1% (v/v) Tween 20 and incubated with polyclonal anti-phosphorylated IκBα antibody (Cell Signaling Technologies, Danvers, USA) or anti-IκBα antibody (Millipore, Billerica, USA), and subsequently incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, England). Enhanced chemiluminescence (GE Healthcare) was used for detection.

NF-κB-DNA binding assay

NF-κB-DNA binding was detected by an electrophoretic mobility shift assay. HeLa cells grown in a 24-well plate were starved with serum-free medium for 1 day. The cells were pretreated with various concentrations of Compound D for 15 min, followed by stimulation with 10 ng/mL of TNFα for 30 min. The cells were harvested and washed with Buffer H (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM NaF, 1 mM dithiothreitol, 0.1 mM Pefablock and 10 μg/mL of leupeptin). After removal of the cytosolic fraction by Buffer H containing 0.2% (v/v) Nonidet P-40, a nuclear pellet was vigorously mixed with Buffer H containing 420 mM NaCl and 20% (v/v) glycerol for 2 h. The supernatant was obtained by centrifugation (13,000*g* at 4°C for 5 min) as nuclear extracts. The nuclear extracts were incubated with 2 units/mL of poly(dI-dC)

in 10 mM HEPES, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol and 0.1% (v/v) Nonidet P-40 for 30 min, followed by incubation for 30 min with 2 pmol/mL of biotinylated oligonucleotides of consensus DNA-binding sites for NF-κB as follows: sense, 5'-AGCTCAAACAGGGGGCTTTCCCTCCTC-3'; antisense, 5'-AGCTGAGGAGGGAAAGCCCCCTGTTTG-3'. The samples were separated using tris(hydroxymethyl)aminomethane-borate-EDTA gel electrophoresis and transferred onto nylon membranes, subsequently crosslinked by UV irradiation. The binding of NF-κB to the oligonucleotides was detected using a Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions.

LPS-induced cytokine production assay using human blood cells

Human blood cells were obtained from the venous blood of healthy adult volunteers in accordance with the ethical approval guidelines of the Daiichi Sankyo Co. Ltd. Ethics Board. The blood cells were suspended to 2×10^6 leukocytes/mL in RPMI-1640 containing 10% FBS, 25 mM HEPES, 50 units/mL of penicillin and 50 μg/mL of streptomycin. The cells were plated in a 96-well plate at a volume of 100 μL/well and pretreated with various concentrations of Compound D just before the stimulation with 1 μg/mL of LPS. After 4 h (TNFα) or 24 h (IL-6) of incubation at 37°C, the culture supernatants were harvested by centrifugation (400*g* at 4°C for 5 min). The concentrations of TNFα and IL-6 in the supernatants were measured using an OptEIA ELISA kit (BD Biosciences) according to the manufacturer's instructions.

LPS-induced TNFα production assay in mice

BALB/c mice were orally given Compound D or vehicle (0.5% methyl cellulose). After 30

min, 0.02 mg/kg of LPS or saline was administered intravenously. After 1 h, blood was collected in the presence of heparin and the plasma was obtained by centrifugation (1,000*g* at 4°C for 30 min). The concentration of TNFα in the plasma was measured using a Quantikine ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Plasma concentrations of Compound D were determined by the LC/MS/MS method.

Induction and assessment of CIA in mice

DBA/1J mice were immunized by an intradermal injection at the base of the tail with 0.1 mL of emulsion containing 1.5 mg/mL of bovine CII (Collagen Gijutsu-Kenshukai) in Freund's complete adjuvant. Twenty-one days later, the mice were secondarily immunized by an intradermal injection at the abdomen with a total of 0.1 mL of the emulsion. Compound D was orally administered once daily beginning with the day of the 2nd immunization for 14 days and all paws of the mice were obtained for measurement of gene expression 24 h after final administration at 14 days after the 2nd immunization. For the evaluation of the efficacy of the compound administered after disease development, Compound D was orally administered once daily from 5 days after the 2nd immunization for 12 days. For pharmacokinetics and gene expression analyses in a single oral administration of the compound, Compound D was orally administered to CIA mice 9-10 days after the 2nd immunization. The plasma and paws were obtained at the indicated time after administration, and gene expression and the concentration of the compound in the tissues were measured. Total RNA was extracted from the paws that had been homogenized in ISOGEN (Nippon gene) using Polytron and reverse transcribed to cDNA using SuperScript III First-Strand Synthesis Super Mix (Life Technologies). A PCR was performed using a 7500 real-time PCR system (Life Technologies) with TaqMan Gene Expression Assays (Life Technologies) and

Premix Ex-Taq (Takara Bio) according to the manufacturer's instructions. The mRNA expression levels were normalized with each GAPDH expression level. The concentrations of the compound in the tissues were determined by the LC/MS/MS method. The severity of arthritis of each paw was scored periodically on a scale of 0-3 according to the following criteria: 0, normal; 0.5, swelling of one digit; 1, swelling of more than two digits or redness of paw; 2, swelling of part of paw; and 3, swelling of entire paw.

Statistical analysis

The data are expressed as mean ± standard deviation (SD) or mean ± SE. Statistical significance in the comparison of TNFα production and gene expressions was determined by a parametric Dunnett's test. Statistical significance in the comparison of arthritis scores was determined by a nonparametric Dunnett's test.

Results

Compound D is a selective inhibitor of IKKβ

Compound D (**Figure 5A**; page 81) was identified as a potent small molecule inhibitor of IKKβ from the chemical optimization of a series of imidazo[1,2-*b*]pyridazine derivatives discovered by high-throughput screening of chemical libraries (Shimizu et al., 2010; Shimizu et al., 2011a; Shimizu et al., 2011b). To evaluate the kinase selectivity of Compound D, I determined the 50% inhibitory concentration (IC_{50}) values against IKKβ and related kinase IKKα. Compound D inhibited kinase activities of IKKβ and IKKα with an IC_{50} of 0.037 and 6.1 μM, respectively, and demonstrated 164-fold selectivity (**Figure 5B**; page 81).

To examine the selectivity of Compound D in cellular signaling pathways, I performed the reporter assays that represent major signaling pathways using 293T cells. Compound D inhibited NF-κB promoter activity with an IC_{50} of 0.15 μM, while the compound weakly inhibited promoter activities of other pathways, showing over 100-fold selectivity compared to the NF-κB pathway (**Table 3**; page 71). In addition, 20 μM of the compound did not cause 50% inhibition of the cell viability at 24 h (**Table 3**; page 71).

Compound D inhibits the NF-κB signaling pathway

To confirm whether Compound D inhibits the IKK-mediated signal transduction to NF-κB, I performed IκBα phosphorylation and degradation assays and an NF-κB-DNA binding assay using HeLa cells. Compound D inhibited IκBα phosphorylation and subsequent degradation induced by TNFα in a concentration-dependent manner (**Figure 6A**; page 82). Additionally, Compound D inhibited NF-κB-DNA binding as well (**Figure 6B**; page 82). The IC_{50} values against these signals were in the range of 0.1 to 1 μM, which were comparable to that against

NF-κB promoter activity.

Compound D inhibits NF-κB-driven cytokine production

LPS is known to induce NF-κB-driven production of proinflammatory cytokines such as TNFα and IL-6 (Guha and Mackman, 2001). To examine whether Compound D inhibits NF-κB-driven production of proinflammatory cytokines other than TNFα, I performed LPS-induced IL-6 production assay using human blood cells. Compound D inhibited the productions of TNFα and IL-6 comparably (**Figure 7A**; page 83). The calculated IC_{50} values were 0.22 and 0.27 μM, respectively. Thus, Compound D inhibited NF-κB-driven production of multiple proinflammatory cytokines. In addition, The IC_{50} value for TNFα was consistent with that in mouse blood cells with an IC_{50} of 0.23 μM as described in the previous report (Shimizu et al., 2011a), indicating that Compound D had no species difference between humans and mice in its inhibitory activity on LPS-induced TNFα production.

The inhibition of TNFα production correlates with the plasma concentration of Compound D *in vivo*

To examine the relationship between the effect on cytokine production and plasma concentration of Compound D, I performed an LPS-induced TNFα production assay *in vivo*. The mice were pretreated with Compound D for 30 min, followed by an LPS injection, and 1 h later, blood was collected to measure the TNFα and Compound D levels. Orally administered Compound D inhibited LPS-induced TNFα production in a dose-dependent manner (**Figure 7B**; page 83). Compound D inhibited 30% of TNFα production with a dose of 10 mg/kg, and completely inhibited it with a dose of 100 mg/kg. The concentrations of Compound D in the plasma 1.5 h after administration were 0.39, 2.9 and 9.4 μM at doses of

10, 30 and 100 mg/kg, respectively, showing dose-dependent elevation (**Table 4**; page 72). Thus, the inhibition of TNFα production correlated with the plasma concentration of Compound D *in vivo*.

