

# Effect of Nuclear Factor- $\kappa$ B Inhibition on Rheumatoid Fibroblast-like Synoviocytes and Collagen Induced Arthritis

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**ABSTRACT. Objective.** The nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway has been implicated as a molecular target for the treatment of various inflammatory diseases, such as rheumatoid arthritis (RA). In particular, I $\kappa$ B kinase (IKK) is considered an important molecular target because the majority of inflammatory signaling pathways mediated by NF- $\kappa$ B involve IKK activation. We investigated the effect of NF- $\kappa$ B inhibition on rheumatoid fibroblast-like synoviocytes (FLS) and collagen induced arthritis.

**Methods.** We evaluated the effect of IMD-0560, an inhibitor of IKK, on rheumatoid FLS *in vitro* and on collagen type II induced arthritis in mice.

**Results.** IMD-0560 suppressed the nuclear translocation of NF- $\kappa$ B and phosphorylation of I $\kappa$ B $\alpha$  induced by tumor necrosis factor- $\alpha$  in FLS. In addition, this compound suppressed the production of inflammatory cytokines, including interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein-1. IMD-0560 also inhibited the proliferation of FLS without showing cellular toxicity. Finally, this compound was effective against collagen induced arthritis in mice.

**Conclusion.** Based on these results, IMD-0560 could be a new therapeutic agent for RA. (J Rheumatol 2005;32:1440–7)

*Key Indexing Terms:*

COLLAGEN-INDUCED ARTHRITIS  
RHEUMATOID ARTHRITIS

NUCLEAR FACTOR- $\kappa$ B  
FIBROBLAST-LIKE SYNOVIOCYTES

Rheumatoid arthritis (RA) is characterized by synovial hyperplasia with infiltration of various inflammatory cells and high expression of nuclear factor  $\kappa$ -B (NF- $\kappa$ B). Expression of NF- $\kappa$ B in the synovium of patients with RA is much higher than in healthy subjects or patients with osteoarthritis<sup>1,2</sup>. Within the joints, activation of NF- $\kappa$ B leads to the expression of inflammatory mediators, such as cytokines, chemokines, and adhesion molecules, that play an important role in the pathogenesis of RA<sup>3</sup>. Therapeutic

strategies for inhibiting the NF- $\kappa$ B pathway are considered promising because activation of NF- $\kappa$ B helps mediate the prolonged inflammation of RA<sup>4,5</sup>.

NF- $\kappa$ B is a dimeric transcription factor of the Rel family that is thought to play an important role in a variety of inflammatory responses<sup>6</sup>. This protein is usually found in the cytoplasm in an inactive state, bound to the inhibitory protein I $\kappa$ B<sup>7</sup>. Extracellular stimuli activate the I $\kappa$ B kinase (IKK) complex, which consists of at least 3 subunits: IKK- $\alpha$ , IKK- $\beta$ , and the regulatory subunit IKK- $\gamma$ <sup>8</sup>. IKK phosphorylates I $\kappa$ B at specific N-terminal serine residues, and phosphorylated I $\kappa$ B is then ubiquitinated and degraded by the 26S proteasome. The degradation of I $\kappa$ B results in the translocation of free NF- $\kappa$ B from the cytoplasm to the nucleus, where it induces transcription of proinflammatory genes<sup>9</sup>.

Several antirheumatic drugs currently in clinical use can inhibit the activation of NF- $\kappa$ B. This supports the concept that NF- $\kappa$ B is involved in the pathogenesis of RA. For instance, glucocorticoids can increase the production of I $\kappa$ B<sup>10</sup>, and aspirin<sup>11</sup>, sodium salicylate<sup>11</sup>, gold compounds<sup>12</sup>, sulfasalazine<sup>13</sup>, methotrexate<sup>14</sup>, and the active metabolite of leflunomide, A771726<sup>15</sup>, can prevent I $\kappa$ B degradation by inhibiting IKK activation. Further, specific inhibition of NF- $\kappa$ B activities *in vivo*, either by intraarticular administration of NF- $\kappa$ B decoy oligonucleotides<sup>16</sup> or by

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a gene therapy approach using a dominant negative IKK- $\beta$  gene<sup>17</sup>, leads to amelioration of collagen type II induced arthritis (CIA) in mice. Therefore, NF- $\kappa$ B targeted therapeutic strategies are thought to be effective for RA.

We investigated the potential of IMD-0560, an inhibitor of IKK, to treat RA. We examined the effects of this compound on the function of rheumatoid fibroblast-like synoviocytes (FLS) and on the development of arthritis in the mouse CIA model.

## MATERIALS AND METHODS

**Drugs.** IMD-0560 (N-[2,5-bis-trifluoromethylphenyl]-5-bromo-2-hydroxybenzamide; molecular weight 428.1) was kindly provided by the Institute of Medical Molecular Design, Tokyo, Japan. The drug was dissolved in dimethyl sulfoxide (DMSO). Controls for each experiment contained DMSO alone.

