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*J Immunol* 2000; 165:1652-1658; doi: 10.4049/jimmunol.165.3.1652

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The Effect of a T Cell-Specific NF-κB Inhibitor on In Vitro Cytokine Production and Collagen-Induced Arthritis

Danielle M. Gerlag,2* Lynn Ransone,2† Paul P. Tak,3* Zuoning Han,* Moorthy Palanki,† Miguel S. Barbosa,† David Boyle,* Anthony M. Manning,† and Gary S. Firestein4*  

NF-κB plays a key role in the production of cytokines in inflammatory diseases. The effects of a novel T cell-specific NF-κB inhibitor, SP100030, were evaluated in cultured Jurkat cells and in murine collagen-induced arthritis (CIA). Chemical libraries were screened for NF-κB-inhibitory activity. SP100030, a compound identified in this process, inhibited NF-κB activation in PMA/PHA-activated Jurkat cells by EMSA at a concentration of 1 μM. Jurkat cells and the monocytic cell line THP-1 were transfected with an NF-κB promoter/luciferase construct and activated. SP100030 inhibited luciferase production in the Jurkat cells (IC50 = 30 nM). ELISA and RT-PCR confirmed that IL-2, IL-8, and TNF-α production by activated Jurkat and other T cell lines were inhibited by SP100030. However, cytokine expression was not blocked by the compound in THP-1 cells, fibroblasts, endothelial cells, or epithelial cells. Subsequently, DBA/1J mice were immunized with type II collagen. Treatment with SP100030 (10 mg/kg/day i.p. beginning on day 21) significantly decreased arthritis severity from onset of clinical signs to the end of the study on day 34 (arthritis score, 5.6 ± 1.7 for SP100030 and 9.8 ± 1.5 for control; p < 0.001). Histologic evaluation demonstrated a trend toward improvement in SP100030-treated animals. EMSA of arthritic mouse ankles in CIA showed that synovial NF-κB binding was suppressed in the SP100030-treated mice. SP100030 inhibits NF-κB activation in T cells, resulting in reduced NF-κB-regulated gene expression and decreased CIA. Its selectivity for T cells could provide potent immunosuppression with less toxicity than other NF-κB inhibitors. The Journal of Immunology, 2000, 165: 1652–1658.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology, affecting synovial tissue in multiple diarthrodial joints. Hallmarks of this disease include synovial hyperplasia, angiogenesis, and erosion of articular cartilage and subchondral bone. Rheumatoid synovial tissue is characterized by marked intimal lining hyperplasia due to increased numbers of both intimal macrophages and fibroblast-like synoviocytes (FLS) and by the accumulation of T cells, plasma cells, macrophages, and other cells in the synovial sublining (1, 2). Infiltrating synovial cells produce various cytokines, predominantly IL-1, TNF-α, and IL-6, promoting induction of adhesion molecule and proteinase gene expression (3). These factors play an important role in attracting and activating other inflammatory cells and in the degradation of cartilage and bone.

Cytokine and adhesion molecule gene expression is regulated by a variety of transcription factors. One of these, NF-κB, plays an especially important role in inflammatory processes (4). The regulation of this particular transcription factor has been extensively explored in RA in both tissues and cultured cells. For instance, its expression and activity is markedly increased in the RA synovium (5, 6). NF-κB also plays a pivotal role in IL-6 production by cultured FLS (7). This critical factor is a potential therapeutic target for the treatment of RA patients.

To assess the possible therapeutic role of NF-κB in arthritis, we investigated a novel T cell-specific transcription factor inhibitor, SP100030 (8). SP100030 was shown to inhibit NF-κB activation in vitro as well as suppress expression of NF-κB-driven genes. Subsequently, we demonstrated efficacy of this compound in a chronic arthritis model. These studies suggest that NF-κB inhibition in T cells could be beneficial in inflammatory arthritis.