Compound D inhibits expression of proinflammatory cytokines in arthritic paws by consecutive treatment

Compound D is efficacious in experimental arthritis models as described previously (Shimizu et al., 2011a). However, the molecular mechanisms of the anti-arthritic effect remain unclear. To investigate the mechanisms, I analyzed the arthritic paws of the compound-administered CIA mice in detail. First, to confirm the efficacy of Compound D, multiple dosages of Compound D or vehicle were administered once daily beginning with the day of the 2nd immunization for 14 days and the severity of arthritis was scored periodically. As shown in **Figure 8A** (page 84, 85), Compound D significantly decreased the mean arthritis scores in a dose-dependent manner. The repeated administration of the compound did not affect body weight gain (**Figure 8B**; page 84, 85). Considering the inhibitory effect of LPS-induced TNFα production *in vivo* by Compound D, it is suggested that one of the mechanisms of its efficacy in CIA is downregulation of proinflammatory cytokines in the arthritic paws. To examine this, I measured the gene expression levels of proinflammatory cytokines and other inflammatory mediators associated with RA in the arthritic paws. The paws of CIA mice were harvested 24 h after final administration at 14 days after the 2nd immunization and the gene expression levels were measured by quantitative PCR. I measured the mRNA levels of proinflammatory cytokines, TNFα, IL-1β and IL-6, and various mediators related to the destruction of bone and cartilage, RANKL, MMP-3 and MMP-9. The arthritic mice administered with only vehicle displayed higher levels of the mRNA expressions of the proinflammatory cytokines and

inflammatory mediators compared to disease-free normal mice (**Table 5**; page 73). In contrast, the expression levels of the genes, except for TNFα, tended to be low in Compound D-treated mice (**Table 5**; page 73). Although due to the considerable variation it did not reach statistical significance, the expression levels decreased in dose-dependent manner. Taken together, these data suggest the possibility that the consecutive treatment of Compound D inhibits the gene expression of proinflammatory cytokines and inflammatory mediators in the arthritic paws of CIA mice.

Compound D distributes to arthritic paws and downregulates proinflammatory cytokines

I investigated the relationship between pharmacokinetics and the inhibition of gene expression by Compound D in arthritic mice. Compound D levels in plasma and paws of normal and arthritic mice were measured at the indicated time after oral administration of the compound at a dosage of 30 mg/kg. In the normal mice, Compound D levels in both plasma and paw tissue decreased from 1 h after administration and disappeared at 24 h. In contrast, Compound D in the arthritic paws was detected at a higher level compared to plasma, which remained at more than 1,000 pmol/g at 24 h, while the plasma compound level of arthritic mice exhibited only slight elevation compared to normal mice (**Figure 8C**; page 84, 85). These data indicates that Compound D distributes to the arthritic paws compared to healthy paws.

I further investigated whether Compound D functions in the arthritic paws because the consecutive treatment has a potential to affect various cells and organs. To exclude the indirect effect of Compound D on gene expression of proinflammatory cytokines in arthritic paws, I measured the mRNA levels of TNFα and IL-1β in the paws of CIA mice 2 h after a

single oral administration of the compound. The gene expression levels of TNFα and IL-1β in the arthritic paws were elevated compared to normal mice, and Compound D decreased the gene expression levels of TNFα and IL-1β at a dose of 100 mg/kg (**Figure 8D**; page 84, 85). The Compound D levels in the paws 2 h after administration at dosages of 30 and 100 mg/kg were 1,166 ± 554 and 24,727 ± 3,594 pmol/g (mean ± SD), respectively, showing rapid distribution of the compound into the arthritic paws in a dose-dependent fashion. These data indicates that Compound D rapidly distributes to the arthritic paws and where the compound directly downregulated the gene expression of proinflammatory cytokines.

Compound D inhibits arthritis progression by the treatment after disease development

Finally, to evaluate the efficacy of Compound D administered after disease development, the compound was orally administered once daily from 5 days after the 2nd immunization for 12 days and arthritis scores were periodically measured. As shown in **Figure 8E** (page 85), Compound D at a dosage of 100 mg/kg completely inhibited the progression of arthritis under the treatment during this experiment.

Discussion

In the present study, I validated the selectivity of Compound D in the inhibition of IKKβ kinase activity and the blockade of the NF-κB signaling pathway. In addition, I confirmed the concentration-dependent inhibition of NF-κB-driven production of proinflammatory cytokines by Compound D *in vitro* and *in vivo*. Furthermore, I demonstrated that Compound D preferentially distributes to arthritic paws compared to healthy paws and where it downregulates proinflammatory cytokines in the arthritic joints.

The results of the *in vitro* analyses demonstrate that Compound D is a selective inhibitor of IKKβ. Compound D inhibited IKKβ kinase activity with an IC_{50} of 0.037 μM with 160-fold selectivity against related kinase IKKα. Studies using genetically manipulated mice implied that IKKβ, but not IKKα, was a key driver of NF-κB activation (Li et al., 1999; Ghosh and Karin, 2002); therefore, high selectivity for IKKβ over IKKα should be effective for the downregulation of proinflammatory cytokines in inflammatory conditions where NF-κB is preferentially activated. The NF-κB pathway mediated by IKKβ is activated in response to inflammatory stimuli, such as TNFα and IL-1, leading to IκB phosphorylation, subsequent IκB degradation, NF-κB nuclear translocation and DNA binding (DiDonato et al., 1996; Karin, 1999). Compound D inhibited TNFα-induced IκBα phosphorylation, degradation and NF-κB-DNA binding in HeLa cells in a concentration-dependent fashion with comparable inhibitory potency against NF-κB promoter activity in 293T cells (**Table 3**). Although the inhibitory activities of Compound D in the cellular assays were 10 times lower than that in the kinase assay, the reduction in the activity is reasonable because over 90% of the compound may bind to serum proteins in the cellular assays containing 10% FBS, as suggested by its protein binding property (Shimizu et al., 2011a). These findings suggest that

the blockade of the NF-κB pathway by Compound D is due to its inhibitory activity of IKKβ. Notably, Compound D affected pathways other than the IKKβ/NF-κB pathway only at high concentrations more than 100-fold. These results indicate that Compound D selectively blocks the NF-κB pathway among major cellular pathways. Compound D did not change the cell viability at even 100-fold concentration of NF-κB inhibition. These findings suggest that the high selectivity of Compound D could contribute to fewer side effects.

LPS activates the NF-κB signaling pathway mediated by IKKβ (Hawiger et al., 1999; Guha and Mackman, 2001). Compound D potently inhibited TNFα productions in a concentration-dependent manner in LPS-stimulated human blood cells *in vitro* and LPS-injected mice *in vivo*. The effective plasma concentrations *in vivo* (**Table 4**; page 72) are likely to correlate with the inhibitory concentrations in human blood cells *in vitro*. As expected, the inhibitory concentration range on TNFα production in human blood cells was comparable to that of NF-κB promoter activity as well as the signal transduction to NF-κB in HeLa cells. Consistent with this, Compound D inhibited production of IL-6, one of the proinflammatory cytokines regulated by NF-κB, as potently as TNFα in human blood cells. These findings suggest that the inhibition of the cytokine production is based on the blockade of the NF-κB pathway *via* IKKβ inhibition. These inhibitory profiles of Compound D should contribute its efficacy in inflammatory conditions such as arthritis where multiple proinflammatory cytokines are simultaneously elevated (Houssiau et al., 1988; Tetta et al., 1990).

Compound D displayed a unique pharmacokinetic feature manifested as preferential distribution to arthritic paws compared to healthy paws. Previous studies have shown that vascular permeability increased preferentially in the arthritic joints (Binstadt et al., 2006; Blanchet et al., 2010; Cloutier et al., 2012). This enhanced vascular permeability has been

associated with mast cells, neutrophils and endothelial cells in response to immune complexes in the joints (Binstadt et al., 2006). These findings suggest that Compound D may preferentially distribute to the arthritic paws by immune complex-triggered alterations in the local joint environment. Notably, Compound D retained in the arthritic paws at a higher level even though the compound disappeared from plasma. The retention period of Compound D seems to be much longer in comparison with other small-molecular agents, such as methotrexate (Stewart et al., 1987). This finding suggests that the mechanisms of the characteristic tissue localization of Compound D would depend on its chemical property. It is probable that the compound binds to arthritic joint contents such as joint structural components, inflammation-induced proteins and immune complexes.