**Fibroblast-like synoviocytes.** Synovial tissues were obtained from patients with RA at the time of joint surgery. All patients satisfied the 1987 revised criteria of the American College of Rheumatology<sup>18</sup>. Written informed consent was obtained from all patients. Synovial tissues were minced into small pieces and treated with 0.1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO, USA; C2674), 0.1 mg/ml hyaluronidase (Sigma-Aldrich; H4272), and 0.1 mg/ml DNase I in 50 ml RPMI-1640 (Sigma-Aldrich) for 1 h at 37°C. Dissociated cells were passed through a metal filter, centrifuged, and washed twice. The cells were then suspended with culture medium consisting of RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 50 U/ml penicillin, 50 U/ml streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA). The cells were seeded in 80 cm<sup>2</sup> culture flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells used in the experiments were from the fourth through tenth passages.

**Preparation of cytoplasmic and nuclear extracts.** FLS ( $1.5 \times 10^6$ ) were treated with various concentrations of IMD-0560 for 2 h and then stimulated with 10 ng/ml tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; Techne-Genzyme, Minneapolis, MN, USA). Cytoplasmic and nuclear extracts were prepared as described<sup>19</sup>. Briefly, cells were detached with a scraper, resuspended in 100  $\mu$ l of cytoplasmic lysis buffer, and incubated on ice for 15 min. After centrifugation, the supernatants (cytoplasmic fraction) were stored at -80°C. The pellets, which contained the nuclei, were resuspended in 25  $\mu$ l nuclear lysis buffer and incubated on ice for 30 min. After centrifugation, supernatants (nuclear fractions) were collected and stored at -80°C until use. Protein concentrations were determined using the Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO, USA).

**Electrophoretic mobility shift assay (EMSA).** EMSA was performed using the Gelshift kit for NF- $\kappa$ B p65 (Geneka Biotechnology, Montreal, QC, Canada). The sequence of the DNA probe for the NF- $\kappa$ B binding site was 5'-AGC TTG GGG TAT TTC CAG CCG-3' (underlined letters represent the consensus binding sequence). The NF- $\kappa$ B probe was labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (Invitrogen) for 30 min at 37°C. The kinase reaction was stopped with 1% sodium dodecyl sulfate (SDS)/100 mM EDTA solution. Labeled probe was purified with a MicroSpin™ G25 column (Geneka Biotechnology). The protein-bound DNA probe was resolved by nondenaturing electrophoresis in a 5% polyacrylamide-Tris-glycine buffered gel. In some experiments, a supershift assay was performed using polyclonal antibodies specific for each NF- $\kappa$ B subunit.

**Western blot analysis.** Identical amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Amersham Life Sciences, Cleveland, OH, USA). The membrane was incubated with polyclonal rabbit antibodies to human I $\kappa$ B $\alpha$  or phospho-I $\kappa$ B $\alpha$  (Cell Signaling Technology, Beverly, MA, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit IgG antibodies were

used as secondary antibodies and were visualized with the Phototope-HRP Western detection system (Cell Signaling Technology) and detected with Hyperfilm (Amersham).

**In vitro kinase assay.** Immunoprecipitation and *in vitro* kinase assay was performed as described<sup>19</sup>. Briefly, the cytoplasmic extracts were immunoprecipitated with anti-IKK- $\alpha$  antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Half of the immunoprecipitates were suspended in 20  $\mu$ l kinase buffer containing 2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, 10  $\mu$ M unlabeled ATP, and 2.5  $\mu$ g substrate GST-I $\kappa$ B $\alpha$ (1-317) (Santa Cruz Biotechnology), and incubated at 30°C for 30 min. Samples were resolved on 10% SDS-polyacrylamide gels, and the level of phosphorylation of GST-I $\kappa$ B $\alpha$  was analyzed by autoradiography. To evaluate the amount of IKK- $\alpha$  and IKK- $\beta$  in each sample, the other half of each of the immunoprecipitates was resolved on 10% SDS-polyacrylamide gels, and Western blot analysis was performed using anti-IKK- $\alpha$  or anti-IKK- $\beta$  (Cell Signaling Technology).

**Reporter gene assay.** Human full-length IKK- $\beta$  sequences were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned in-frame into the mammalian amino-terminal FLAG-expression vector, pFLAG-CMV (Sigma-Aldrich). Site directed mutagenesis was performed to make a constitutively active mutant (S177E/S181E) vector.

Human embryonic kidney (HEK293T) cells ( $1.5 \times 10^5$ ) were transiently transfected with 30 ng p-FLAG-CMV-IKK- $\beta$  (S177E/S181E) vector, 120 ng p-NF- $\kappa$ B-Luc vector (Stratagene, La Jolla, CA, USA), and 10 ng pCMV- $\beta$ Gal vector (Clontech, Palo Alto, CA, USA) using Fugene6 (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. After 24 h, the cells were treated with fresh medium containing various concentrations of IMD-0560. Cell lysates were prepared 48 h post-transfection, and luciferase activity was measured with Picagene LT2.0 (Toyo Ink, Tokyo, Japan) according to manufacturer's instructions.  $\beta$ -galactosidase activity was assayed with 4-methylumbelliferyl- $\beta$ -D-galactoside (Sigma-Aldrich) as a substrate, and the fluorescence was determined with an excitation wavelength 360 nm and an emission wavelength 465 nm. Activity was expressed as relative light units and normalized for  $\beta$ -galactosidase activity.

