The Journal of Immunology

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J Immunol 2000; 165:7199-7206; ;
doi: 10.4049/jimmunol.165.12.7199
http://www.jimmunol.org/content/165/12/7199

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Regulation of IL-6 and IL-8 Expression in Rheumatoid Arthritis Synovial Fibroblasts: the Dominant Role for NF-κB But Not C/EBPβ or c-Jun[1]

Constantinos Georganas,[2]* Hongtao Liu,* Harris Perlman,* Alexander Hoffmann,† Bayar Thimmapaya,‡ and Richard M. Pope[3]*

Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) produce IL-6 and IL-8, which contribute to inflammation and joint damage. The promoters of both cytokines possess binding sites for NF-κB, C/EBPβ, and c-Jun, but the contribution of each to the regulation of IL-6 and IL-8 in RA FLS is unknown. We employed adenoviral-mediated gene delivery of a nondegradable IκBα, or dominant-negative versions of C/EBPβ or c-Jun, to determine the contribution of each transcription factor to IL-6 and IL-8 expression. Inhibition of NF-κB activation significantly reduced the spontaneous and IL-1β-induced secretion of IL-6 and IL-8 by RA FLS and the IL-1β-induced production of IL-6 and IL-8 by human dermal fibroblasts. Inhibition of C/EBPβ modestly reduced constitutive and IL-1β-induced IL-6 by RA FLS, but not by human dermal fibroblasts, and had no effect on IL-8. Inhibition of c-Jun/AP-1 had no effect on the production of either IL-6 or IL-8. Employing gel shift assays, NF-κB, C/EBPβ, and c-Jun were constitutively activated in RA FLS, but only NF-κB and c-Jun activity increased after IL-1β. The reduction of cytokines by IκBα was mediated through inhibition of NF-κB activation, which resulted in decreased IL-6 and IL-8 mRNA. NF-κB was essential for IL-6 expression, because fibroblasts in which both NF-κB p50/p65 genes were deleted failed to express IL-6 in response to IL-1. These findings document the importance of NF-κB for the regulation of the constitutive and IL-1β-stimulated expression of IL-6 and IL-8 by RA FLS and support the role of inhibition of NF-κB as a therapeutic goal in RA. *The Journal of Immunology,* **2000, 165: 7199–7206.**

Rheumatoid arthritis (RA)[4] is characterized by inflammation of the synovial membrane and proliferation of the synovial lining, which results in erosion of cartilage and bone. Fibroblast-like synoviocytes (FLS) and macrophage-like synoviocytes play critical roles in the destructive process (1, 2). RA FLS synthesize and secrete mediators of inflammation including IL-6 and IL-8 (2–6). IL-1β, produced by synovial lining macrophages (7, 8), contributes to the joint damage (9), in part by promoting the expression of these proinflammatory mediators. The mechanisms responsible for the constitutive and IL-1β-stimulated expression of IL-6 and IL-8 by RA FLS have not been fully elucidated.

The transcription factors C/EBPβ, AP-1, and NF-κB are constitutively activated in RA synovial tissue (10–16). Binding sites for each of these transcription factors have been identified in the promoter regions of the IL-6 and IL-8 genes, and under certain conditions each factor has been shown to activate both proinflammatory genes (17–25). Furthermore, each of these transcription factors has been implicated in IL-6 and IL-8 expression in RA synovial tissue (26–29). However, prior studies have not directly examined, employing specific inhibitors, the contribution of each of the transcription factors to the constitutive and IL-1β-stimulated expression of these genes by isolated FLS.

In the present study, we employed adenoviral vectors expressing dominant-negative (DN) versions of C/EBPβ or c-Jun (30–33), and a nondegradable IκBα (34), to determine the contribution of each transcription factor to the constitutive and IL-1β-stimulated expression of IL-6 and IL-8 by RA FLS. More IL-6 and IL-8 was produced by RA FLS, both constitutively and following IL-1β stimulation, compared with control normal human dermal fibroblasts (HDF). The IκBα-expressing adenovirus significantly reduced the spontaneous and IL-1β-stimulated expression of IL-6 and IL-8 by the RA FLS, and of IL-1β-stimulated HDF. The DN C/EBPβ resulted in modest reduction of the spontaneous and IL-1β-stimulated IL-6 secretion by RA FLS. In contrast, the DN C/EBPβ did not suppress the IL-1β-stimulated IL-6 secretion by HDF, nor did it affect IL-8 secretion by either cell type. Inhibition of c-Jun had no effect in either IL-6 or IL-8, by RA FLS or HDF, despite the fact that AP-1/c-Jun was strongly activated. The inhibition of IL-1β-stimulated IL-6 and IL-8 by the IκBα was due to inhibition of NF-κB activation, as determined by EMSA, and to suppression of transcriptional activation. Supporting the critical role of NF-κB, mouse embryonic fibroblasts with both NF-κB p50 and p65 genes deleted failed to express the IL-6 gene in response to IL-1. These observations demonstrate that NF-κB activation is the major contributor to both IL-6 and IL-8 secretion by RA FLS, both constitutively and following IL-1β stimulation. These observations support the role of inhibition of NF-κB as a novel therapeutic target in RA.

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Received for publication April 5, 2000. Accepted for publication September 13, 2000.

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[1] This work was supported in part by Grants AR43642, AR30692, and contract AR62229, each from the National Institute of Arthritis, Musculoskeletal and Skin Diseases.

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; HDF, human dermal fibroblasts; DN, dominant-negative; moi, multiplicity of infection; Ad, adenovirus; MAP, mitogen-activated protein.

0022-1767/00/$02.00

Materials and Methods

Cell culture

Normal HDF (CRL 1475) were purchased from American Type Culture Collection (ATCC, Manassas, VA). RA FLS were isolated from synovial tissue of patients undergoing scheduled total joint arthroplasty who met the American College of Rheumatology (former American Rheumatism Association) classification criteria for RA (35). Isolated synovial tissues were digested with collagenase, dispase, and DNAase I, and single-cell suspensions were obtained as previously described (36, 37). The cells were cultured at 37°C with 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine (all from Life Technologies, Gaithersburg, MD). The RA FLS were used between passages 4 and 10 and were free of contaminating cells (36, 37).