Materials and Methods

Reagents

SP100030 was obtained from Signal Pharmaceuticals (San Diego, CA). The chemical formula is 2-chloro-4-(trifluoromethyl)pyrimidine-5-N-(3′, 5′-bis(trifluoromethyl)phenyl)-carboxamide. PMA and PHA were obtained from Sigma (St. Louis, MO). A Bandshift kit was purchased from Promega (Madison, WI). Oligonucleotides and Abs cross-reactive to rat p50, p65, c-Rel, RelB, and p52 subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells

Human Jurkat T cells and THP-monocytes were obtained from the America Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Media Tech, West Chester, PA) containing 2 mM l-glutamine and 50 U/ml penicillin plus 50 μg/ml streptomycin (Life Technologies, Grand Island, NY) and either 10% FCS (Jurkat) or 20% FCS (Gemini Biological Products, Calabasas, CA) and 2-ME (Sigma) (THP-1). Normal human endothelial cells were obtained from Clonetics (Walkersville, MD), and other cell lines were purchased from American Type Culture Collection (Manassas, VA).

Received for publication January 3, 2000. Accepted for publication May 17, 2000.

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1 This work was supported in part by grants from the National Institutes of Health, the Arthritis Foundation, and Signal Pharmaceuticals.

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5 Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; CIA, collagen-induced arthritis; IKK, IκB kinase.
**Mice**

Six- to 8-wk-old male DBA/1J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Type II collagen and CFA were obtained from Chondrex (Seattle, WA), IFA and LPS (Escherichia coli serotype 0111:B4) from Sigma.

**RT-PCR**

Total RNA was isolated using Tri reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Semi-quantitative RT-PCR was conducted using 2 μg of RNA, 0.4 μM each primer (Clontech; Palo Alto, CA), 0.2 mM each dNTP, 5 μl 10 × PCR buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3), 0.5 μM RNAse (Promega), 0.5 μM avian myeloblastosis virus reverse transcriptase (25 U/μl; Promega), and 0.5 μl Taq polymerase (5 U/μl; Boehringer Mannheim, Chicago, IL) in a 50-μl reaction volume. Reverse transcription was conducted at 42°C for 1 h. The temperature was then adjusted to 95°C for 2 min to inactivate the avian myeloblastosis virus reverse transcriptase. The PCR was amplified for 35 cycles. The PCR cycle consisted of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. After the final amplification, the reaction was completed at 72°C for 7 min. Twenty microliters of each reaction mix was separated by electrophoresis by using 1.5% agarose gels, followed by staining with ethidium bromide.

**NF-κB assay**

Human Jurkat T cells were stably transfected with a synthetic promoter comprising three copies of an NF-κB binding site (from the HMC promoter) fused to a minimal SV40 promoter driving luciferase (9). Cells were cultured, resuspended in fresh medium containing 10% Serum-Plus (HyClone, Logan, UT) at a density of 1 × 10⁵ cells/ml, and plated in 96-well round-bottom plates (200 μl per well) for 18 h. SP100030 was dissolved in 0.2% DMSO/H₂O and then added at the appropriate concentrations to the microwell plates containing the cells, and the plates were incubated at 37°C for 20 min. To induce transcriptional activation, 50 ng/ml of PMA and 1 μM NaF were added to each well, and the cells were incubated for an additional 5 h at 37°C. Control cells were treated with identical amounts of DMSO alone. The plates were centrifuged at 2200 rpm for 1 min at room temperature followed by removal of the media. Sixty microliters of cell lysis buffer (1 × Cell Culture Lysis Reagent, 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 10% glycerol, 1% Triton X-100; Promega) was added to each well, and cells were lysed for 15 min. Then 40 μl of each cell lysate was transferred to a black 96-well plate, and 50 μl of luciferase substrate buffer was added. Luminescence was immediately measured using Packard TopCount (Packard Instruments, Meriden, CT). The β-actin assay was run as described above except for that the Jurkat T cells were transfected with a plasmid that contained the β-actin promoter driving luciferase.

**Cytokine production assay**

After centrifugation, supernatants were collected from each well in the above luciferase experiments and stored at −20°C until assay. Approximately 20- to 50-μl aliquots were removed and the levels of IL-2, IL-6, IL-8, IFN-γ, and TNF-α were determined by ELISA (Biosource International, Camarillo, CA).