**Enzyme linked immunosorbent assay.** FLS ( $6 \times 10^4$ ) were incubated with various concentrations of IMD-0560 for 1 h. The cells were further incubated for 24 h in the presence or absence of TNF- $\alpha$  (10 ng/ml). Culture supernatants were obtained, and the cytokine concentrations were determined by ELISA. Interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1) were measured in duplicate using an OptiEIA kit (BD Pharmingen, San Diego, CA, USA).

**Collagen induced arthritis.** On Day 1, male DBA/1J (6 weeks old) mice were immunized with bovine type II collagen (Chondrex, Seattle, WA, USA) emulsified with an equal volume of complete Freund's adjuvant. The mice were administered a booster injection of bovine type II collagen and incomplete Freund's adjuvant on Day 21. IMD-0560 (1 or 3 mg/kg every 48 h) or vehicle (10  $\mu$ l/g every 48 h) was administered intraperitoneally from the first immunization until the end of the study (n = 10). IMD-0560 was not administered at 10 mg/kg because it was not stable at this concentration. Clinical arthritis scores were evaluated using a scale of 0 to 4 for each paw, giving a maximum score of 16 per mouse: Grade 0, normal; grade 1, erythema and swelling in single joint; grade 2, erythema and swelling in > 2 joints; grade 3, erythema and swelling of the entire paw; grade 4, deformity and/or ankylosis. The clinical arthritis scores were assessed twice per week. Forty-nine days after the first immunization, mice were sacrificed, and both knees and hind paws from selected groups were fixed in 10% formalin solution, decalcified, and embedded in paraffin. Paraffin sections of the tissues were stained with hematoxylin and eosin (H&E). As described<sup>20</sup>, the severity of pathological findings of CIA was evaluated using 4 measures: synovial hypertrophy, pannus formation, and cartilage and bone erosion. Each indicator was scored on a scale from 0 to 3. An investigator blinded to the treatment performed clinical and histological assessments.

**Cell proliferation assay.** The effect of IMD-0560 on the proliferation of

FLS was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation using a Biotrak cell proliferation ELISA system (Amersham). Briefly,  $5 \times 10^3$  FLS were incubated 36 h with various concentrations of IMD-0560 in triplicate wells of a 96-well plate. Next, BrdU was added to the wells, and after 4 h incubation cells were fixed and the DNA was denatured by addition of fixative. Peroxidase labeled anti-BrdU antibody was added and peroxidase activity was detected using 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate. The resultant color was read at 450 nm using a microplate photometer (Model 550; BioRad, Hercules, CA, USA).

**Cell-cycle analysis.** FLS ( $5 \times 10^5$ ) were precultured 24 h in RPMI-1640 containing 0.3% bovine serum albumin (Sigma-Aldrich). Serum-starved FLS were subsequently divided into 4 samples. One was immediately fixed with 70% ethanol, and the others were further incubated in culture medium containing 10% FBS for 24 h with various concentrations of IMD-0560. Cells were resuspended in phosphate-buffered saline containing 40  $\mu$ g/ml propidium iodide and 200  $\mu$ g/ml RNase type IIA (Sigma-Aldrich), and their DNA contents were measured by flow cytometry using an Epics XL (Coulter, Hialeah, FL, USA) as described<sup>21</sup>. The cell-cycle distribution was determined using the MultiCycle program (Coulter).

**Cytotoxicity assay.** FLS ( $1 \times 10^5$ ) cultured in 96 well plates were treated with IMD-0560 (10  $\mu$ M) for 24 h. The cytotoxicity was evaluated by lactate dehydrogenase (LDH) release from injured cells. LDH activity in the culture medium was determined using a commercial kit (Wako, Osaka, Japan) as described by the manufacturer.

**Statistical analyses.** All values are expressed as the mean  $\pm$  standard error. Statistical analyses were performed using Dunnet's multiple comparison test or the Mann-Whitney U test. P values < 0.05 were considered significant.

## RESULTS

**Inhibition of NF- $\kappa$ B activation.** To investigate the pharmacological characteristics of IMD-0560 mediated inhibition of I $\kappa$ B phosphorylation, we performed a reporter gene assay using a constitutively active IKK- $\beta$  mutant. IMD-0560 inhibited in a dose-dependent manner the activated expression of NF- $\kappa$ B in HEK293T cells transfected with the p-FLAG-CMV-IKK- $\beta$  (S177E/S181E) vector (Figure 1A). Further, as shown in Figure 1B, pretreatment with IMD-0560 dose-dependently suppressed the DNA binding activity of NF- $\kappa$ B. Supershift experiments confirmed that p65 and p50 were the predominant subunits involved (data not shown). Treatment with IMD-0560 dose-dependently suppressed TNF- $\alpha$  induced I $\kappa$ B $\alpha$  phosphorylation. Western blotting showed that 20  $\mu$ M MG132 completely blocked I $\kappa$ B $\alpha$  degradation and that IMD-0560 had no effect on I $\kappa$ B $\alpha$  protein levels (Figure 1C). IMD-0560 also suppressed TNF- $\alpha$  induced IKK activity in a dose-dependent manner. IMD-0560 had no effect on IKK- $\alpha$  and IKK- $\beta$  protein levels, as shown by Western blot analysis (Figure 1D). Together, these findings suggest that IMD-0560 can inhibit the phosphorylation of I $\kappa$ B $\alpha$  by reducing IKK- $\beta$  activity, thereby preventing subsequent NF- $\kappa$ B activation.