The consecutive treatment of Compound D resulted in reduction of the gene expression of proinflammatory cytokines and inflammatory mediators in the paws of CIA mice, accompanied with the dose-dependent efficacy to the disease. Although Compound D did not markedly decrease TNFα gene expression compared to the other genes in the experiment of consecutive treatment (**Table 5**; page 73), the compound resulted in reduction of the gene expression of TNFα as well as IL-1β in the established arthritic paws after 2 h of a single administration. It is known that TNFα plays an important role only in the development of CIA at an early stage and its expression is reduced in late stages of the disease (Williams et al., 1992; Wooley et al., 1993; Joosten et al., 1996). Indeed, the TNFα mRNA level was low at the time point used in my present study, consistent with previous reports (Rioja et al., 2004; Medicherla et al., 2006). These observations imply that the elevation of TNFα expression might be too low to evaluate the inhibitory effect of the compound even though the expression is higher than unimmunized normal mice. Alternatively, it is possible that TNFα expression has recovered at the vehicle-treated level at 24 h after administration due to the decline of the

concentration of the compound in the paw, even though the expression was inhibited 2 h after administration as observed in the present study. This is because TNFα expressed more rapidly than other cytokines by proinflammatory stimulation in the paws (Marinova-Mutafchieva et al., 1997; Thornton et al., 1999; Kagari et al., 2002). Nevertheless, the efficacy of Compound D was observed despite upregulation of the TNFα expression. In contrast, the other genes may be continuously downregulated at least until 24 h after administration and thereby markedly suppressed by the consecutive treatment. Consistent with this, Compound D completely inhibited arthritis progression even when treatment occurred after disease development, suggesting that the downregulation of proinflammatory cytokines in the arthritic joints contributes to the anti-arthritic effect.

The treatment of Compound D reduced gene expression of inflammatory mediators involved in the destruction of bone and cartilage, such as RANKL, MMP-3 and MMP-9, as well as proinflammatory cytokines in the paws of CIA mice (**Table 5**; page 73). MMP-3 and MMP-9, which are overexpressed as a result of aspects of joint inflammation such as proliferation of synovial cells and infiltration of inflammatory cells, degrade the extracellular matrix and cartilage (Yoshihara et al., 2000; Goldring and Marcu, 2009). MMPs were detected at high levels in the synovial fluid or serum of RA patients (Yoshihara et al., 2000; Tchetverikov et al., 2004). RANKL is known to contribute to bone erosion and destruction by differentiation and activation of osteoclasts (Goldring, 2002). Suppression of bone destruction by Compound D was observed in the rat arthritis model, accompanied with delayed onset and relieved symptoms of paw swelling (Shimizu et al., 2011a). Based on these findings, Compound D has a potential to suppress the destruction of bone and cartilage in RA.

CIA is commonly used as an RA model because of its similarities in both etiology and pathology (Williams, 2007). In the joint tissue of CIA mice, synovial hyperplasia and

infiltration of inflammatory cells have been observed (Williams, 2007). These synovial cells secrete proinflammatory cytokines, which amplify inflammation through further generation of various inflammatory mediators. Involvement of proinflammatory cytokines in the pathogenetic process of CIA is evident by the effectiveness of neutralizing antibodies against TNFα, IL-1β and IL-6 (Williams et al., 1992; Takagi et al., 1998; Williams et al., 2000). Additionally, NF-κB activation has been demonstrated in the synovial tissue of mice in the development of CIA (Tak and Firestein, 2001; Podolin et al., 2005). The intra-articular gene transfer of wild-type IKKβ into the joints of normal rats causes paw swelling and synovial inflammation, while the transfer of a dominant-negative IKKβ ameliorates the severity of adjuvant arthritis (Tak and Firestein, 2001). These findings suggest that IKKβ plays an important role in synovial inflammation in arthritic joints. The role of IKKβ in inflammatory diseases has been demonstrated in multiple disease-relevant cells and animal models using selective IKKβ inhibitors (McIntyre et al., 2003; Podolin et al., 2005; Schopf et al., 2006; Mbalaviele et al., 2009; Gamble et al., 2012). However, the mechanisms of the action of IKKβ inhibitors are complicated because NF-κB plays an important role in immune response, inflammation, cell differentiation, proliferation and survival in various cells and tissues (Baeuerle and Henkel, 1994; Hayden and Ghosh, 2004; Perkins, 2007). An IKKβ inhibitor could induce a variety of functional alterations in various cells through suppression of the NF-κB signaling as contribution to the efficacy or other side effects, in addition to inhibiting proinflammatory cytokine productions in arthritic joints. In this regard, Compound D may be a useful agent for understanding the mechanisms of the anti-arthritic effect of IKKβ inhibitors in arthritic joints because Compound D may function in local inflammatory sites due to its preferential distribution in the arthritic paws compared to healthy ones. Supporting this, Compound D did not affect the body weight gain of arthritic mice by consecutive treatment,

implying the compound has less systemic toxicity. The inhibitory activity of Compound D against generation of proinflammatory cytokines in the arthritic joint tissue could be led by a blockade of the NF-κB pathway through IKKβ inhibition and break the amplification loop of inflammation in CIA. Taking these findings together, one of the mechanisms underlying the anti-arthritic effect of Compound D should be the direct downregulation of proinflammatory cytokines in the arthritic joints.

In summary, I have characterized a novel small molecule IKKβ inhibitor, Compound D, exhibiting selective inhibition of the IKKβ kinase activity and the NF-κB signaling pathway. Furthermore, I have demonstrated that Compound D preferentially distributed to the arthritic paws, compared to healthy paws, where it directly downregulated the gene expression of proinflammatory cytokines, thereby exhibiting an anti-arthritic effect. These findings suggest that NF-κB-dependent proinflammatory cytokines in local joint tissues play an important role on the development of CIA.

General Discussion

In the present study, in order to elucidate the role of proinflammatory cytokines in joint tissues during development of arthritis, I analyzed pathogenetic mechanisms of LPS-induced exacerbation of arthritis and the role of NF-κB in the regulation of proinflammatory cytokines expression in joint tissues using a mouse CIA model. Recently, studies of development and pathophysiology of RA are performed on the experimental arthritis models applied to several kinds of knockout mice to clarify the role of the target genes. In such cases, emphasis tends to be placed on characterization of phenotypes; thereby molecular mechanisms before the onset of the disease have been poorly investigated. In addition, immunity is established by the network formed by a variety of immune cells interacting with each other in the whole body; therefore, it is difficult to analyze tissue-specific mechanisms. From these aspects, a spatiotemporal-specific approach is necessary to gain further insight into the mechanisms of RA. Therefore, I focused on the mechanisms specifically in local joint tissues during development of arthritis.

I demonstrated in the present study that the timing of the expression of proinflammatory cytokines, such as TNFα and IL-1β, in the joint tissues of LPS-CIA mice was accelerated; nevertheless, the sequence was likely consistent with other animal arthritis models (Marinova-Mutafchieva et al., 1997; Thornton et al., 1999; Rioja et al., 2004). I also demonstrated here that these proinflammatory cytokines are regulated by NF-κB in arthritic joint tissues and contributed to the progression of arthritis. In particular, the expression pattern of TNFα was implicated as a key factor in the early stages of arthritis development as previously reported (Joosten et al., 1996). Especially, the mechanism of production of anti-CII antibodies induced by TNFα is of great interest. Recombinant TNF receptor Fc fusion protein inhibited anti-collagen antibody production in CIA mice (Wooley et al., 1993), supporting involvement of TNFα in the mechanism on anti-collagen antibody production. The

mechanisms can be explored by studying B cells *in vitro* in the future. The study also raises the possibility that involvement of other proinflammatory cytokines could be necessary for the development of LPS-CIA because TNFα injection accompanied by subsequent increase of anti-CII antibodies is not sufficient to induce arthritis. It has been reported that the injection of IL-1β is able to accelerate CIA (Caccese et al., 1992). Therefore, TNFα and IL-1β, although having similar properties, act in distinctive manners to develop arthritis. Additionally, MIP-2 could play an important role in the recruitment of neutrophils, which is highlighted as a possible contributor in the early phases of RA and animal arthritis models (Thornton et al., 1999; Wipke and Allen, 2001; Tanaka et al., 2006; Cascao et al., 2010; Wright et al., 2010; Dominical et al., 2011). Notably, NF-κB also regulates the gene expression of MIP-2 (Kim et al., 2003). The proinflammatory cytokines activate inflammatory cells including synovial cells, and induce production of autoantibodies and inflammatory mediators, such as cytokines, chemokines and proteases. The inflammatory responses induce further production of proinflammatory cytokines through formation of immune complexes and recruitment of inflammatory cells into the synovium. Their interplay might promote the generation of inflammatory loop responsible for progression of joint inflammation. Taken together, proinflammatory cytokines regulated by NF-κB in local joint tissues are one of the key factors in the development of arthritis *via* amplification of joint inflammation through acceleration of the inflammatory loop.