Viruses

Replication-defective adenovirus (Ad) vectors were propagated in the 293 embryonic kidney cell line (ATCC) and purified by ultracentrifugation through cesium chloride gradients. Plaque assay was used for the determination of the titers of viral stocks. The adenoviral vector AdIκBα (kindly provided by Dr. Jobin, University of North Carolina) expresses a nondegradable form of human IκBα, with hemagglutinin peptide added, in which serines 32 and 36 were replaced by alanine residues (S32A/S36A), thereby blocking its inducible phosphorylation and degradation (34, 38). Adenoviral vectors expressing DN versions of C/EBPβ (AdDNC/EBPβ) and c-Jun (AdDNc-Jun) were also employed. Each of these DNs lacked the transactivation domain, but retained the ability to dimerize and bind DNA, and inhibited the function of the respective wild-type transcription factor (30, 31, 33, 39). Infection of macrophages with either the AdDNC/EBPβ or the AdDNc-Jun significantly suppressed PMA-induced TNF-α secretion by macrophages (40).

Infections and collection of samples

A total of 1×10^4 cells/well were plated in 24-well plates (Costar, Cambridge, MA) in DMEM 10%FBS, and allowed to attach for 1–2 days. Cells were infected with AdIκBα, AdDNC/EBPβ, AdDNc-Jun, or the control vector expressing β-galactosidase (Adβgal) at a multiplicity of infection (moi) of 100 or 200 and incubated overnight. After washing twice with PBS, fresh medium without or with 1 ng/ml of recombinant human IL-1β (R&D Systems, Minneapolis, MN) was added. After 48 h the supernatants were collected for quantification of IL-6 and IL-8. All the experiments were performed in triplicate.

EMSA

RA FLS (1.5×10^5 cells/100 mm plate) were infected, and nuclear extracts were prepared 48 h later, as previously described (31, 33), either following IL-1β (1 ng/ml) stimulation for 30 min or without IL-1β treatment. ^{32}P-labeled oligonucleotides containing the IL-6 κB binding sequence (5′-TCGACATGTGGGATTTTCCCATGAC-3′), the IL-8 κB binding sequence (5′-TCGAGCGTGGAATTTCCTCTGG-3′), the IL-6 C/EBPβ binding sequence (5′-TCGAGACATTGCACAATCTG-3′) (41), or an AP-1 binding sequence from the collagenase promoter (32), were used as probes for EMSA.

DNA-binding reactions were performed by incubation for 20 min at room temperature in a final volume of 20 μl. The reaction mixture contained 100 mmol/L NaCl, 20 mmol/L HEPES, 1 mmol/L EDTA, 4% glycerol, 5% (w/v) Ficoll, 0.25 μg BSA, 1 μg poly(dI-dC), 1 ng ^{32}P-labeled oligonucleotide, and 5–10 μg of the nuclear extract. Protein:DNA complexes were separated from free probe by electrophoresis on 5% polyacrylamide gels in 0.5× TBE at 160 V for 2–3 h. Gels were dried onto Whatman 3 M paper (Whatman, Maidstone, U.K.) and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). For supershift assays 1–2 μl of mouse mAb against NF-κB p65 (Transduction Laboratories, Lexington, KY), or rabbit polyclonal Abs against NF-κB p50 (NLS, sc114 X; Santa Cruz Biotechnology, Santa Cruz, CA), NF-κB p52 (K-27, sc-298 X; Santa Cruz Biotechnology), c-Rel (N, sc70 X, Santa Cruz), C/EBPβ (C-19, sc-150 X; Santa Cruz Biotechnology), or a goat polyclonal Ab against c-Jun/AP-1 (N, sc-45 X; Santa Cruz Biotechnology), were incubated with the nuclear extract on ice for 30 min before the addition of the labeled oligonucleotide to the binding reaction.

Western blots

Cells were harvested, washed with PBS, to which lysis buffer (150 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris, 2 mM EDTA (pH 8.0)) containing protease inhibitors (2 ng/ml leupeptin, 0.5 mM PMSF, 15 ng/ml aprotinin) were added, to prepare cell extracts as previously described (15, 42). Twenty micrograms of protein from each sample was boiled and analyzed on 12.5% polyacrylamide gels for 3 h, transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA), and blocked with PBS containing 0.2% Tween 20/5% nonfat milk for 1 h. The membranes were incubated overnight at 4°C with rabbit monospecific Abs to IκBα (C-21, sc 371; Santa Cruz Biotechnology), C/EBPβ (C-19, sc 150; Santa Cruz Biotechnology), or c-Jun (PC06L; Oncogene Research Products, Cambridge, MA). Also, a mouse anti-tubulin mAb (Calbiochem, La Jolla, CA) was used for the examination of equal loading. The filters were washed three times with PBS containing 0.2% Tween 20 and 5% nonfat milk and incubated for 2 h with donkey anti-rabbit or anti-mouse secondary Abs (1:2000 dilution) conjugated to HRP (Amersham, Piscataway, NJ). After washing, the blots were developed using enhanced ECL (Amersham).

RT-PCR

RNA was isolated by the RNAzol B method (Tel-Test, Friendwood, TX) as described by the manufacturer. One microgram of total RNA was incubated in reaction buffer containing oligo(dT) primer, avian myeloblastosis virus reverse transcriptase and RNase inhibitor (recombinant RNasin RNase inhibitor), and dNTP mixture for 1 h at 42°C. The reaction was stopped by incubation at 94°C for 5 min. PCR was performed using primers for IL-6 (forward 5′-ATGAACTCCTTCTCCACAAGCGC-3′, reverse 5′-GAAGAGCCCTCAGGCTGGACTG-3′) and for IL-8 (forward 5′-CCAAGGAAAACTGGGTGCAGAG-3′, reverse 5′-GGCACAGGGGAACAAGGACTTG-3′), and β-actin (Clontech, Palo Alto, CA). Cycling conditions included: one initial denaturation cycle for 5 min at 94°C, 25 cycles of amplification for 2 min at 72°C, 1 min at 94°C, 1 min at 60°C, and a final extension phase consisting of 1 cycle of 10 min at 72°C. Ten microliters of the PCR product and 2 μl of loading buffer were run on a 1% agarose gel stained with ethidium bromide.