**Inhibition of proinflammatory cytokine production.** TNF- $\alpha$  stimulation induced the production of IL-6, IL-8, and MCP-1 from rheumatoid FLS by 4-, 50-, and 8-fold, respectively. Further, as shown in Figure 2, IMD-0560 inhibited this production of IL-6, IL-8, and MCP-1. The concentrations of IMD-0560 causing 50% inhibition (IC<sub>50</sub>) of IL-6, IL-8, and

MCP-1 production were roughly 8, 6, and 2  $\mu$ M, respectively.

**Suppression of mouse CIA by IMD-0560.** As shown in Figures 3A and 3B, treatment with IMD-0560 dose-dependently decreased the incidence and severity of CIA. These results were confirmed by histological assessment of H&E stained sections of knee joints from control and IMD-0560 (3 mg/kg) treated mice (Figure 3C). The histological evaluation of the control mice (untreated) showed severe arthritis, with synovial cell proliferation, pannus formation, cartilage and bone erosion, and massive mixed cell infiltration. In contrast, in the IMD-0560 treated mice, the degree of pathological findings was significantly reduced (Figure 3D).

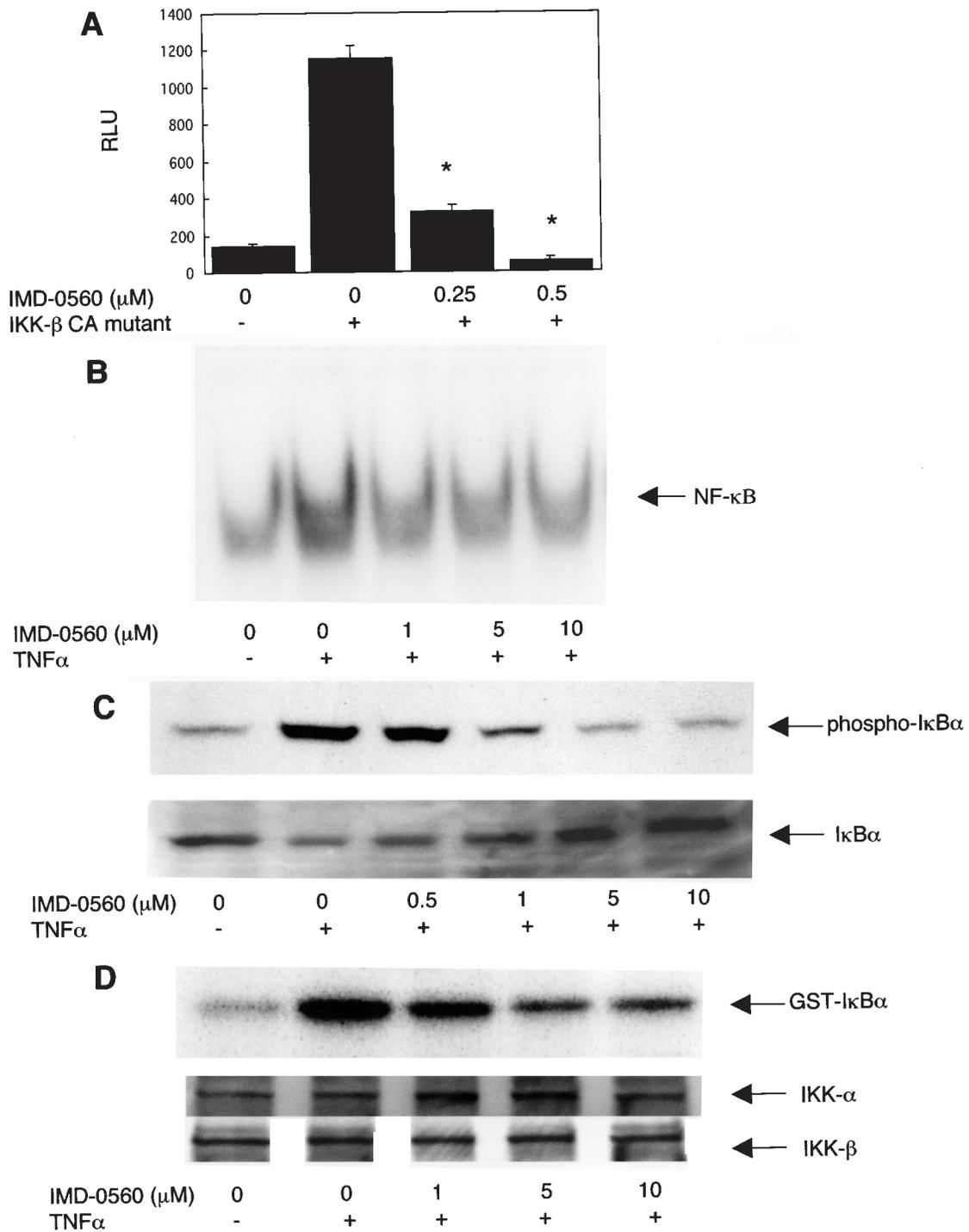
**Inhibition of FLS proliferation and cell-cycle progression.** As shown in Figure 4A, IMD-0560 dose-dependently inhibited FLS proliferation. The IC<sub>50</sub> for inhibition of BrdU incorporation was 2  $\mu$ M. Serum starvation of FLS resulted in the arrest of the cell cycle in the G0/G1 phase (Figure 4B). The entry of FLS from the G0/G1 into the S/G2/M phase of the cell cycle after addition of serum was completely blocked by the addition of 10  $\mu$ M IMD-0560, suggesting that this compound can inhibit proliferation of FLS before their entry into the S phase.