The NF-κB signaling pathway is important and necessary to the mechanisms underlying the function and pathogenesis of arthritis; however, the idea to apply NF-κB inhibitors, such as IKKβ inhibitors, for the therapy of RA is not yet validated. One of the reasons is that the systemic inhibition of the NF-κB pathway by chemicals should lead to tremendous numbers of side effects throughout the body because the NF-κB signaling

pathway functions in a variety of mammalian tissues other than pathogenic cells in patients with RA (Tak and Firestein, 2001; Senftleben and Karin, 2002; Viatour et al., 2005; Brown et al., 2008). Therefore, a rational approach is necessary to use the NF-κB inhibitor as a beneficial drug for a specific disease. Based on the findings in the present study, regulation of IKKβ in locally inflamed joint tissues may be a promising approach for the therapeutics of RA. Although several small molecule inhibitors of IKKβ have been identified, and the efficacy of each inhibitor has been clearly demonstrated in arthritis models (McIntyre et al., 2003; Podolin et al., 2005; Schopf et al., 2006; Mbalaviele et al., 2009), there is no report on IKKβ inhibitors which have been analyzed from the perspective of the relationship between the distribution of the compounds in tissue and the regulation of proinflammatory cytokines in the arthritic joints. In this regard, Compound D may be a useful agent because the compound may function in local inflammatory sites due to its preferential distribution in the arthritic paws compared to healthy ones.

In conclusion, I demonstrated that the NF-κB-dependent proinflammatory cytokines in local joint tissues amplify inflammation and contribute to the development of arthritis (summarized in **Figure 9**; page 86).

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Tables

Table 1 Fold change of mRNA of inflammatory mediators on Days 3 and 6.

Gene	Day 3	Day 6	
IL-1β	8.0 ± 2.2	21.5 ± 2.8	***
IL-6	21.2 ± 8.8	32.9 ± 6.6	*
IL-17A	7.2 ± 2.6	13.0 ± 2.9	**
RANKL	5.9 ± 1.2	20.4 ± 2.7	***
MIP-2	4.9 ± 1.6	16.4 ± 2.8	***
MMP-3	14.2 ± 3.8	48.3 ± 6.9	***
MMP-9	2.3 ± 0.5	19.5 ± 2.5	***

The experiment was performed as described in the legend to Figure 2A. The data are expressed as mean ± SE of fold changes over pre-LPS injection (*n*, pre = 8, the others = 12). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; vs. pre-LPS group by a Dunnett's test.

Table 2 Correlation between arthritis scores and expression level of inflammatory mediators.

Gene	Score 0	Score 1	Score 2	Score 3	*R*	
IL-1β	0.1 ± 0.0	0.7 ± 0.2	1.3 ± 0.5	2.1 ± 0.9	0.82	***
IL-6	0.1 ± 0.0	1.3 ± 0.4	1.4 ± 0.6	3.0 ± 1.2	0.75	***
IL-17A	0.1 ± 0.0	0.3 ± 0.1	0.7 ± 0.3	1.4 ± 0.6	0.62	***
RANKL	0.0 ± 0.0	0.3 ± 0.1	0.6 ± 0.3	1.2 ± 0.5	0.84	***
MIP-2	0.1 ± 0.0	0.2 ± 0.1	0.6 ± 0.3	1.6 ± 0.6	0.79	***
MMP-3	1.3 ± 0.5	8.5 ± 2.4	10.0 ± 4.1	26.3 ± 10.7	0.89	***
MMP-9	0.6 ± 0.2	2.8 ± 0.8	5.6 ± 2.3	12.3 ± 5.0	0.78	***

The experiment was performed as described in the legend to Figure 2B. The data are expressed as mean ± SE of relative expression values against each GAPDH expression level (*n*, score 0 = 8, score 1 = 12, scores 2 and 3 = 6). The correlations between the mRNA levels and arthritis scores are determined using a Spearman's rank order correlation test. *R*, correlation coefficient (***, $P < 0.001$).

Table 3 Signaling pathway selectivity of Compound D in reporter assay.

Target	IC_{50} (μM)
NF-κB	0.15
AP-1	>20
NFAT	>20
SRE	19
CRE	>20
ISRE	16
Viability (24 h)	>20

293T cells transfected with each reporter luciferase plasmid were treated with various concentrations of Compound D and stimulated with each stimulant as described in Materials and Methods. After 6 h (TNFα) or 24 h (the others), the luciferase activities were measured. Viability of untransfected 293T cells treated with Compound D was evaluated at 24 h using WST-8.

Table 4 Concentrations of Compound D in the plasma 1.5 h after a single oral administration.

Dose (mg/kg)	Plasma concentration of Compound D (μM)
10	0.39 ± 0.18
30	2.9 ± 0.95
100	9.4 ± 4.3

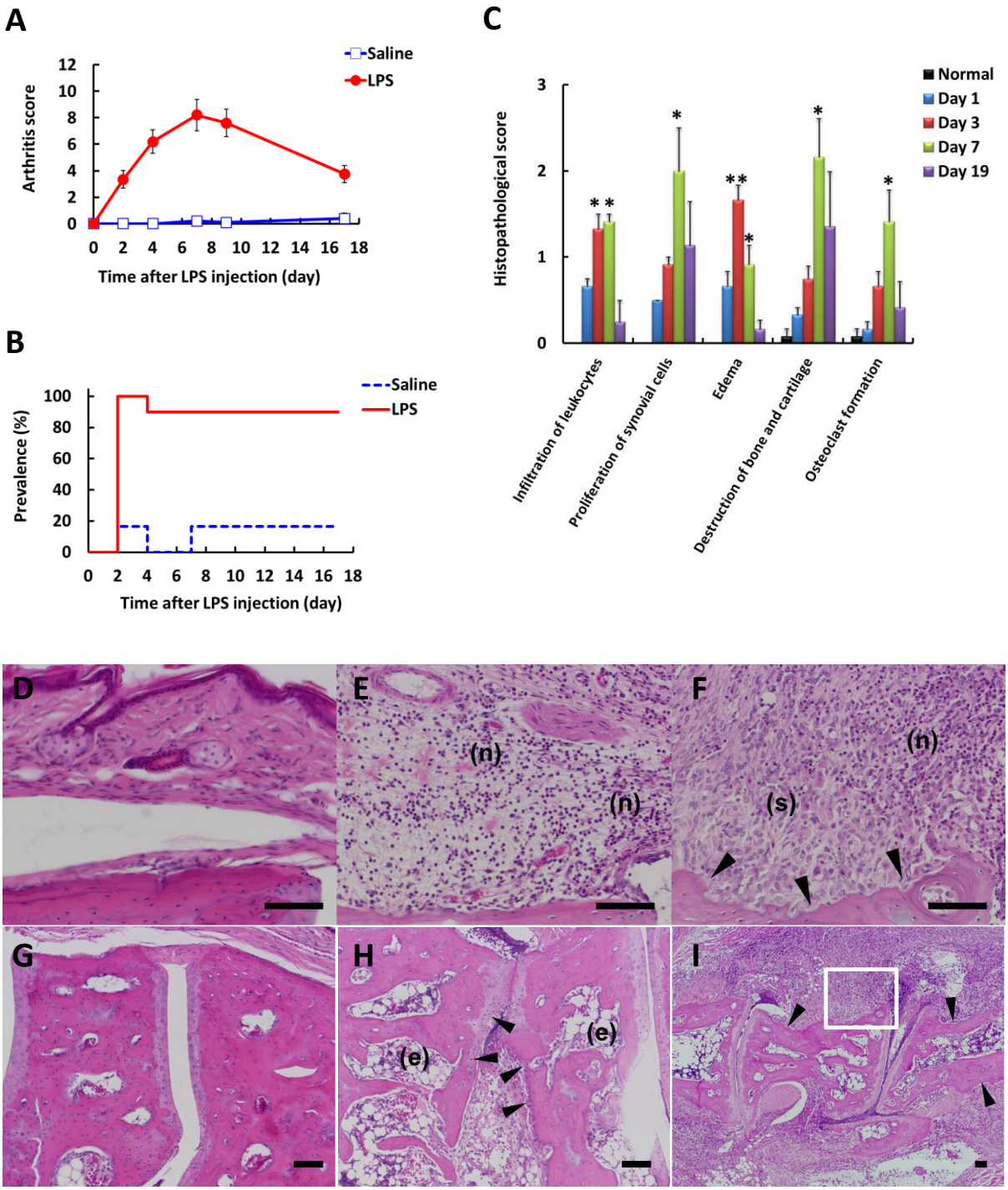
The experiment was performed as described in the figure legend to Figure 7B. The data are expressed as mean ± SD of five mice in each group.

Table 5 Gene expressions in paws of CIA mice after the consecutive treatment of Compound D.