RNase protection assay

Untreated and IL-1-stimulated wild-type and p65/p50$^{-/-}$ mouse embryonic fibroblasts (43) were harvested for RNA preparation employing Tri-Reagent (Molecular Research Center, Cincinnati, OH). RNase protection assay was performed according to the manufacturer's specifications (PharMingen, San Diego, CA).

ELISA

For IL-6 and IL-8, sandwich ELISAs were performed, according to the manufacturer's instructions, employing commercially available kits (DY206 and DY208 DuoSet kits; R&D Systems). The OD were read by a Microplate Autoreader (Bio-Tek, Burlington, VT).

Statistical analysis

To estimate the effect of IL-1β stimulation or viral infections on the production of cytokines, the mean values of the experiments done with the cells of individual patients or HDF were analyzed using a two-tailed paired Student's *t* test. To compare the levels of cytokines produced by HDF and RA FLS, an unpaired Student's *t* test was used.

Results

RA FLS produce higher levels of IL-6 and IL-8 than HDF, spontaneously and after IL-1β-stimulation

Normal HDF produced very low levels of IL-6 and IL-8 spontaneously, but the production increased significantly following IL-1β stimulation (IL-6 from 0.02 ± 0.02 ng/ml to 1.07 ± 0.41 ng/ml, $p < 0.04$; and IL-8 from 0.09 ± 0.05 ng/ml to 5.33 ± 0.85 ng/ml, $p < 0.002$). The concentrations of the constitutively secreted IL-6 and IL-8 were significantly ($p < 0.005$ and $p < 0.03$, respectively) greater in the supernatants of the RA FLS compared with the HDF (Fig. 1). The production of both cytokines by RA FLS increased significantly following IL-1β stimulation (IL-6 from 0.67 ± 0.19 to 11.58 ± 3.08 ng/ml, $p < 0.03$; and IL-8 from 1.44 ± 0.56 to 33.27 ± 7.50 ng/ml, $p < 0.02$). Following IL-1β treatment, the concentrations of both cytokines were greater in the supernatants of the RA FLS compared with the HDF ($p < 0.005$). Thus, although the RA FLS spontaneously secreted significantly more IL-6 and IL-8 than the HDF, they remained highly responsive to IL-1β.

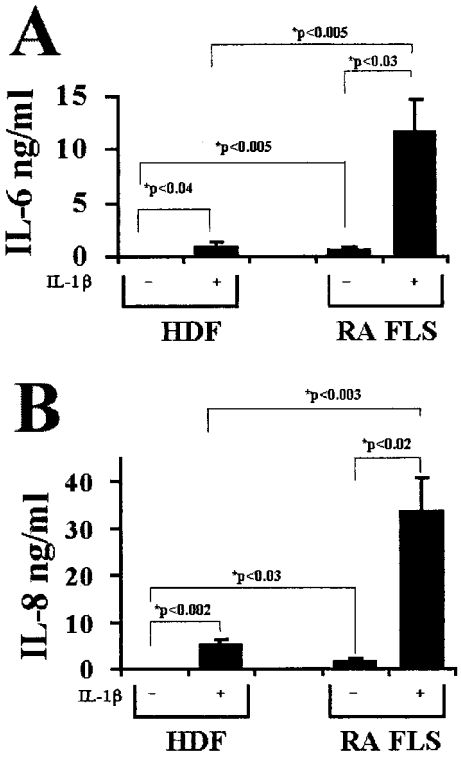

FIGURE 1. Spontaneous and IL-1β-stimulated secretion of IL-6 and IL-8 by normal HDF and RA FLS. RA FLS and HDF were cultured as described in *Materials and Methods*. Control (IL-1β^-), or IL-1β (IL-1β^+) (1 ng/ml)-containing medium was added to the cells for 48 h. The culture supernatants were harvested and employed to quantitate IL-6 and IL-8 by ELISA, which is expressed in nanograms per milliliter. The values for the HDF represent the mean ± SE of five independent experiments, performed in triplicate. The values for the RA FLS represent the mean ± SE of five individual patients, each performed in triplicate. The significance of the differences between the values are indicated in the figure: *, differences were determined by unpaired Student's *t* test; **, Student's *t* test for matched pairs was employed.

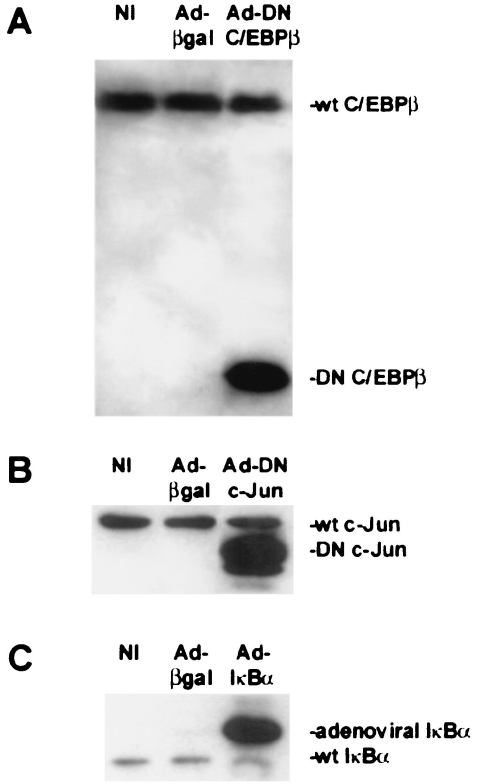

FIGURE 2. Expression of the DN proteins by the adenoviral vectors. HDF were infected with the adenoviral vectors indicated at the top of each panel as described in *Materials and Methods*. After 48 h the cells were harvested, and whole-cell extracts were analyzed by Western blotting using monospecific Abs against C/EBPβ (*A*), c-Jun (*B*), or IκBα (*C*). The location of the wild-type and DN version of each protein are indicated on the right of each panel. Uninfected cells are present where indicated (NI).