**IMD-0560 is not cytotoxic.** Treatment of FLS *in vitro* with 10  $\mu$ M IMD-0560 caused no increase in the release of LDH compared to untreated cells (Figure 4C). Thus, IMD-0560 showed no cellular toxicity at the concentrations used in this study.

## DISCUSSION

We examined the effect of blocking I $\kappa$ B phosphorylation with IMD-0560 on rheumatoid fibroblast-like synoviocytes and collagen induced arthritis. We first characterized the pharmacological activity of IMD-0560 by investigating the effect of IMD-0560 on the NF- $\kappa$ B signaling pathway. We observed that IMD-0560 inhibits TNF- $\alpha$  induced DNA binding activity of NF- $\kappa$ B in FLS. Phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  has been reported to be essential for NF- $\kappa$ B activation, and recent evidence suggests that I $\kappa$ B $\alpha$  phosphorylation is regulated by IKK<sup>8</sup>. Consistent with this concept, using a reporter gene assay, Western blot analysis, and *in vitro* kinase assay, we further found that IMD-0560 suppressed IKK- $\beta$  activation and I $\kappa$ B phosphorylation.

We also found that IMD-0560 inhibited the production of proinflammatory cytokines and chemokines from TNF- $\alpha$  stimulated rheumatoid FLS. IL-6 is a pleiotropic inflammatory cytokine involved in a variety of physiologic processes, including production of autoantibodies, induction of acute phase responses, and activation of osteoblasts<sup>22</sup>. IL-8 is a CXC chemokine (CXCL8) that acts as a chemoattractant for neutrophils and promotes angiogenesis. MCP-1, a CC chemokine (CCL2), plays a major role in attracting monocytes to the synovium<sup>23</sup>. Because high concentrations of IL-



**Figure 1.** A. IMD-0560 inhibits NF-κB transcriptional activation induced by the transfection of a constitutively active IKK-β mutant. Luciferase activity was expressed as relative light units (RLU) and normalized according to β-galactosidase activity. Values are shown as mean ± SEM of 3 independent experiments. \**p* < 0.01. B. Inhibition of TNF-α induced NF-κB binding activity by IMD-0560. FLS were pretreated with various concentrations of IMD-0560 for 2 h and then stimulated with TNF-α (10 ng/ml) for 15 min. Nuclear extracts were isolated and incubated with <sup>32</sup>P-labeled DNA probes containing a binding site for p65. The resulting complexes were analyzed by EMSA. C. IMD-0560 blocks TNF-α induced phosphorylation of IκBα. FLS were pretreated with 20 μM MG132 and various concentrations of IMD-0560 for 2 h and then stimulated with TNF-α (10 ng/ml). Cell lysates were analyzed by Western blotting using an anti-phospho-IκBα antibody. The presence of an equal amount of the endogenous IκBα protein was verified using antibodies against IκBα. Representative findings from 3 experiments are shown. D. IMD-0560 blocks TNF-α induced IKK activities. FLS were pretreated with IMD-0560 for 2 h and then stimulated with TNF-α (10 ng/ml) for 10 min. Cytoplasmic proteins were extracted and used for *in vitro* kinase assay using GST-IκBα as substrate (upper panel). Equal amount of the endogenous IKK-α and IKK-β protein was verified by Western blot using antibodies against IKK-α and IKK-β (lower panel). Representative findings from 3 experiments are shown.