Gene	Normal	Arthritis + Vehicle	Arthritis + 30 mg/kg Compound D	Arthritis + 100 mg/kg Compound D
TNFα	0.03 ± 0.01	0.47 ± 0.19	0.49 ± 0.20	0.27 ± 0.09
IL-1β	0.01 ± 0.01	2.55 ± 1.36	0.59 ± 0.35	0.53 ± 0.26
IL-6	0.00 ± 0.00	4.79 ± 3.07	0.47 ± 0.26	0.26 ± 0.11
RANKL	0.00 ± 0.00	2.13 ± 1.34	0.34 ± 0.20	0.13 ± 0.10
MMP-3	0.00 ± 0.00	2.29 ± 1.12	0.54 ± 0.33	0.21 ± 0.08
MMP-9	0.00 ± 0.00	6.80 ± 4.05	1.14 ± 0.76	0.72 ± 0.34

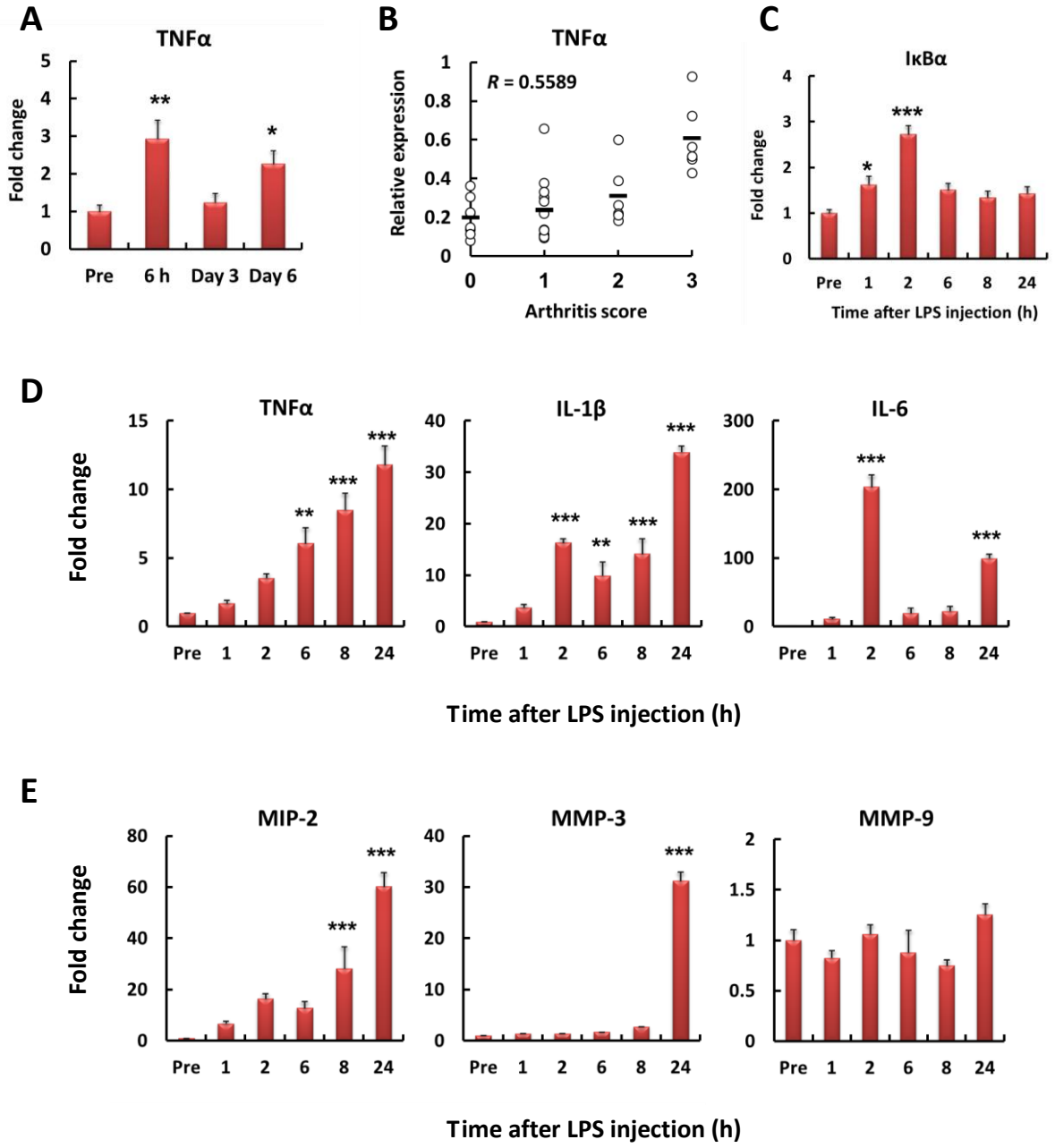
The experiment was performed as described in the figure legend to Figure 8A. Gene expressions in the paws of CIA mice 14 days after the 2nd immunization are shown as relative expression values against each GAPDH expression level. The data are expressed as mean ± SE of seven mice in each group. No values were statistically significant compared to vehicle-treated group ($P > 0.05$, by a parametric Dunnett's test).

Figures


A
Saline
LPS
Arthritis score
Time after LPS injection (day)
B
Saline
LPS
Prevalence (%)
Time after LPS injection (day)
C
Normal
Day 1
Day 3
Day 7
Day 19
Histopathological score
Infiltration of leukocytes
Proliferation of synovial cells
Edema
Destruction of bone and cartilage
Osteoclast formation
D
E
(n)
(n)
F
(n)
(s)
G
H
(e)
(e)
I

(Figure 1)

Figure 1 Time course of arthritis development induced by an LPS injection in CII-immunized mice. The mice were injected with LPS or saline 17 days after CII immunization. Arthritis severity and histopathology were periodically evaluated. (A) Arthritis scores are expressed as mean ± SE of five (saline) or ten (LPS) mice in each group. (B) Prevalence of paw inflammation is expressed as a percentage of the number of mice in each group with at least one affected paw. (C) Histopathological scores of tarsal joints. The data are expressed as mean + SE (n = 3). *, $P < 0.05$; **, $P < 0.01$; vs. normal group by a nonparametric Dunnett's test. (D-I) Representative histologies of synovium (D-F) and bone and cartilage (G-I) of the tarsal joints stained with hematoxylin and eosin of normal mice (D, G), LPS-CIA mice on Day 3 (E, H) and Day 7 (F, I). Neutrophils (n), edema (e), synovial cell (s) and bone destruction (arrowhead) are indicated. Original magnification, (D-F) 200×, (G, H) 100×, (I) 40×. (F) is a photograph of the inset in (I) at higher magnification. Scale bars, 100 μm.


A
TNFα
Fold change
Pre
6 h
Day 3
Day 6
B
TNFα
R = 0.5589
Relative expression
Arthritis score
C
IκBα
Fold change
Time after LPS injection (h)
D
TNFα
IL-1β
IL-6
Fold change
Time after LPS injection (h)
E
MIP-2
MMP-3
MMP-9
Fold change
Time after LPS injection (h)

(Figure 2)

Figure 2 Gene expression level in paws of LPS-CIA mice. Expression levels of various mRNAs in the paws of LPS-CIA mice were measured by quantitative real-time PCR in specimens obtained pre- or on various days after LPS injection. The data were normalized with expression level of GAPDH. (A) The mRNA level of TNFα on pre-LPS injection, at 6 h, and on Days 3 and 6. The data are expressed as mean + SE of fold changes over pre-LPS injection (*n*, pre = 8, the others = 12). *, $P < 0.05$; **, $P < 0.01$; vs. pre-LPS group by a Student's *t*-test. (B) Correlation between arthritis scores and expression level of mRNA of TNFα on Days 0, 3 and 6. Horizontal bars represent the mean of mRNA expression for each score (*n*, score 0 = 8, score 1 = 12, scores 2 and 3 = 6). The *R*-values represent Spearman's rank order correlation coefficients. (C-E) The mRNA levels of IκBα and inflammatory mediators at indicated times after LPS injection. The data are expressed as mean + SE of fold changes over pre-LPS injection ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; no asterisk, not significant vs. pre-LPS group by a parametric Dunnett's test.