**Collagen-induced arthritis (CIA)**

Six- to 8-wk-old male DBA/1J mice were immunized s.c. at the base of the tail with 100 μl of a solution containing type II collagen (1 mg/ml) in CFA and IFA. On day 21, 100 μg of type II collagen in 100 μl PBS was injected i.p. On day 28, 50 μg of LPS in 100 μl PBS was injected i.p. to synchronize and exacerbate arthritis, as previously described (10). Using this protocol, the incidence of arthritis was 100% and peaked at day 34–35. Clinical arthritis scores were evaluated using a scale of 0–4 for each paw, (0, normal; 1, minimal erythema and mild swelling; 2, moderate erythema and mild swelling; 3, marked erythema and severe swelling, digits not yet involved; 4, maximal and swelling, digits involved).

**Study design**

The animals were treated daily starting on day 21 till the end of the study by i.p. injection of either SP100030 (10 mg/kg, dissolved in polyethylene glycol 200 (ICN, Aurora, OH) in a total volume of 50 μl) or 50 μl polyethylene glycol 200 only. The clinical arthritis scores were assessed three times a week in a blinded manner. On day 35, the mice were sacrificed and the two hind paws and one fore paw were harvested from each animal for nuclear protein and RNA extraction, as described below.

**Histologic analysis**

On day 35, the animals were sacrificed and one forepaw of each animal was fixed in a 10% formalin solution, decalcified, and embedded in paraffin for histologic analysis. Five-micrometer sections were cut, mounted on a glass slide, and stained with hematoxylin and eosin. The tissue was evaluated using a semiquantitative scoring system (0–3+) for synovial hyperplasia, cartilage erosion, and extra-articular inflammation. The maximum score per paw was 9.

**Preparation of nuclear extracts of mouse tissue**

The mouse paws were cut just above and below the ankle and the skin was removed. The joints were then snap frozen in liquid nitrogen and pulverized. Nuclear extracts were prepared according to the method described by Schreiber et al. with modifications (11). Homogenization of the tissue was performed using 3 ml buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, pH 8.0, 1 mM DTT, and 1 mM PMSF; 0.1% Nonidet P-40). The supernatant was discarded after incubation on ice for 15 min and centrifugation at 850 × g at 4°C. The pellet was resuspended in 4 ml buffer A without Nonidet P-40, the samples were centrifuged, and the supernatant was again discarded. Buffer C (25% (v/v) glycerol, 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 sodium EDTA, pH 8.0, 1 mM DTT, and 1 mM PMSF; 100 μl) was added to the pellets, and the samples were rocked for 30 min at 4°C. After centrifugation at 4°C, the supernatants were aliquoted and stored at −80°C. The protein concentration was measured using the method of Bradford using a protein dye reagent (12).

**EMSA**

The Bandshift kit was used according to the manufacturer’s instructions as previously described (10). Double-stranded NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCAGGGC-3′) or the mutant oligonucleotide probe (5′-AGTTGAGGGAGCTTCCCCAGGC-3′) were end-labeled with [γ-32P]ATP (50 μCi at 222 TBq/mmol; Amersham Life Science, Arlington Heights, IL). For the experiments using the Jurkat T cells, binding reactions contained 35 fmol of oligonucleotide and 5 μg of nuclear protein. The reactions were conducted at room temperature for 20 min in a total volume of 10 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol (v/v), and 0.5 μg poly(dI-dC) (Pharmacia, Peapack, NJ)). Unlabeled oligonucleotide was added 5 min before addition of radiolabeled probe in case of the competition reactions. For supershift analysis, 1 μg of each Ab was added 5 min before the reaction mixtures immediately after addition of radiolabeled probe. Following the binding reactions, 1 μl of 10× gel loading buffer was added. For the experiments with the nuclear extracts of mouse tissue, the samples of the treated and the untreated groups were pooled. Two microliters of the labeled oligonucleotides were added to the nuclear extracts (4 μg of protein) in a total volume of 20 μl, and the samples were incubated 20 min at room temperature. For the negative control samples, mutant or a 15-fold excess of cold consensus oligonucleotide was added. To perform supershift experiments, 2 μg of Abs to NF-κB p50 and p65 were added. Samples were incubated for 15 min at room temperature and loaded onto a 4% polyacrylamide gel. After electrophoresis, the gel was transferred to Whatman paper (Whatman International, Maidstone, U.K.) or vacuum dried and visualized by autoradiography.