Expression of functional IκBα and DNs of C/EBPβ and AP-1/c-Jun

Expression of the AdβGal was examined by x-Gal staining 48 h after infection. Although only about 50% of cells were positive at 100 moi, at 200 moi of AdβGal >95% of the HDF and RA FLS expressed β-galactosidase (data not shown). Because cell death was not observed with any of the viruses at 200 moi, this concentration was used for subsequent experiments. Western blot analyses using whole-cell extracts of RA FLS and HDF demonstrated that DN proteins encoded by the viruses (AdIκBα, AdDNC/EBPβ, and AdDNc-Jun) were highly expressed (Fig. 2). EMSAs, using nuclear extracts of RA FLS, were employed to document the function of each ectopically expressed molecule (discussed below, see Figs. 6 and 7). The AdIκBα inhibited the binding of NF-κB to the IL-6 κB (Fig. 6*A*) and IL-8 κB (data not shown) oligonucleotides, and the DN C/EBPβ and DN c-Jun bound to their respective oligonucleotides (see Fig. 7).

Effects of the viruses on the IL-1β-stimulated production of IL-6 and IL-8 by normal HDF

The concentrations of IL-6 and IL-8 constitutively secreted by the normal HDF were too low to characterize the effects of the DN-expressing adenoviruses. Therefore, we examined the effect of these vectors on the IL-1β-stimulated secretion of IL-6 and IL-8 by HDF. Compared with the control virus (Adβgal), infection of normal HDF by AdIκBα resulted in >95% suppression of IL-1β-stimulated IL-6 ($p < 0.005$) and IL-8 ($p < 0.004$) (Fig. 3). In contrast, infection of HDF with AdDNC/EBPβ or AdDNc-Jun had no effect on the production of either IL-6 or IL-8 following IL-1β stimulation. These findings suggest that NF-κB is essential for the IL-1β-stimulated expression of both cytokines by normal HDF.

Effects of the-DN expressing vectors on the spontaneous and IL-1β-induced production of IL-6 and IL-8 by RA FLS

The effect of the DN-expressing adenoviral vectors on the IL-6 and IL-8 constitutively secreted by RA FLS was examined. AdIκBα infection resulted in significant suppression of the spontaneous secretion of both IL-6 (51%, $p < 0.001$) and IL-8 (82%, $p < 0.002$) by RA FLS, compared with Adβgal infection (Fig. 4). Expression of the DN C/EBPβ resulted in a modest but statistically significant reduction of the spontaneous secretion of IL-6 (19%, $p < 0.02$), compared with the control β-galactosidase (Fig. 4*A*). However, there was no difference in the concentration of IL-6 between the AdDNC/EBPβ-infected cells and those that were uninfected (Fig. 4*A*). In contrast, infection with the AdDNC/EBPβ had no effect on the spontaneous secretion of IL-8 by the RA FLS, compared with Adβgal-infected or uninfected cells (Fig. 4*B*). Additionally, expression of the DN c-Jun had no effect on constitutive expression of either IL-6 or IL-8 by RA FLS. These observations document that activation of NF-κB is critical for the constitutive expression of IL-6 and IL-8 by RA FLS. C/EBPβ contributed to the secretion of IL-6 but not IL-8, while AP-1/c-Jun did not play a role.

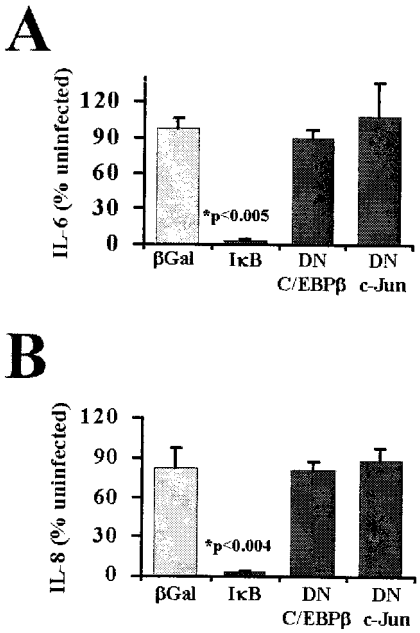

FIGURE 3. IκBα suppresses the IL-1β induced secretion of IL-6 and IL-8 by normal HDF. Normal HDF were infected with adenoviral vectors expressing β-galactosidase (βgal), IκBα, DN C/EBPβ, and DN c-Jun at 200 moi as indicated in each panel. The cells were incubated with IL-1β for 48 h, and the supernatants were harvested to measure IL-6 (*A*) and IL-8 (*B*). The concentration of IL-6 and IL-8 is presented as the percentage of control uninfected cells treated with IL-1β. The asterisk represents the difference from Adβgal-infected cells.

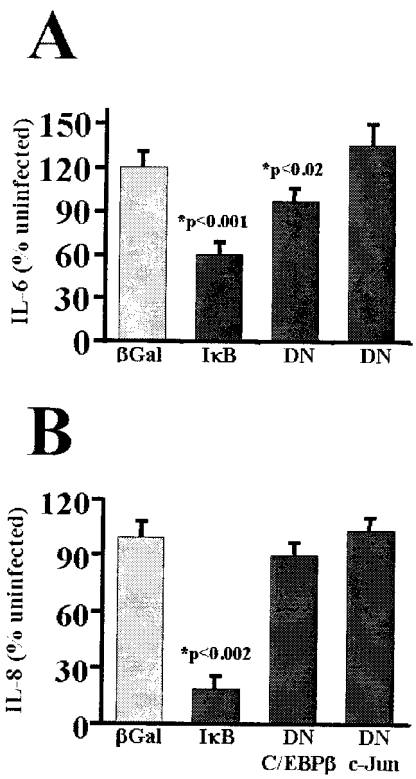

FIGURE 4. NF-κB regulates the constitutive secretion of IL-6 and IL-8 by RA FLS. RA FLS were infected with AdIκBα, AdDNC/EBPβ, AdDNc-Jun, or the control Adβgal as described in *Materials and Methods*. After infection, the cells were incubated in medium without added IL-1β for 48 h, the supernatants were harvested, and the constitutively secreted IL-6 (*A*) and IL-8 (*B*) was determined by ELISA. The data are presented as the percentage of cytokine secreted by uninfected cells. The data are presented as the mean ± SE of experiments performed in triplicate with FLS from five different patients. ∗, Values of p as determined by comparing the differences between the cells infected with AdIκBα or AdDNC/EBPβ and the control Adβgal, employing a paired two-tailed Student's t test ($n = 5$).

