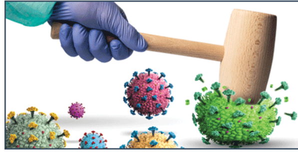

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Immunosuppressive Leflunomide Metabolite (A77 1726) Blocks TNF-Dependent Nuclear Factor-κB Activation and Gene Expression[1]

Sunil K. Manna and Bharat B. Aggarwal[2]

Leflunomide is a novel immunosuppressive and antiinflammatory agent currently being tested for treatment of autoimmune diseases and transplant rejection. NF-κB is a transcription factor activated in response to a wide variety of inflammatory stimuli, including TNF, but whether leflunomide blocks NF-κB activation is not known. In the present report we demonstrate that treatment of a human T cell line (Jurkat) with leflunomide blocks TNF-mediated NF-κB activation in a dose- and time-dependent manner, with maximum inhibition at 5–10 μM. Inhibition was not restricted to TNF-induced activation, because leflunomide also inhibited NF-κB activation induced by other inflammatory agents, including phorbol ester, LPS, H_2O_2, okadaic acid, and ceramide. Leflunomide blocked the degradation of IκBα and subsequent nuclear translocation of the p65 subunit, steps essential for NF-κB activation. This correlated with inhibition of dual specificity-mitogen-activated protein kinase kinase as well as an Src protein tyrosine kinase, p56*lck*, by leflunomide. Reducing agents did not reverse the effect of leflunomide. Leflunomide also suppressed the TNF-activated NF-κB-dependent reporter gene expression. Our results thus indicate that leflunomide is a potent inhibitor of NF-κB activation induced by a wide variety of inflammatory stimuli, and this provides the molecular basis for its anti-inflammatory and immunosuppressive effects. *The Journal of Immunology,* **1999, 162: 2095–2102.**

Nuclear factor-κB is a ubiquitous transcription factor that plays a critical role in cells of the immune system. This transcription factor is sequestered in its inactive state in the cytoplasm by a noncovalent association with the inhibitory protein called IκBα[3] (for references, see Ref. 1). NF-κB is activated by a wide variety of inflammatory stimuli, including TNF, IL-1, okadaic acid, phorbol ester, H_2O_2, ceramide, endotoxin, and γ-radiation. Most of these agents induce the phosphorylation-dependent degradation of IκBα, thus unmasking the nuclear localization signals on p65 and activating NF-κB. Indeed, IκBα-deficient mice exhibit constitutive NF-κB activation, severe runting, dermatitis, and extensive granulopoiesis and die during the neonatal period (2).

NF-κB regulates the expression of many genes that play essential roles in immune and inflammatory responses. It regulates the expression of genes for various cytokines, the MHC, viral replication (e.g., type I HIV), and cell proliferation (1, 2). The inappropriate regulation of NF-κB and its dependent genes has been associated with various pathological conditions, including septic shock, graft-vs-host reaction, acute inflammatory conditions, acute phase response, transplant rejection, autoimmune diseases, viral replication, radiation damage, atherosclerosis, and cancer (1, 2).

Agents that can block NF-κB activation have potential for treatment of the pathological situations indicated above. Leflunomide (HWA-486; *N*-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide) is one such agent, in that it exhibits antiinflammatory, antiproliferative, and immunosuppressive effects through mechanisms that are not fully understood (3–7). HWA-486 is a prodrug that is rapidly converted in the cell to an active metabolite, *N*-(4-trifluoromethylphenyl-2,2-cyano-3-hydroxycrotoamide), named A77 1726. The initial conversion involves the opening of the isoxazole ring to produce A77 1726, which constitutes >95% of the drug in the circulation. Early experiments suggest that A77 1726 blocks T cell proliferation stimulated by anti-CD28 and PMA, anti-CD3, and IL-2 (5, 8). It also prevents the proliferation of B cells and Ab production by B cells (4).

Since NF-κB activation is critical for inflammation, and leflunomide exhibits antiinflammatory and immunosuppressive effects, we investigated whether it inhibits NF-κB activation. Our results show that the active form of leflunomide, A77 1726, is a potent inhibitor of NF-κB activation induced by TNF and other inflammatory agents.