**Statistical analysis**

The clinical and histologic scores were compared using the ANOVA and Student t tests.

**Results**

**DNA binding activity of NF-κB in Jurkat cells is reduced by SP100030**

Chemical libraries were screened to identify compounds that inhibit induction of NF-κB binding activity in stimulated Jurkat cells. After identification of a lead and subsequent optimization, SP100030 was identified as a potent NF-κB inhibitor and characterized more completely. To assess the effect of SP100030 on NF-κB DNA binding activity, an EMAS was performed on nuclear extracts of Jurkat cells that had been stimulated with PMA and PHA for 6 h. As shown in Fig. 1, the DNA binding activity was reduced in the SP100030-treated cells compared with the untreated cells (lanes 2 and 3, respectively). In supershift experiments, the DNA protein complex appeared to contain both p50 and p65.
SP100030 decreased the intensity of both the p50 and p65 super-shifted bands. Nonspecific bands with a higher electromobility were seen in all lanes.

Dose-response effect of SP100030 on Jurkat cells

Having demonstrated that SP100030 decreased NF-κB binding activity, we then determined whether the compound inhibited transcription of a gene regulated by NF-κB. For these experiments, Jurkat cells were transfected with either an NF-κB or β-actin promoter coupled to luciferase and activated with PHA/PMA for 5 h in the presence of increasing concentrations of SP100030. Luciferase expression is shown as percentage of control.

Jurkat cells were transfected with a construct containing the NF-κB promoter and the luciferase cDNA. Cells were then stimulated with PMA and PHA to activate NF-κB. There was a dose-response-dependent inhibition of luciferase expression in the transfected Jurkat cells (see Fig. 2 for a representative experiment). The IC₅₀ for SP100030 was 30 nM. The compound had no effect on the β-actin promoter coupled to luciferase, demonstrating its specificity for NF-κB.

SP100030 inhibits NF-κB driven cytokine gene expression

We subsequently determined whether SP100030 inhibited expression of NF-κB-regulated cytokines in T cells. Jurkat cells were stimulated with PMA/PHA for 5 h in the presence of SP100030 or vehicle for 0.5–5 h, and cytokine mRNA was determined by RT-PCR analysis. Fig. 3, a representative experiment, shows the effect of SP100030 on gene expression for IL-2, IL-8, and TNF-α in Jurkat cells. SP100030 markedly decreased IL-2, TNF-α, and IL-8 mRNA levels compared with control cells. Notably, the PCR method used permits only a semiquantitative assessment of mRNA abundance due to potential differences in primer efficiency and the relative abundance of the control gene compared with the cytokine genes. These results suggest that SP100030 inhibits NF-κB-regulated cytokine production at the transcriptional level.

Specificity of SP100030 for T cell cytokine production

The effect of SP100030 on Jurkat cell cytokine protein release was then determined. PMA/PHA-stimulated Jurkat cells were incubated for 0–5 h in the presence of either SP100030 or vehicle, and
the levels of IL-2, TNF-α, and IL-8 were measured in the supernatant by ELISA. Fig. 4A shows that SP100030 inhibited cytokine production in a concentration-dependent fashion in Jurkat cells. Similar effects were observed in other T cell lines using several methods to stimulate cells (PMA/PHA; soluble anti-CD3 plus anti-CD28 Ab; Con A; PMA plus ionophore (A23187)) (see Table I). For each of these lines, complete dose-responses were evaluated from 0.1 to 30 μM of SP100030. At least 80% inhibition of