The effect of the DNs for NF-κB, C/EBPβ, and c-Jun on the IL-1β-stimulated secretion of IL-6 and IL-8 by RA FLS was examined. Similar to the results observed with the HDF, the IL-1β-stimulated production of IL-6 by RA FLS was significantly inhibited by AdIκBα (by 86%, $p < 0.001$) (Fig. 5*A*). In contrast to the results obtained with the HDF, the DN C/EBPβ resulted in a modest reduction of IL-1β-stimulated IL-6 (23%, $p < 0.005$), compared with the control β-galactosidase (Fig. 5*A*). Similar to the results of the unstimulated cultures, no difference was observed between the AdDNC/EBPβ infected and the uninfected RA FLS. Additionally, the ectopic expression of the DN c-Jun had no effect on IL-1β-stimulated IL-6 secretion.

The results were somewhat different for IL-8. The IL-1β-stimulated secretion of IL-8 was suppressed only by AdIκBα infection, which resulted in 90% ($p < 0.001$) reduction (Fig. 5*B*). Expression of neither the DN C/EBPβ nor the DN c-Jun had any effect on IL-1β-stimulated IL-8 secretion by RA FLS (Fig. 5*B*). These observations suggest that NF-κB is the critical transcription factor responsible for both the spontaneous and the IL-1β-stimulated expression of the IL-8 gene by RA FLS.

Activation of wild-type transcription factors and the effect of DNs in RA FLS

To further characterize the mechanism of suppression observed in this study, EMSAs were employed, using radiolabeled oligonucleotides capable of binding NF-κB, C/EBPβ, and AP-1. NF-κB, C/EBPβ, and c-Jun were each constitutively activated and bound to their respective oligonucleotides, employing nuclear extracts from unstimulated RA FLS (Figs. 6 and 7). Following stimulation with IL-1β, NF-κB and c-Jun binding were each enhanced (Figs. 6 and 7). In contrast, binding by C/EBPβ was somewhat diminished following stimulation with IL-1β (Fig. 7*A*). In each instance, specificity was demonstrated by inhibition with excess unlabeled oligonucleotide and with monospecific Abs (Figs. 6 and 7). Monospecific Ab to C/EBPβ supershifted its respective complex (Fig. 7*A*). The AP-1 oligonucleotide-bound complex was partially supershifted by monospecific anti-c-Jun, indicating that additional factors may also be bound (Fig. 7*B*).

The complex binding to the IL-6 promoter NF-κB binding site, following treatment with IL-1β, contained principally NF-κB p65 because monospecific Abs to NF-κB p65 resulted in a supershift of the majority of the complex (Fig. 6*A*). No effect was observed with anti-NF-κB p52, or with c-Rel or the irrelevant control (anti-c-Jun) Ab. Employing the monospecific anti-NF-κB p50 Ab, supershift of a portion of the complex was observed (Fig. 6*A*). This same Ab caused a dramatic supershift of NF-κB p50 homodimers employing macrophage nuclear extracts (data not shown, and Ref. 33). Combining Abs to p65 and p50 failed to supershift the entire complex, suggesting the presence of an unidentified component. These findings indicate that the IL-6 κB site bound mainly NF-κB p65 and, to a lesser extent, NF-κB p50.

Because the sequence of the IL-8 NF-κB binding site was slightly different from the IL-6 κB site (see *Materials and Methods*), we examined binding to the IL-8 κB site. NF-κB in the RA FLS nuclear extracts bound constitutively, and the binding increased following IL-1β stimulation (Fig. 6*B*). Only the Ab recognizing NF-κB p65 inhibited the binding of the complex (Fig.

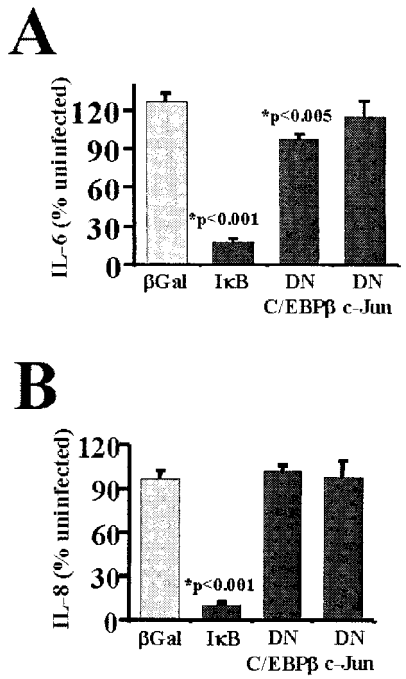

FIGURE 5. NF-κB regulates the IL-1β-stimulated secretion of IL-6 and IL-8 by RA FLS. RA FLS were uninfected or infected with AdIκBα, AdDNC/EBPβ, AdDNc-Jun, or the control Adβgal as described in *Materials and Methods*. After infection, the cells were incubated in medium with added IL-1β for 48 h, the supernatants were harvested, and IL-6 (*A*) and IL-8 (*B*) secreted following stimulation by IL-1β was determined by ELISA. The data are presented as the percentage of cytokine secreted by uninfected cells. The data are presented as the mean ± SE of experiments performed in triplicate with FLS from five different patients. *, Values of *p* as determined by comparing the differences between the cells infected with AdIκBα or AdDNC/EBPβ and the control Adβgal, employing a paired two-tailed Student's *t* test.