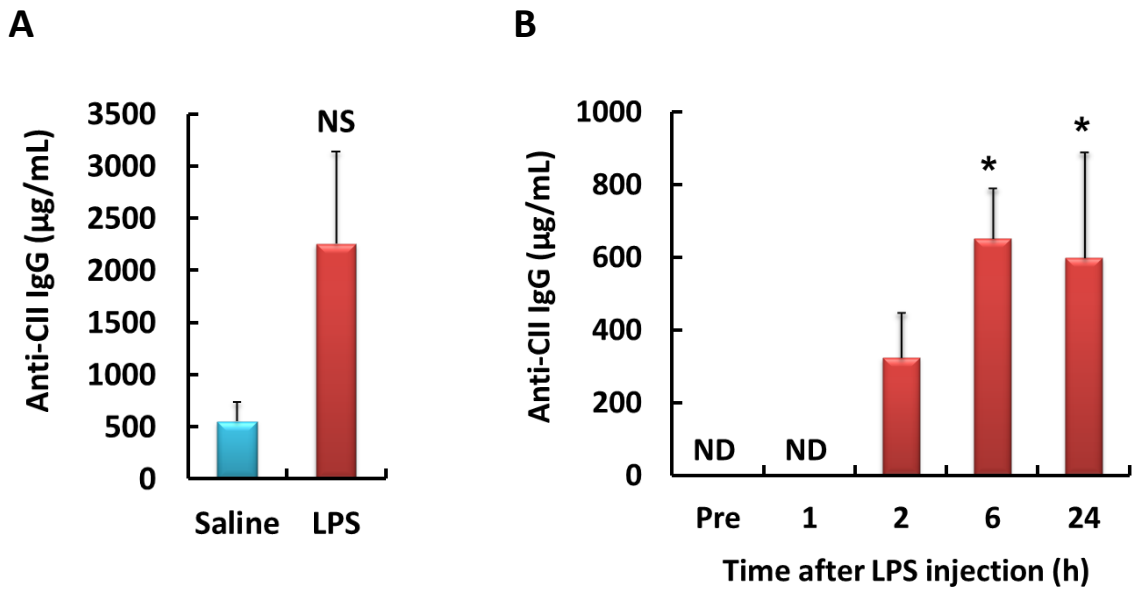


Figure 3 Serum anti-CII antibody concentrations in LPS-CIA mice. Serum was obtained at the indicated time points after LPS injection. Anti-CII IgG concentrations in the serum were measured by ELISA. (A) Anti-CII IgG concentrations 6 days after LPS or saline injection. The data are expressed as mean + SE (n = 6). NS, not significant vs. pre-LPS group by a Student's t-test. (B) Anti-CII IgG concentrations at indicated times after LPS injection. The data are expressed as mean + SE (n = 5). *, $P < 0.05$; vs. pre-LPS group by a parametric Dunnett's test. ND, not detected.

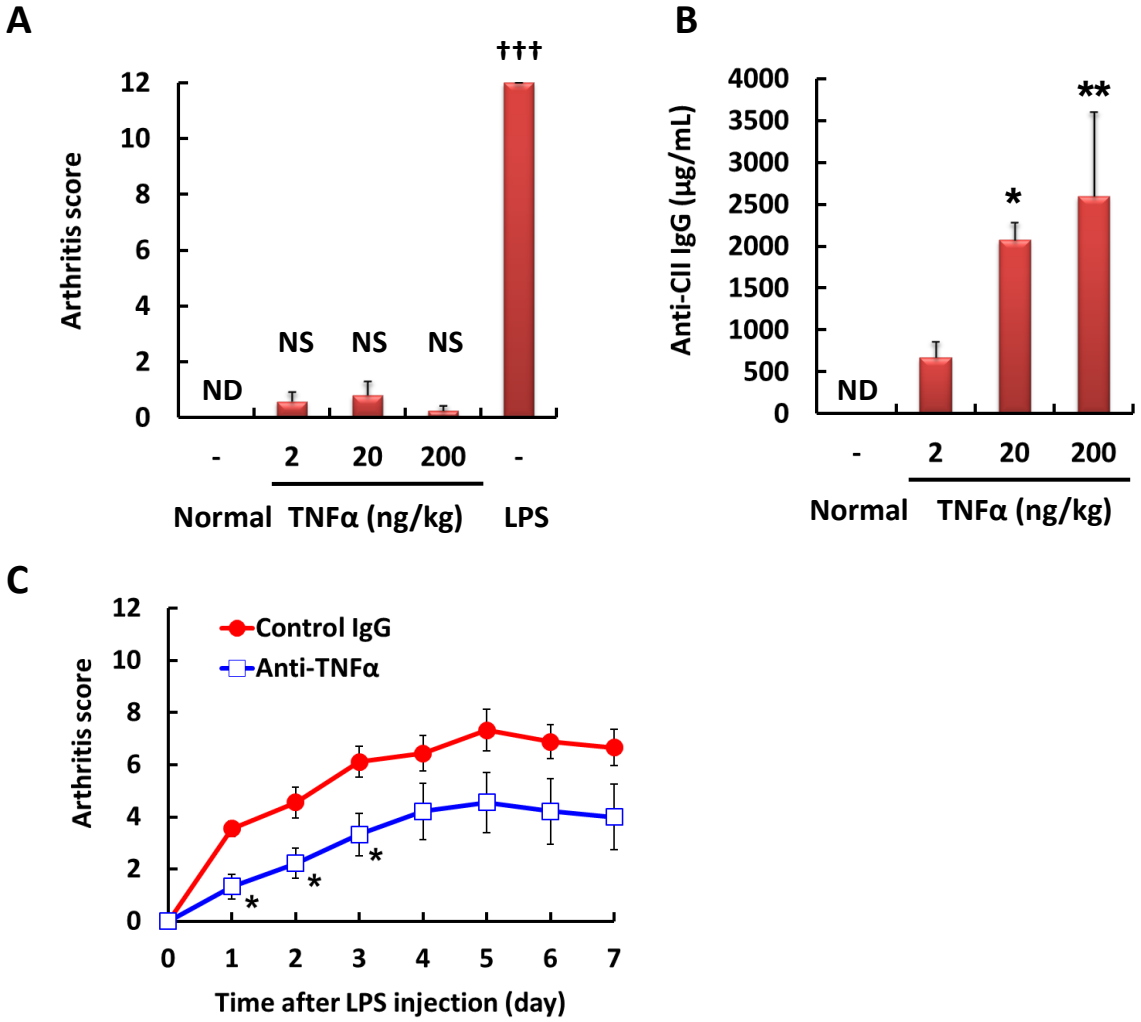


Figure 4. Involvement of TNFα in development of LPS-CIA. (A, B) Recombinant mouse TNFα was injected into CII-immunized mice. Arthritis scores (A) and serum anti-CII IgG concentrations (B) on Day 6 are expressed as mean + SE (*n*, normal = 8, 20 ng/kg TNFα = 5, the others = 6). NS, not significant; vs. normal group by a nonparametric Dunnett's test, †††, $P < 0.001$; vs. normal group by a Wilcoxon's rank sum test, *, $P < 0.05$; **, $P < 0.01$; vs. normal group by a parametric Dunnett's test. ND, not detected. (C) Anti-TNFα antibodies or control IgG (25 mg/kg) were administered intraperitoneally once daily from the day before to 6 days after LPS injection. Arthritis scores are expressed as mean ± SE ($n = 9$). *, $P < 0.05$; vs. control IgG group by a Wilcoxon's rank sum test.

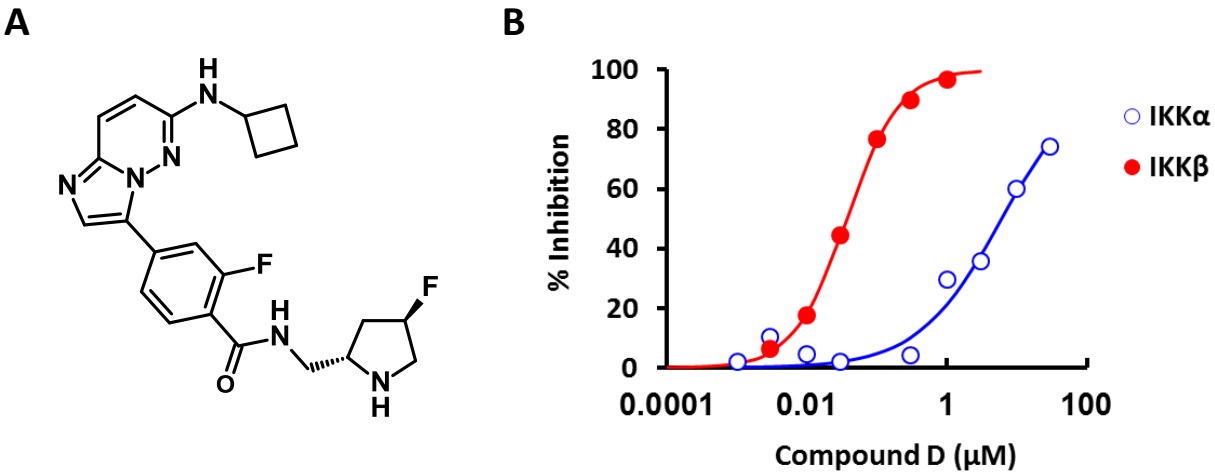


Figure 5 Compound D is a selective inhibitor of IKKβ. (A) Chemical structure of Compound D. (B) Inhibition of kinase activities of IKKβ and IKKα measured by an IMAP^{TM} assay using ATP at the concentrations around *Km* for each kinase. The data are expressed as the mean percentage of inhibition over the vehicle control group.