Table I. Effect of SP100030 on T cell cytokine production

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Name of Line</th>
<th>Source</th>
<th>Stimulus</th>
<th>Cytokines Assayed</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell</td>
<td>Jurkat</td>
<td>T cell leukemia</td>
<td>PMA + PHA, PMA + A23187, CD3 + CD28, Con A</td>
<td>IL-2, IL-8</td>
<td>+</td>
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<tr>
<td>T cell</td>
<td>HUT78</td>
<td>T cell leukemia</td>
<td>PMA + PHA, PMA + A23187</td>
<td>IL-2, IL-8</td>
<td>+</td>
</tr>
<tr>
<td>T cell</td>
<td>MOLT-4</td>
<td>Acute lymphoblastic leukemia</td>
<td>PMA + A23187</td>
<td>IL-2</td>
<td>+</td>
</tr>
<tr>
<td>T cell</td>
<td>CCRF-HSB-2</td>
<td>T cell leukemia</td>
<td>PMA + A23187</td>
<td>IL-2, IL-8, IFN-γ</td>
<td>+</td>
</tr>
<tr>
<td>T cell</td>
<td>LBRM-33</td>
<td>Mouse T cell lymphoma</td>
<td>PMA + PHA, PMA + A23187, Con A</td>
<td>IL-2</td>
<td>+</td>
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<tr>
<td>T cell</td>
<td>H9</td>
<td>Cutaneous T cell lymphoma</td>
<td>PMA + PHA, PMA + A23187, Con A</td>
<td>IL-2, IFN-γ</td>
<td>+</td>
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</tbody>
</table>

*Samples were incubated in the presence or absence of SP100030 for 6–24 h, and supernatants were assayed by ELISA. PMA, 50 ng/ml; PHA, 1 μg/ml; A23187, 1 μg/ml; Con A, 10 μg/ml.

* +, >80% inhibition at 3 μM; −, <5% inhibition at 10 μM.
cytokine production was observed at a concentration of 3 μM or less (see Table I). In contrast, cytokine expression was not inhibited by 10 μM in THP-1 cells (Fig. 4B), other monocytic cell lines, endothelial cells, fibroblasts, synoviocytes, osteoblasts, and epithelial cell lines (see Table II). Several different methods of stimulating these cells was tested, including PMA, LPS, IL-1, or TNF-α, and IL-1 plus TNF-α. Although IL-8 was the primary cytokine evaluated, SP100030 also had no effect on IL-6 and TNF-α production in selected cell lines.

To confirm that the compound had no effect on NF-κB activation, EMSA experiments were performed on U937 and THP-1 cells. SP100030 had no effect on NF-κB binding in the activated monocytic cells. Furthermore, expression of the NF-κB-luciferase construct was not inhibited by the compound in transfected U937 and U2OS cells (data not shown).

Table II. Effect of SP100030 on cytokine production by non-T cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Name of Line</th>
<th>Source</th>
<th>Stimulus</th>
<th>Cytokines Assayed</th>
<th>Inhibition</th>
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<td>PMA, LPS, TNFα</td>
<td>IL-6, IL-8, TNF-α</td>
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<tr>
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<td>Acute monocytic leukemia</td>
<td>PMA, LPS, TNFα</td>
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<tr>
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<td>PMA, LPS, TNFα</td>
<td>IL-6, IL-8, TNF-α</td>
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<td>Primary cells</td>
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<td>Rabbit FLS</td>
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<td>IL-8</td>
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<td>Osteoblast</td>
<td>U2OS</td>
<td>Osteosarcoma</td>
<td>IL-1 + TNFα</td>
<td>IL-6</td>
<td>–</td>
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<td>GM637</td>
<td>Skin fibroblast</td>
<td>PMA</td>
<td>IL-8</td>
<td>–</td>
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</tbody>
</table>

* Samples were incubated in the presence or absence of SP100030 for 6–24 h, and supernatants were assayed by ELISA. PMA, 50 ng/ml; LPS, 1 μg/ml; TNFα, 20 ng/ml; IL-1, 10 ng/ml.
 1 +, >80% inhibition at 3 μM, –, <5% inhibition at 10 μM.
 2 Transcriptional activity via luciferase reporters also performed and confirmed cytokine results.

FIGURE 5. Effect of SP100030 on paw swelling of mice with CIA. Mice with CIA were treated daily with 10 mg/kg i.p. SP100030 or vehicle starting at day 21 after immunization. Clinical arthritis scores are on a scale of 0–4 for each paw. Statistical evaluation was performed by ANOVA; p < 0.001 comparing vehicle to SP100030-treated animals.