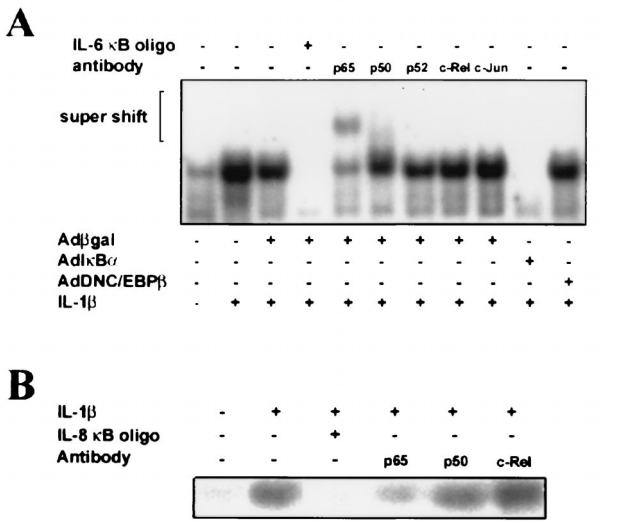

FIGURE 6. NF-κB is constitutively activated in RA FLS and is increased following stimulation with IL-1β. *A*, IL-6 κB binding site and the effect of IκBα. Nuclear extracts were prepared from RA synovial fibroblasts that were untreated or stimulated with IL-1β (1ng/ml) for 30 min, as indicated. Before treatment, the cells were infected for 72 h with Adβgal, AdIκBα, and AdDNC/EBPβ, where indicated. The EMSA was performed as described in *Materials and Methods*, employing a ^{32}P-labeled oligonucleotide representing the NF-κB binding site of the IL-6 promoter. Before running the gel, nuclear extracts were incubated with unlabeled IL-6 κB oligonucleotide (IL-6 κB oligo), or Abs to NF-κB p65, p50, p52, c-Rel or an irrelevant control Ab (anti-c-Jun), as identified. The supershifted complexes are identified to the left of the figure. *B*, The IL-8 κB promoter site binds NF-κB p65, but not p50. The experiments were performed as described in *A*, except that a ^{32}P-labeled oligonucleotide representing the NF-κB binding site of the IL-8 promoter was employed. With this oligonucleotide, the NF-κB p65-containing complexes were identified by inhibition of binding, and no discrete supershift was observed.

6*B*). Abs recognizing NF-κB p50, c-Rel (Fig. 6*B*), and NF-κB p52 (not shown) did not decrease binding of the complex to the κB IL-8 oligonucleotide. These observations suggest that the NF-κB p65 homodimers were the principal complex contributing to the expression of the IL-8 promoter in RA FLS.

The effect of each of the inhibitory molecules was examined by EMSA, employing nuclear extracts from RA FLS. Infection with the AdIκBα inhibited spontaneous (data not shown) and IL-1β-induced binding to the IL-6 κB (Fig. 6*A*) and IL-8 κB (data not shown) promoters. The expression of the DN C/EBPβ (Fig. 6*A*) or the DN c-Jun (data not shown) did not affect NF-κB activation, as determined by EMSA. Expression of the DN C/EBPβ resulted in a dramatic increase of the transcriptionally inactive version of C/EBPβ (Fig. 7*A*). This complex was also supershifted by the monospecific Ab (data not shown). The bound DN C/EBPβ overlapped with the wild-type C/EBPβ, precluding evaluation of the effect of the DN on the wild type in this experiment. Previous studies have demonstrated that this DN may inactivate the wild-type C/EBPβ by heterodimerizing with and inactivating wild-type C/EBPβ or by binding as a homodimer, displacing the wild type from its DNA binding site (31). Expression of the DN c-Jun resulted in inhibition of binding by the wild-type complex, with binding of the transcriptionally inactive version of c-Jun, which migrated more rapidly in the gel (Fig. 7*B*). In contrast, the ectopic expression of the IκBα did not effect the migration of the AP-1 binding complex (Fig. 7*B*). These data document the functional effects of IκBα, DN C/EBPβ, and c-Jun by EMSA.

IκBα suppresses the transcriptional activation of IL-6 and IL-8 in RA FLS

To further characterize the mechanism by which IκBα functions in RA FLS, RT-PCR was performed (Fig. 8*A*). As expected, following stimulation with IL-1β, increased mRNA for both IL-6 and IL-8 was observed. AdIκBα infection resulted in reduced IL-6 and IL-8 m-RNA compared with infection with Adβgal, constitutively and following treatment with IL-1β (Fig. 8*A*). The control β-actin was expressed comparably following infection with either virus. These observations indicate that infection with the AdIκBα results in inhibition of NF-κB activation and subsequent suppression of the transcriptional activation of the IL-6 and IL-8 genes in RA FLS.

NF-κB is essential for IL-6 expression

Our observations (Figs. 4 and 5) and those of others (26, 44) suggest that factors other than NF-κB may be necessary for the expression of IL-6, at least in RA FLS. To determine whether NF-κB was necessary for IL-6 expression, we employed mouse embryonic fibroblasts in which both NF-κB p50 and p65 genes had been deleted. Following stimulation with IL-1, the IL-6 gene was expressed in wild-type mouse embryonic fibroblasts, as determined by an RNase protection assay (Fig. 8*B*). In contrast, no expression of the IL-6 gene was detected following IL-1 stimulation of cells lacking the NF-κB p50 and p65 genes. These observations indicate that NF-κB activation is essential for the expression of the IL-6