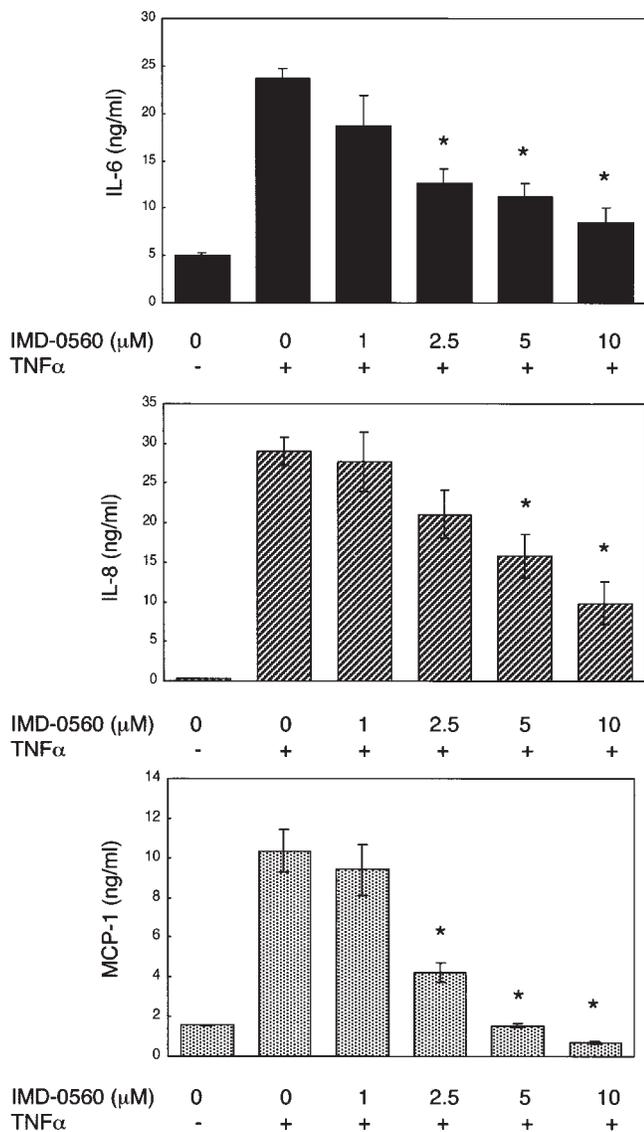


Figure 2. Effect of IMD-0560 on the production of IL-6, IL-8, and MCP-1 in FLS. FLS were incubated with various concentrations of IMD-0560 (0, 1, 2.5, 5, or 10  $\mu$ M) for 1 h and further incubated for 24 h in the presence or absence of TNF- $\alpha$  (10 ng/ml). IL-6, IL-8, and MCP-1 were measured in culture supernatants by ELISA. Values represent mean  $\pm$  SEM of 3 independent experiments. \* $p < 0.05$ .

IL-6, IL-8, and MCP-1 have been detected in the synovial fluid of patients with RA<sup>23</sup>, it appears that they contribute to the pathogenesis of RA. These cytokines have been shown to be regulated through a NF- $\kappa$ B-dependent mechanism<sup>24,25</sup>. Using FLS derived from 3 RA patients, we confirmed that TNF- $\alpha$  leads to an increase in IL-6, IL-8, and MCP-1 and that their production is dose-dependently inhibited by IMD-0560.

Previous studies revealed that several NF- $\kappa$ B inhibitors are effective in models of arthritis. Systemic administration of NF- $\kappa$ B inhibitors, such as the T cell-specific NF- $\kappa$ B

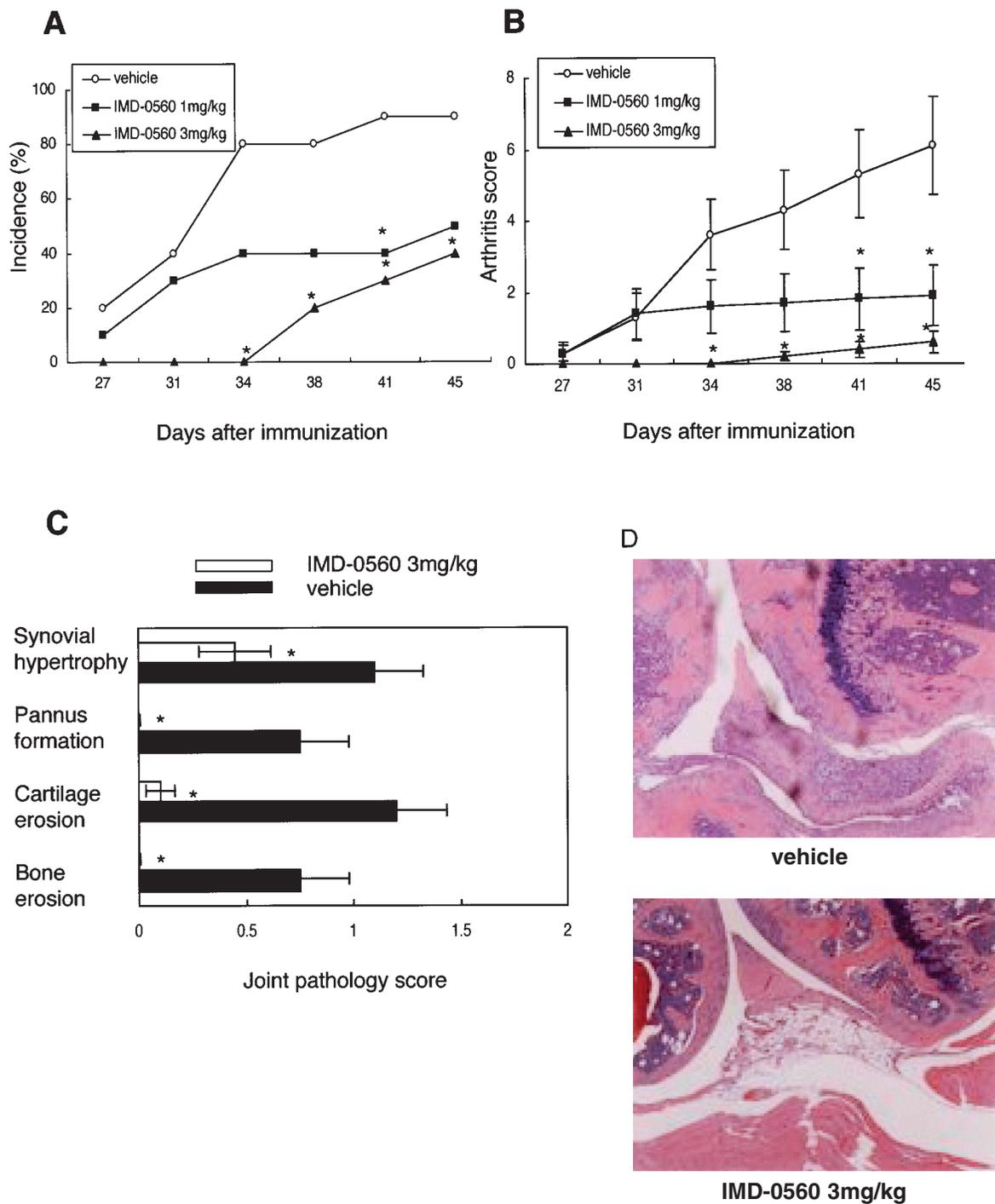
inhibitor SP100030<sup>26</sup> and a proteasome inhibitor<sup>27</sup>, have proven to be effective in CIA models. Local injection, for example, intraarticular injection of NF- $\kappa$ B decoy oligonucleotides<sup>16</sup> or of a dominant-negative IKK- $\beta$  gene<sup>17</sup>, also leads to an improvement in arthritis. However, because RA patients have multiple joint inflammations, systemic administration of a NF- $\kappa$ B inhibitor would be more useful than intraarticular injection.