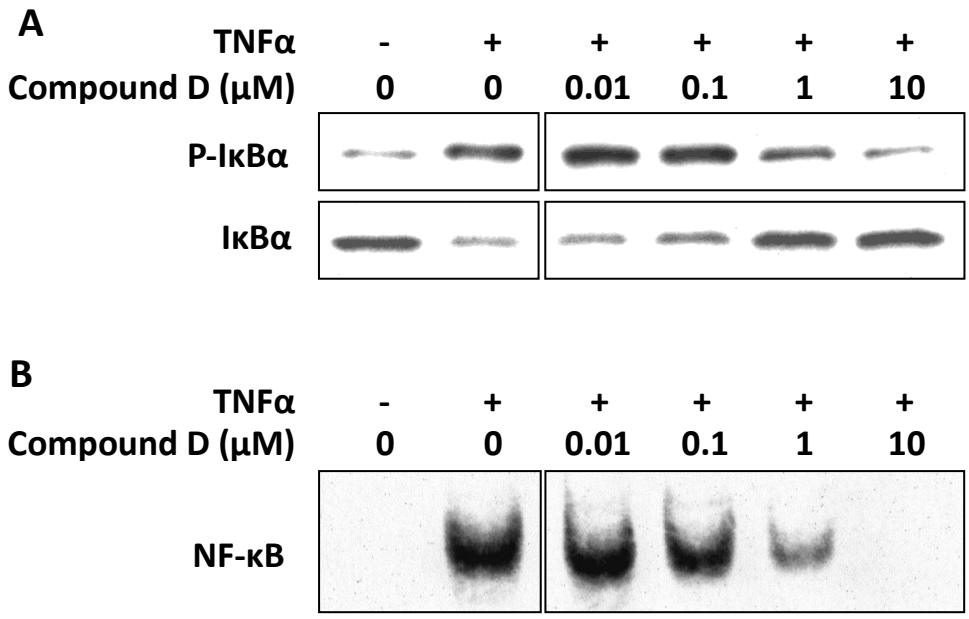


Figure 6 Compound D inhibits the NF-κB signaling pathway. (A) Inhibition of IκBα phosphorylation and degradation induced by TNFα. HeLa cells were starved with serum-free medium for 1 day and pretreated with or without Compound D, followed by stimulation with 10 ng/mL of TNFα for 10 min (IκBα phosphorylation) or 15 min (IκBα degradation). Phosphorylated IκBα (upper panel) and total IκBα (lower panel) in the cell lysates were detected by Western blotting. (B) Inhibition of NF-κB-DNA binding induced by TNFα. HeLa cells prepared and pretreated with Compound D as described above were stimulated with 10 ng/mL of TNFα for 30 min. Nuclear extracts were prepared and NF-κB-DNA binding was detected by an electrophoretic mobility shift assay.

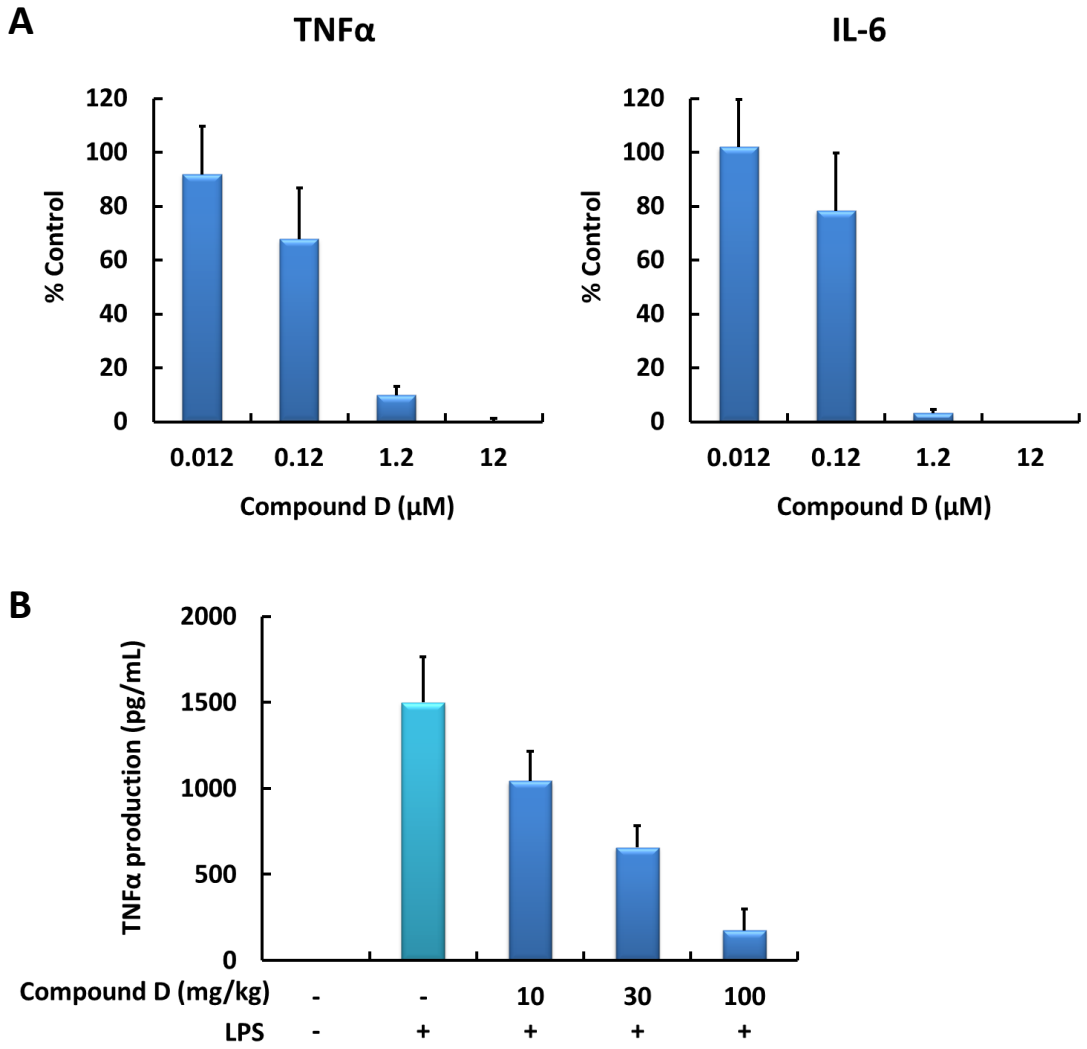
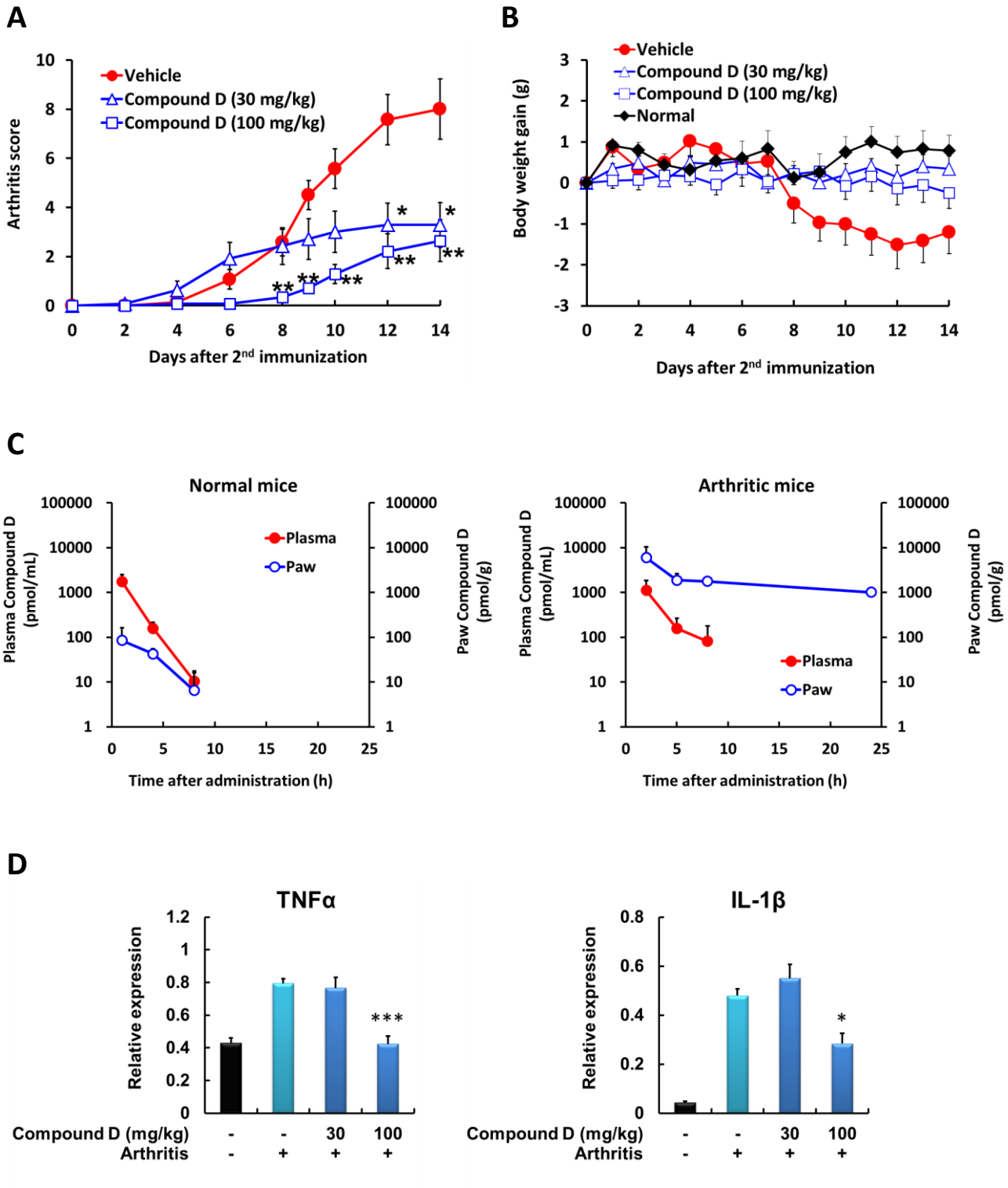