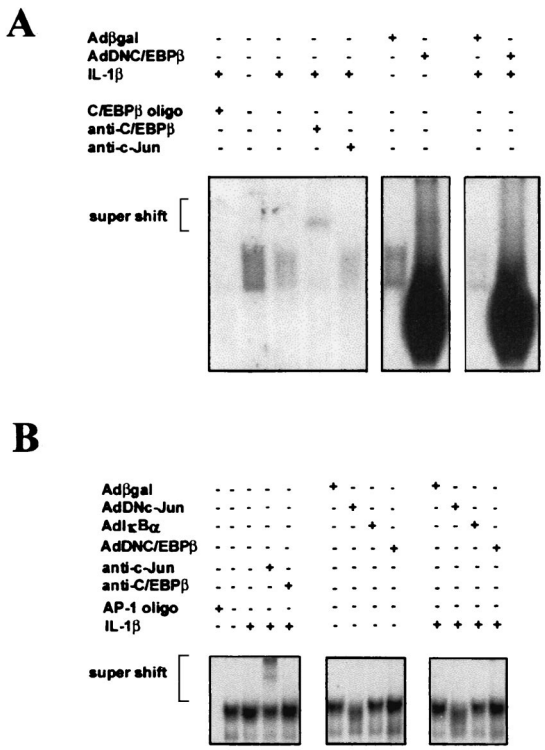

FIGURE 7. C/EBPβ and AP-1/c-Jun activation in RA FLS. *A*, IL-1β does not increase C/EBPβ activity in RA FLS, and the DN C/EBPβ expressed in RA FLS binds avidly to the IL-6 promoter. RA FLS were cultured as described in Fig. 6. Cells were uninfected, or infected with Adβgal or AdDN/EBPβ, as indicated. IL-1β (1 ng/ml) or control medium were added, the cells were harvested after 30 min, and nuclear extracts were prepared as described. The extracts were incubated with a ^{32}P-labeled oligonucleotide representing the C/EBPβ binding site of the IL-6 promoter. Before running the gel, extracts were incubated with unlabeled C/EBPβ oligonucleotide (C/EBPβ oligo) or with a C/EBPβ-specific or a control (anti-c-Jun) Ab. Supershifted complex is identified to the left of the figure. *B*, AP-1/c-Jun is constitutively activated in RA FLS, and the DN c-Jun inhibits binding to the AP-1 binding oligonucleotide. Cells were uninfected, or infected with Adβgal, AdDNc-Jun, or AdIκBα, as indicated. Control or IL-1β (1 ng/ml)-containing media were added, the cells were harvested after 30 min, and nuclear extracts were prepared, as described. The extracts were incubated with a ^{32}P-labeled AP-1 binding oligonucleotide. Before running the gel, extracts were incubated with unlabeled AP-1 oligonucleotide (AP-1 oligo) or with a c-Jun-specific or a control (anti-C/EBPβ) Ab. Supershifted complex is identified to the left of the figure.

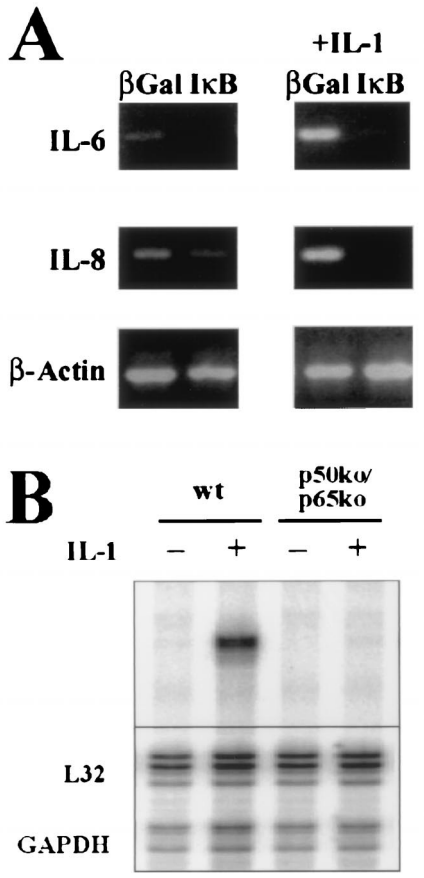

FIGURE 8. NF-κB activation regulates IL-6 and IL-8 gene transcription: *A*, IκBα suppresses the constitutive and IL-1β-stimulated expression of IL-6 and IL-8 by RA FLS at the transcriptional level. RA FLS were infected at 200 moi by AdIκBα or the control Adβgal. The cells were incubated with control medium or IL-1β as indicated at the top of each panel. The cells were harvested after 48 h, and RNA was isolated and used to perform RT-PCR for IL-6, IL-8, and β-actin as described in *Materials and Methods*. *B*, NF-κB p65 and p50 are essential for expression of the IL-6 gene in fibroblasts. Wild-type (wt) or NF-κB p50/p65 double knockout (p50ko/p65ko) mouse embryonic fibroblasts, which had been serum depleted, were incubated with IL-1α (5 μg/ml) for 2 h. The cells were harvested, and an RNase protection assay for murine IL-6 was performed as described in the *Materials and Methods*. GAPDH and L32 served as the controls.

gene mouse embryonic fibroblasts, supporting the observations obtained with the normal HDF (Fig. 3).

Discussion

This study has characterized the transcriptional mechanisms responsible for the spontaneous and the IL-1β-stimulated secretion of IL-6 and IL-8 by RA FLS. RA FLS produced significantly greater concentrations of both cytokines compared with normal HDF, both spontaneously and following IL-1β stimulation, consistent with earlier observations (2–6). Although the composition of the κB complexes binding the IL-6 and IL-8 promoters was somewhat different, expression of IκBα inhibited the nuclear localization and activation of all IL-6 and IL-8 κB binding complexes. Additionally, expression of the IκBα resulted in suppression of the transcription of both the IL-6 and IL-8 genes and in the marked reduction of the constitutive and IL-1β-stimulated secretion of IL-6 and IL-8 by RA FLS. The observed results were not due to decreased cell numbers or apoptosis (data not shown). These observations document the critical role of NF-κB in the expression of IL-6 and IL-8 by RA FLS.

Other studies have examined the potential contribution of NF-κB to the expression of IL-6 and IL-8 by RA FLS. NF-κB activation was temporally associated with IL-6 and IL-8 expression in RA FLS (4, 26, 29, 45, 46). Inhibition of NF-κB by *N*-acetyl-L-cysteine or aurothioglucose was associated with inhibition of IL-6 and IL-8 secretion by RA FLS (45, 46). Although these observations suggest that NF-κB activation may be related to the expression of IL-6 and IL-8 by RA FLS, none has specifically defined the role of NF-κB in isolated RA FLS. Our study is novel because it has directly documented the contribution of NF-κB to the constitutive and the IL-1β-stimulated secretion of IL-6 and IL-8, employing isolated RA FLS. Employing whole RA synovial tissue digests possessing fibroblasts, macrophages, and T cells, the constitutive secretion of IL-6 and IL-8 was inhibited by 85% and 40%, respectively, by a porcine IκBα-expressing adenovirus (47). However, these results are difficult to compare with ours because

the porcine IκBα employed was not modified to prevent degradation following activation, and cytokines, including TNF-α and IL-1, were present in the culture supernatants. Additionally, both macrophages and FLS from RA synovial tissue produce IL-6 and IL-8, and this study did not document the cell type(s) responsible, nor did it address the potential contribution of other transcription factors (47). Nonetheless, both observations, using different experimental conditions, have documented the important role of NF-κB in the expression of IL-6 and IL-8 in RA synovial tissue.