The *in vitro* efficacy of IMD-0560 prompted us to investigate its *in vivo* effects in a mouse model of arthritis. Intraperitoneal injection of DBA/1J mice with IMD-0560 (1 or 3 mg/kg every 48 h beginning at the first immunization) resulted in a significant and dose-dependent reduction in the incidence, severity, and pathological abnormalities of CIA. We therefore have clearly demonstrated a prophylactic effect of IMD-0560 on mouse CIA. We have now begun studies of the effect of this compound administered to mice after the onset of CIA, but we have not yet observed a significant therapeutic effect (data not shown).

Importantly, cartilage and bone erosion was almost completely prevented by administration of IMD-0560. Recent studies suggest that bone-resorbing osteoclasts in the synovium play an important role in bone destruction in RA<sup>28</sup>. Osteoclast differentiation and activation is regulated by receptor activator of NF- $\kappa$ B (RANK) ligand and RANK signaling. Activation of RANK by its ligand leads to activation of various signaling pathways in osteoclasts. Among them, NF- $\kappa$ B signaling pathway appears to be crucial for osteoclast differentiation. In agreement with this, a cell-permeable peptide inhibitor of the IKK complex inhibits osteoclastogenesis and bone destruction in CIA<sup>29</sup>. Thus, it is possible that IMD-0560 also inhibited NF- $\kappa$ B activity in osteoclasts and suppressed osteoclast differentiation and activation, resulting in a minimal erosion of bone.

In some cells and tissues, NF- $\kappa$ B activation prevents apoptosis through the induction of survival genes. Intraarticular injection of MG132 or intraarticular adenoviral gene transfer of super-repressor I $\kappa$ B $\alpha$  has been reported to enhance apoptosis in the synovium of rat arthritis<sup>30</sup>. Although the induction of apoptosis in synoviocytes by local injection can lead to suppression of synovial hyperplasia, systemic administration may induce apoptosis in other tissues. Therefore, the adverse effects of systemic NF- $\kappa$ B inhibition need further consideration, especially with respect to the induction of apoptosis. IMD-0560 did not appear to have significant toxicity for rheumatoid FLS *in vitro*, but further examination of the *in vivo* safety of this compound is necessary.