FIGURE 6. NF-κB DNA binding in synovial tissue of mice with CIA treated with SP100030 or vehicle. EMSA results of nuclear extracts from pooled synovial samples from mice with CIA treated with SP100030 (“SP”) or vehicle (“Veh”) using a radiolabeled oligonucleotide containing the NF-κB consensus binding site are shown. Lane 1 (P) was a human positive control (which has additional nonspecific bands as in Fig. 1), and lane 2 (CC) included competition with cold oligos. In supershift assays, anti-p50 Ab (lanes 5 and 6) and anti-p65 Ab (lanes 7 and 8) were present in the sample. Arrows show the specific complexes. There is a clear reduction of NF-κB activity in the samples of the SP100030-treated mice. A portion of the NF-κB binding activity appears to be due to p50, and this was decreased by SP100030.
**Therapeutic effect of SP100030 in CIA**

NF-κB activation and subsequent cytokine production are known to play a role in RA as well as animal models of inflammatory arthritis. To explore the possibility that T cell-selective NF-κB suppression might have therapeutic efficacy in arthritis, the effect of SP100030 was determined in the murine CIA model. DBA/1J mice were immunized with type II collagen and treated with either vehicle (PEG 200; n = 13) or SP100030 (10 mg/kg/day; n = 14) beginning on day 21. Fig. 5 shows that the SP100030-treated mice had significantly decreased arthritis scores compared with controls (p < 0.001, ANOVA). Histologic evaluation of the paws from mice treated from day 20 to 34 showed a trend toward decreased inflammation (mean ± SEM scores: SP100030, 2.86 ± 1.19; vehicle, 5.73 ± 1.12; p > 0.05).

**Synovial DNA binding activity of NF-κB is inhibited by SP100030 in mice with CIA**

We have previously shown that NF-κB activity in this model gradually increases from day 10 to intense NF-κB suppression at day 35 (10). To determine the effect of SP100030 on NF-κB binding activity in joints, we performed an EMSA on pooled samples from joints of treated and control mice (Fig. 6). In the group of control animals, NF-κB binding was increased on day 35 (lane 3). However, the treatment group showed clearly diminished NF-κB binding activity. A portion of the functional NF-κB protein consisted of p50, as determined by supershift experiments, although the supershifted bands were not as clear as in the human cell line studies.

**Discussion**

RA is a synovial inflammatory disease marked by accumulation of mononuclear cells, increased numbers of blood vessels, and hyperplasia of the invasive intimal lining (2). Production of inflammatory cytokines is a key feature of this process, and recent advances in anti-cytokine therapy confirm that these factors play a critical role in the pathogenesis of RA (13, 14). Development of novel treatment strategies that alter the cytokine milieu would be greatly facilitated by dissecting the regulatory elements that control mediator production in the joint.

Of the many transcription factors involved in joint inflammation, NF-κB appears to be especially important. For instance, production of IL-1 and TNF-α by synovial macrophages is regulated by NF-κB, as is the expression of TNF-α and IL-6 in FLS (15–20). In addition, constitutive IL-6 production by RA FLS clones is NF-κB dependent (7). Increased cytokine production driven by NF-κB can enhance expression of vascular adhesion molecules that attract leukocytes into the joint, as well as matrix metalloproteinases, which help degrade the extracellular matrix (21–23). NF-κB protein is readily detected in rheumatoid synovium, especially in the nuclei of intimal lining cells (5, 6, 20). Although the proteins are also detected in osteoarthritis synovium, NF-κB DNA binding activity is much greater in RA than osteoarthritis (10). These data suggest that this signal transduction pathway can play a pivotal role in the synovial cytokine cascade.

NF-κB is a ubiquitous transcription factor that is primarily comprised of homo- or heterodimeric combinations of proteins belonging to the Rel family that shares a highly homologous N-terminal region. This domain contains sequences required for DNA binding, protein dimerization, and nuclear localization. Several Rel-containing dimers exist, of which p50/p65 (NF-κB1/RelA) heterodimers and the p50 homodimers are the most common. NF-κB normally resides as an inactive protein in the cytoplasm, where it is bound to an inhibitory protein, IκB. Activation is initiated by many stimuli, including IL-1, TNF-α, platelet-derived growth factor, and phorbol esters. Signal transduction proceeds through activation of two IκB kinases, IKK-1 (IKK-α) and IKK-2 (IKK-β), which phosphorylate IκB. These kinases are constitutively expressed by RA FLS, and IKK-2 stimulation is necessary and sufficient for NF-κB activation in these cells (20). After phosphorylation and degradation of IκB, NF-κB is translocated to the nucleus, where it binds to its target genes to initiate transcription.