The role of C/EBPβ and AP-1/c-Jun in the expression of IL-6 and IL-8 in RA FLS was also examined. Earlier studies have documented that C/EBPβ and AP-1 may contribute to the activation of the IL-6 and IL-8 genes (19–21, 28). Our study employed a novel approach to specifically inhibit the function of each potentially relevant transcription factor. Neither C/EBPβ nor AP-1/c-Jun contributed to the expression of the IL-8 gene, in either RA FLS or HDF, despite the activation of both C/EBPβ and AP-1 in RA FLS. It is possible that C/EBPβ or c-Jun may contribute to the expression of IL-8 in other cell types or when the cells are stimulated differently (24, 48). Nonetheless, our observations do not exclude a potential interaction of NF-κB with AP-1 or C/EBPβ (19–21, 24, 28, 48). Because we did not employ AdDNC/EBPβ and AdDNc-Jun simultaneously, it is possible that the expression of the IL-8 gene may require NF-κB plus either C/EBPβ or AP-1 (20, 24, 25).

Although the expression of IL-6 in RA FLS was regulated primarily by NF-κB, the DN C/EBPβ resulted in a 20% reduction, both spontaneously and following IL-1β stimulation, suggesting that C/EBPβ contributes to the activation of the IL-6 gene in this cell type. However, an alternate interpretation, which cannot be excluded from our data, is that adenovirus infection resulted in increased IL-6, but not IL-8, and that this increase was due to C/EBPβ. Arguing against this possibility, adenoviral infection had no effect on the IL-6 secreted by the HDF, and no activation of C/EBPβ was observed following adenoviral infection of RA FLS (Fig. 7*A*). These observations are consistent with previous studies showing that C/EBPβ was not necessary for the expression of the IL-6 gene, because C/EBP$\beta^{-/-}$ knockout mice produced essentially normal IL-6 (49, 50). Following stimulation with IL-1β, inhibition of NF-κB and C/EBPβ accounted for essentially all of the IL-6 secreted. Although NF-κB and C/EBPβ have been shown by transient transfection to synergistically activate the transcription of the IL-6 gene (22), the approach employed in this study does not allow us to determine whether or not these two factors were interacting synergistically in RA FLS. In contrast to the results observed with the RA FLS, employing normal fibroblasts, only NF-κB contributed to IL-1-stimulated IL-6 activation, because IκBα in the HDF and deletion of NF-κB p50 and p65 in mouse embryonic fibroblasts essentially abolished IL-1-stimulated IL-6 gene expression.

An earlier study has suggested that AP-1 may contribute to IL-6 and IL-8 secretion by RA FLS, because *jun* D down-modulated AP-1 and suppressed TNF-α-induced secretion of these cytokines (28). In contrast, our study clearly demonstrated that even though AP-1 activation was suppressed by EMSA following expression of the DN c-Jun, no suppression of IL-6 or IL-8 secretion was observed. Activation of the mitogen-activated protein (MAP) kinase pathway has been shown to contribute to both IL-6 and IL-8 expression (44, 51, 52). Inhibition of the p38 MAP kinase pathway in RA FLS, or the extracellular signal-related kinase-1/2 pathway in monocytes, suppressed IL-6 secretion without affecting NF-κB activation (44, 51). In RA FLS p38, MAP kinase activation by IL-1β contributed to IL-6 expression by stabilizing the IL-6 mRNA (44). Similarly, MKK6, a p38 MAP kinase-specific activator, stabilized IL-8 mRNA, while activation of the c-Jun N-terminal kinase pathway by MKK7 enhanced IL-8 synthesis and IL-8 promoter activity (52). Despite the effects of MKK6 and 7 on the regulation of the IL-8 gene, basal NF-κB activation was always present, suggesting that activation of the MAP kinase pathway may enhance NF-κB-induced IL-8 expression. Although these observations suggest that activation of the p38 and extracellular signal-related kinase-1/2 MAP kinase pathways may modulate IL-6 and IL-8 expression, our observations demonstrate that activated AP-1/c-Jun did not participate in the spontaneous or IL-1β-stimulated activation of the IL-6 or IL-8 genes in either RA FLS or HDF. However, because the activation of NF-κB observed by EMSA was comparable for both cell types (data not shown), it is possible that modulation of gene expression by MAP kinase pathway activation may have contributed to the differences observed between the RA FLS and the HDF, by mechanisms that do not involve the AP-1 complex.

The composition of the κB complexes responsible for the IL-1β-stimulated activation of the IL-6 and IL-8 promoters in RA FLS were somewhat different. NF-κB p65 was the most abundant species binding to the IL-6 κB site, while Abs to NF-κB p50 partially supershifted the IL-6 κB binding complex. These observations suggest the presence of p65 homodimers and p65/p50 heterodimers following IL-1β-stimulation, consistent with earlier observations (26). Although controversy exists in the literature (21, 23), employing RA FLS, only NF-κB p65 bound to the IL-8 κB oligonucleotide, and no NF-κB p50 containing heterodimers or p50 homodimers were observed. In a previous study, using RA FLS, antisense oligonucleotides to NF-κB p65, and c-Rel, but not NF-κB p50, partially inhibited the IL-1β-stimulated IL-8 secretion by RA FLS, suggesting a potential role for c-Rel (53). However, similar to our observations, no c-Rel-containing complexes were identified (53). Additionally, the effectiveness and specificity of the c-Rel antisense oligonucleotides employed in this study were not documented, making the interpretation that c-Rel contributed to the expression of the IL-8 gene in RA FLS inconclusive (53). A recent study employing rabbit FLS identified a major contribution of NF-κB p50 to the regulation of IL-1β-stimulated matrix metalloproteinase 1 (54). In contrast, no NF-κB p50 homodimers were detected with RA FLS nuclear extracts employing the IL-6 κB oligonucleotide, or an HIV/Ig NF-κB binding oligonucleotide, which avidly bound p50 homodimers in macrophages (Ref. 33, and data not shown). These observations document that NF-κB p65 was the dominant species binding to both IL-6 and IL-8 κB binding sites, although NF-κB p65/p50 heterodimers also bound to the IL-6, but not the IL-8, κB site, in IL-1β-treated RA FLS nuclear extracts. Together, these observations identify the central role of NF-κB in the regulation of the IL-6 and IL-8 genes by RA FLS, and they suggest that inhibition of NF-κB may be an effective target in the treatment of RA.

Acknowledgments

We thank Dr. Alisa Koch (Northwestern University Medical School, Chicago, IL) for providing the RA FLS, Dr. Michael Birrer (Biomarkers and Prevention Branch, National Institutes of Health, Rockville, MD) for the AdDNc-Jun, and Dr. Christian Jobin (University of North Carolina, Chapel Hill, NC) for the AdIκBα.

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