Materials and Methods

Materials

Leflunomide (A77 1726) was a gift from Dr. Robert R. Bartlett (Hoechst, Weisbaden, Germany). A 5 mM solution of leflunomide was made in water. Bacteria-derived human rTNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Life Technologies (Grand Island, NY). Tris, glycine, NaCl, SDS, PMA, and BSA were obtained from Sigma (St. Louis, MO). The polyclonal Abs used were as follows: anti-p65, against the epitope corresponding to amino acids mapping within the amino-terminal domain of human NF-κBp65; anti-p50, against a peptide 15 amino acids long mapping at the NLS region of NF-κB p50; anti-IκB-α, against amino acids

Cytokine Research Laboratory, Department of Molecular Oncology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

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[2] Address correspondence and reprint requests to Dr. Bharat B. Aggarwal, Cytokine Research Laboratory, Department of Molecular Oncology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. E-mail address: aggarwal@utmdacc.mda.uth.tmc.edu

[3] Abbreviations used in this paper: IκB, inhibitor of nuclear factor-κB; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase; AP-1, activating protein-1; TPCK, L-*p*-tosylamino-2-phenylethyl chloromethyl ketone; DOC, deoxycholate; MEKK, mitogen-activated protein kinase kinase kinase; PTK, protein tyrosine kinase; DHODH, dihydroorotate dehydrogenase; ROI, reactive oxygen intermediates.

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297–317 mapping at the carboxyl terminus of IκB-α/MAD-3; and anti-c-Rel and anti-cyclin D1 against amino acids 1–295, which represent full-length cyclin D1 of human origin. All these Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Rat mdr1b promoter containing wild-type (MDR1bCAT) and mutated (243RMICAT) NF-κB binding sites and linked to the chloramphenicol acetyltransferase (CAT) gene were supplied by Dr. M. Tien Kuo of the University of Texas M. D. Anderson Cancer Center (Houston, TX). The characterization of these plasmids has been described previously in detail (9).

Cell line

For most studies an acute human T cell leukemia cell line (Jurkat) obtained from American Type Culture Collection (Manassas, VA) was used. Cells were routinely grown in RPMI 1640 medium supplemented with glutamine (2 mM), gentamicin (50 μg/ml), and FBS (10%). The cells were seeded at a density of 1×10^5 cells/ml in T25 flasks (Falcon 3013, Becton Dickinson Labware, Lincoln Park, NJ) containing 10 ml of medium and were grown at 37°C in an atmosphere of 95% air and 5% CO_2. Cell cultures were split every 3 days.

Electrophoretic mobility shift assays (EMSAs)

Cells (2×10^6 cells/ml) were treated separately with different concentrations of activator at 37°C, and nuclear extracts were prepared as previously described (10). Briefly, 2×10^6 cells were washed with cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). Cells were lysed with 12.5 μl of 10% Nonidet P-40. The nuclear pellet was resuspended in 25 μl of ice-cold extraction buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine). The supernatant, i.e., nuclear extract, was used immediately or was stored at −70°C for later use. The protein content was measured by the method of Bradford (11).

EMSAs were performed by incubating 4 μg of protein of the nuclear extract with 16 fmol of ^{32}P end-labeled, 45-mer, double-stranded NF-κB oligonucleotide from the HIV-1 long terminal repeat, 5′-TTGT TACAAG GGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3′ (12), in the presence of 2–3 μg of poly(dI-dC) in a binding buffer (25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl) for 15 min at 37°C as described previously (13, 14). The DNA-protein complex formed was separated from the oligonucleotide on 7.5% native polyacrylamide gel using buffer containing 50 mM Tris, 200 mM glycine (pH 8.5), and 1 mM EDTA (15), and then the gel was dried. An oligonucleotide with mutated binding sites (5′-TTGTTACAACT CACTT TCCGCTGCRCACRRRCCAGGGAGGCGTGG-3′) was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide.

For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with the Abs against either p50 or p65 of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA as described previously in detail (16). Abs against c-Rel B and cyclin D1 and preimmune serum were included as negative controls.

The EMSAs for AP-1 and Oct-1 were performed as described for NF-κB using ^{32}P end-labeled double-stranded oligonucleotides.