The therapeutic potential for NF-κB suppression led us to screen libraries for compounds that inhibit NF-κB activation. After optimization, one compound, SP100030, was an especially potent inhibitor of NF-κB activation in a T cell line with an IC50 of 30 nM for NF-κB inhibition. Preliminary pharmacologic studies with SP100030 in rodents demonstrated anti-inflammatory effects in cutaneous delayed-type hypersensitivity and allogeneic cardiac transplantation (8). The purpose of the experiments described in the current report was to characterize the in vitro activity of SP100030 more completely and evaluate its mechanism of action in a chronic model of inflammatory arthritis in mice.

After confirming the effect of SP100030 on NF-κB activation in Jurkat cells, we demonstrated that the compound inhibited NF-κB-driven expression of a luciferase reporter gene. This effect could not be explained by nonspecific toxicity because it had no effect on β-actin transcription. Furthermore, expression of endogenous NF-κB-regulated cytokines genes was also suppressed at the mRNA and protein levels by SP100030. Although this compound blocked NF-κB activation in T cell lines (including Jurkat, Molt-4, HUT-78, and H9), it had no effect on cytokine production by monocyteid cells, fibroblasts, synoviocytes, endothelial cells, epithelial cells, and osteoblasts. Additional EMSA and NF-κB-driven reporter gene studies in selected non-T cell lines confirmed that SP100030 did not suppress NF-κB activation. Hence, its action appears to be relatively specific to T cells. The mechanism of action is currently under investigation, but does not appear to be related to IKK inhibition or decreased IκB phosphorylation (data not shown). In any case, the selectivity for the T cell lineage suggests that SP100030 does not act at signal transduction pathways that are common to T cells and macrophages.

The effect of SP100030 was then evaluated in murine CIA. We have previously shown that NF-κB is activated in the joints of mice 10 days after systemic immunization with type II collagen even though clinical arthritis does not appear until day 25–30 (10). Joint swelling was significantly decreased in animals that were treated with SP100030 before the onset of joint swelling, although the degree of disease suppression was surprisingly modest. Clinical efficacy was accompanied by diminished NF-κB activation in joint extracts, suggesting that the compound acted through this mechanism in vivo. Although SP100030 only appears to suppress NF-κB activation in T cells, the dependence of synovial inflammation on T cell activation in CIA likely results in decreased NF-κB activity in other cell types as well. Efficacy in this animal model also indicate that SP100030 can block NF-κB expression in normal cells in addition to the cell lines tested in vitro. Of interest, animals with established disease did not appear to benefit when treatment was begun on day 35. At this later time point, many additional transcription factors, such as AP-1, are highly activated in CIA and could interfere with efficacy. Alternatively, T cell-independent processes in established disease might diminish the effectiveness of a selective T cell approach.

These data suggest that modulation of NF-κB activation by compounds like SP100030 could be beneficial in chronic inflammatory diseases like arthritis. Of interest, it recently has been shown that the incidence and severity of CIA was significantly reduced in transgenic mice expressing a constitutive inhibitor of NF-κB/Rel (IκBα) in the T cell lineage (24). Selectivity for T cells...
could also potentially minimize systemic toxicity that could complicate inhibition of a key signal transduction pathway. However, in light of the importance of macrophage-derived cytokines and the relative lack of T cell products in RA, efficacy in this particular disease would depend on the ability of SP100030 to suppress local lymphocyte-dependent responses that support the macrophage-fibroblast cytokine network (3, 25). Other anti-rheumatic drugs, such as salicylates, sulfalazine, and corticosteroids also appear to suppress NF-κB activation, possibly contributing to their clinical efficacy (26–28). Furthermore, NF-κB activation prevents Fas ligand- and TNF-α-mediated apoptosis in arthritis and contributes to synovial hyperplasia (29). Suppression of NF-κB in rat arthritis with NF-κB decoy oligonucleotides increased apoptosis in the inflamed synovium, thereby decreasing swelling. In conjunction with our studies, these data suggest that NF-κB is a potential therapeutic approach in RA that is amenable to small molecule inhibitors.

References