Figure 7 Compound D inhibits LPS-induced production of proinflammatory cytokines *in vitro* and *in vivo*. (A) Inhibition of cytokine production in human blood cells. The blood cells from healthy adult volunteers were incubated with 1 μg/mL of LPS for 4 h (TNFα) or 24 h (IL-6) in the presence or absence of Compound D. TNFα and IL-6 concentrations in the supernatants were measured. The data are shown as a percentage over the vehicle control group and expressed as mean + SD of three independent experiments. (B) Inhibition of TNFα production *in vivo*. BALB/c mice were orally given Compound D or vehicle for 30 min, followed by an injection of 0.02 mg/kg of LPS, and 1 h later, the plasma was collected to measure TNFα levels. The data are expressed as mean + SD of ten (vehicle) or five (Compound D) mice in each group. **, $P < 0.01$; ***, $P < 0.001$; vs. vehicle-treated group by a parametric Dunnett's test.


A
Arthritis score
Vehicle
Compound D (30 mg/kg)
Compound D (100 mg/kg)
Days after 2nd immunization
B
Body weight gain (g)
Vehicle
Compound D (30 mg/kg)
Compound D (100 mg/kg)
Normal
Days after 2nd immunization
C
Normal mice
Plasma Compound D (pmol/mL)
Paw Compound D (pmol/g)
Plasma
Paw
Time after administration (h)
Arthritic mice
Plasma Compound D (pmol/mL)
Paw Compound D (pmol/g)
Plasma
Paw
Time after administration (h)
D
TNFα
Relative expression
Compound D (mg/kg)
Arthritis
IL-1β
Relative expression
Compound D (mg/kg)
Arthritis

(Figure 8)

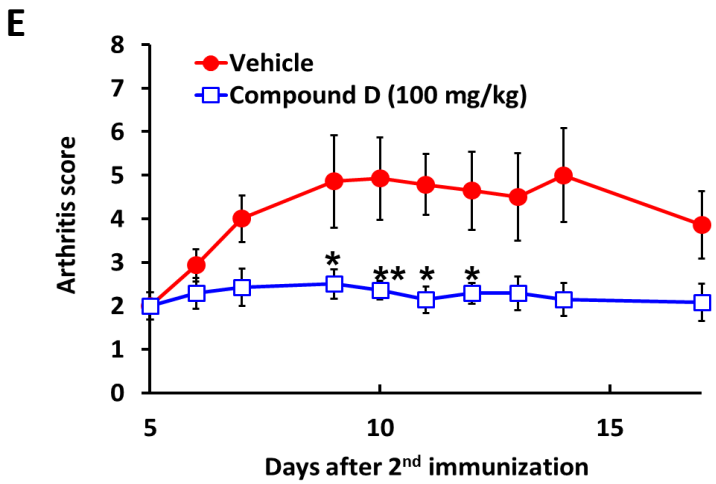


Figure 8 Compound D inhibits CIA in association with downregulation of proinflammatory cytokines in arthritic paws. DBA1/J mice were immunized with CII, and followed 21 days later by a 2nd immunization with CII. (A, B) Compound D or vehicle was orally administered once daily from the day of the 2nd immunization to Day 13. Arthritis scores (A) and body weight gains (B) are expressed as mean ± SE of seven mice in each group. *, $P < 0.05$; **, $P < 0.01$, vs. vehicle-treated group by a nonparametric Dunnett's test. (C) Pharmacokinetic analyses of Compound D (30 mg/kg) in normal and CIA mice (10 days after the 2nd immunization). The plasma and paws were collected at indicated times after a single oral administration of Compound D to measure the compound levels. The data are expressed as mean ± SD of three (normal) or five (arthritic) mice in each group. (D) Gene expressions of proinflammatory cytokines in the paws of arthritic mice treated with a single dosing of Compound D. The paws of CIA mice (9 days after the 2nd immunization) were obtained 2 h after oral administration of Compound D. Gene expressions of TNFα and IL-1β in the paw homogenates were measured by quantitative PCR and shown as relative expression values against each GAPDH expression level. The data are expressed as mean + SE of five mice in each group. *, $P < 0.05$; ***, $P < 0.001$; vs. vehicle-treated group by a parametric Dunnett's test. (E) Compound D or vehicle was orally administered once daily from 5 days after the 2nd immunization to Day 16 and arthritis severities were scored periodically. The data are expressed as mean ± SE of seven mice in each group. *, $P < 0.05$; **, $P < 0.01$, vs. vehicle-treated group by a nonparametric Dunnett's test.

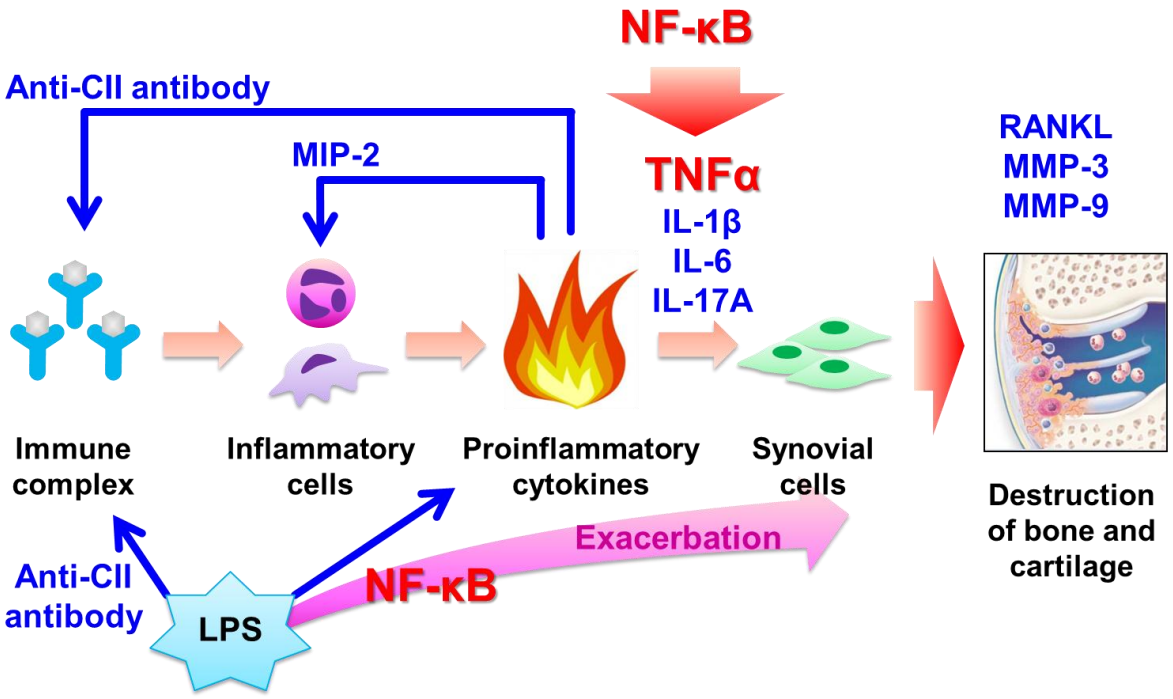


Figure 9 Summary of the role of proinflammatory cytokines in joint tissues during development of arthritis. Immune complexes formed with autoantibodies induce recruitment and activation of inflammatory cells, such as neutrophils and macrophages, into the synovium. These infiltrated inflammatory cells produce proinflammatory cytokines, such as TNFα, IL-1β, IL-6 and IL-17A, which activate inflammatory cells including synovial cells. The synovial cells proliferate and progress to hyperplasia. The proinflammatory cytokines also induce further production of autoantibodies and inflammatory mediators, such as cytokines, chemokines and proteases including MMPs and RANKL, which contribute to destruction of bone and cartilage. The present study has demonstrated that the proinflammatory cytokines in the joint tissues are regulated by NF-κB and involved in amplification of joint inflammation (blue arrows). The proinflammatory cytokines, in particular TNFα, in addition to anti-CII antibodies are rapidly induced during LPS-induced exacerbation of arthritis. TNFα may contribute in the production of anti-CII antibodies. Chemokine MIP-2 is also upregulated in association with neutrophil infiltration in the onset of arthritis. Taken together, the NF-κB-dependent proinflammatory cytokines in arthritic joint tissues are a key factor during the development of arthritis.