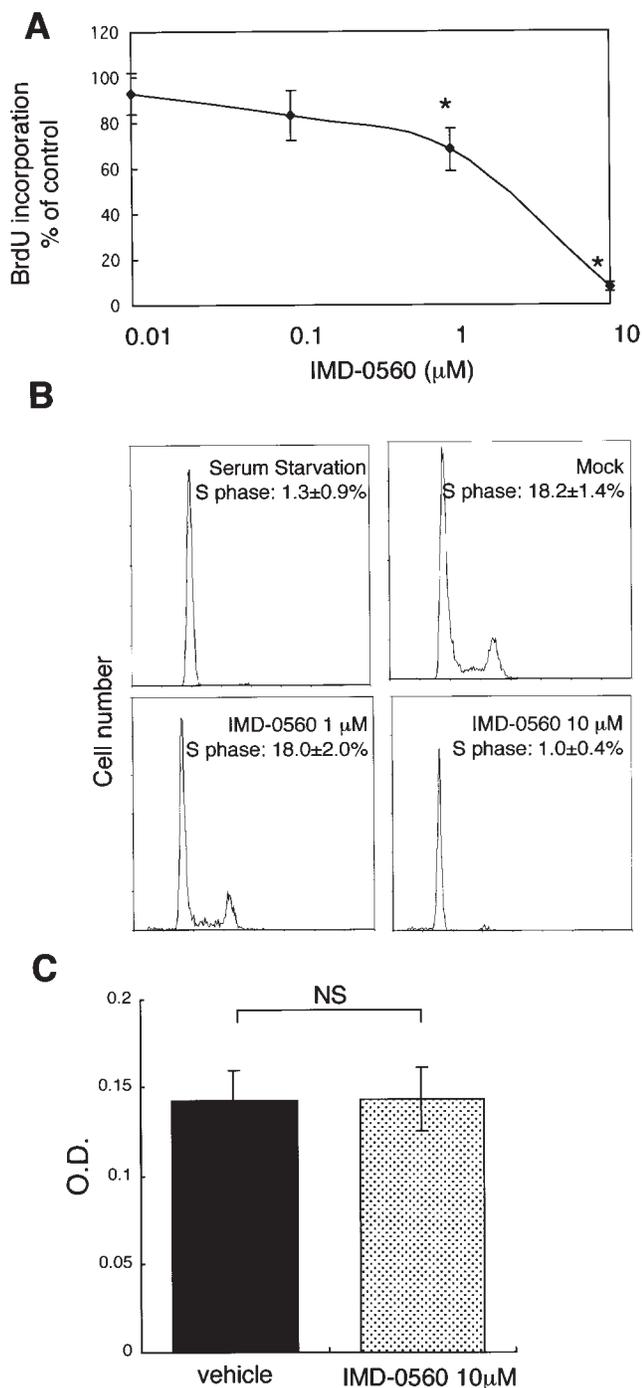
A loss of growth control in FLS may contribute to synovial lining hyperplasia and pannus formation in RA. Therefore, inhibition of the cell cycle in FLS may lead to the suppression of synovial cell overgrowth and subsequent articular destruction in RA. NF- $\kappa$ B is thought to play an important role in cell-cycle regulation, differentiation, and



**Figure 3.** IMD-0560 reduces the incidence and severity of CIA. Mice were immunized with collagen type II on Day 1 and a booster dose on Day 21. Mice were then treated intraperitoneally every 48 h from the day after the first immunization with vehicle (○) or with 1 mg/kg (■) or 3 mg/kg (▲) IMD-0560. Significance (\* $p < 0.05$ ) was determined in comparison with vehicle treated mice for the incidence of arthritis (A) and the arthritis score (B). C. Knee joints from control (vehicle) and IMD-0560 (3 mg/kg) treated mice were scored histologically for the 4 arthritis indicators. Results represent mean  $\pm$  SEM from 10 mice/group. \* $p < 0.05$ . D. Joint histology of IMD-0560 treated and control mice. Representative histological images of knee joints are shown (H&E stain, original magnification  $\times 100$ ).

protection from apoptosis<sup>31</sup>. Our findings clearly showed that IMD-0560 blocked the proliferation of FLS and inhibited cell-cycle progression from G<sub>0</sub>/G<sub>1</sub> to S/G<sub>2</sub>/M. NF- $\kappa$ B

is known to activate the transcription of cyclin D1, a key positive regulator of G<sub>1</sub> to S phase progression<sup>32</sup>. Although the effect of IMD-0560 on the expression of cyclin D1 was



**Figure 4.** A. Effect of IMD-0560 on FLS cell proliferation measured by BrdU incorporation. FLS were incubated with various concentrations of IMD-0560 for 36 h. BrdU was added for an additional 4 h. Color was developed using anti-BrdU peroxidase labeled immune complexes and read at 450 nm. Values represent mean  $\pm$  SEM from 3 independent experiments. \* $p < 0.05$ . B. Effect of IMD-0560 on cell-cycle progression from G0/G1 to S phase. FLS were precultured in serum-free conditions for 24 h. Serum-starved FLS were further incubated in culture medium containing 10% FBS for 24 h with various concentrations (0, 1, or 10  $\mu$ M) of IMD-0560. FLS were then fixed with 70% ethanol and cell-cycle analysis was performed. Percentages of FLS in S phase are expressed as means  $\pm$  SEM from 3 independent experiments. C. The cytotoxicity of IMD-0560 was evaluated by LDH release into the medium from cultured FLS. Values represent mean  $\pm$  SEM of 3 measurements. NS: not statistically significant.

not examined in this study, we suspect that inhibition of NF- $\kappa$ B activity by IMD-0560 blocks cell-cycle progression into the S phase through suppression of cyclin D1 and other cell-cycle regulatory molecules.

We observed the inhibitory effects of IMD-0560 on the NF- $\kappa$ B signaling pathway in rheumatoid FLS as well as on CIA in mice. These findings suggest that IMD-0560 is a promising new therapeutic agent for RA.

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