Visualization and quantitation of radioactive bands were conducted by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Western blotting for IκBα, p50, and p65

After the NF-κB activation reaction described above, postnuclear extracts were resolved on 10% SDS-polyacrylamide gels to assay IκBα. To determine the p50 and p65 levels, nuclear and postnuclear (cytoplasmic) extracts were resolved on 8% SDS-PAGE. After the gels, the proteins were electrotransferred onto nitrocellulose filters and probed with rabbit polyclonal Abs against IκBα, p50, and p65, and bands were detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL) (17). The bands were quantitated by personal densitometer scan v1.30 using ImageQuant software version 3.3 (Molecular Dynamics).

Transient transfection and CAT assay

Jurkat cells were transiently transfected with -243RMICAT (wild-type) and -243RMICAT-κm (mutant) genes for 6 h by the calcium phosphate method according to the instructions supplied by the manufacturer (Life Technologies). After transfection, the cells were incubated for 24 h at 37°C and then treated with leflunomide (10 μM) for 2 h before stimulation with 100 pM TNF for 1 h. Then, the cells were washed with PBS and examined for CAT activity as previously described (18).

*In vitro tyrosine kinase assay for p56^lck^*

The in vitro kinase assay was performed using a modified method as described previously (19). Briefly, the Jurkat cells (5×10^6/ml) were treated with different concentrations of leflunomide for 2 h and then stimulated with TNF (100 pM) for 15 min, cell extracts were prepared by lysing cells in buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine, 1 mM DTT, and 1 mM sodium *o*-vanadate (lysis buffer). Cell extracts (400–500 μg/sample) were immunoprecipitated with 0.3 μg anti-$p56^{lck}$ Ab (Santa Cruz Biotechnology) after incubation for 60 min at 4°C. The immune complex was collected by incubation with protein A/G-Sepharose beads for 45 min at 4°C. The beads were washed four times with lysis buffer (400 μl) and twice with the kinase buffer (400 μl; 20 mM HEPES (pH 7.4), 1 mM DTT, and 25 mM NaCl). Kinase assays were performed for 15 min at room temperature with 5 μg of histone 2B as a substrate in 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 μCi of [$\gamma^{32}P$] ATP. Reaction was stopped by addition of 15 μl of SDS sample buffer (20% glycerol (v/v), 2% SDS, 62.5 mM Tris-Cl (pH 6.8), 5% 2-ME, and 0.0025% bromophenol blue), boiled for 5 min, and subjected to SDS-PAGE (9 or 12%). After electrophoresis, autophosphorylation of $p56^{lck}$ and phosphorylation of exogenous histone 2B were analyzed by visualization and quantitation of radioactive bands on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

MAPK assay

Leflunomide-pretreated Jurkat cells were stimulated with different concentrations of TNF. After incubation for 30 min at 37°C, cells were washed with Dulbecco's PBS and then extracted with lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine, 1 mM DTT, and 1 mM sodium *o*-vanadate. The protein concentration in the supernatant was determined and then resolved with 50 μg of protein/lane on 10% SDS-PAGE. After the electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with the phospho-specific anti-p44/42 MAPK (Thr^{202}/Tyr^{204}) Ab (New England Biolabs, Beverley, MA) raised in rabbits (1/3000 dilution). Then the membrane was incubated with peroxidase-conjugated anti-rabbit IgG (1/3000 dilution), and bands were detected by chemiluminescence (ECL, Amersham).

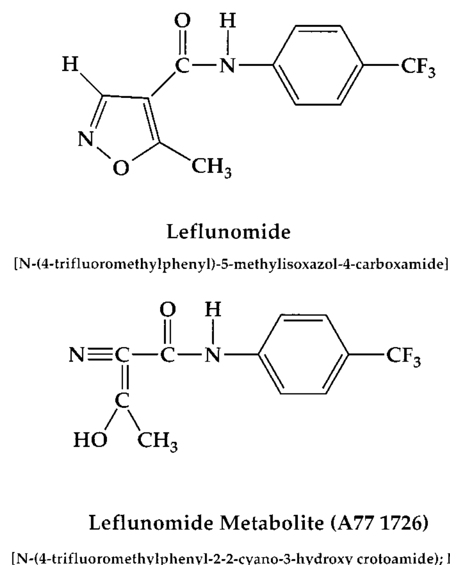

FIGURE 1. Chemical structures of leflunomide and its metabolite A77 1726.

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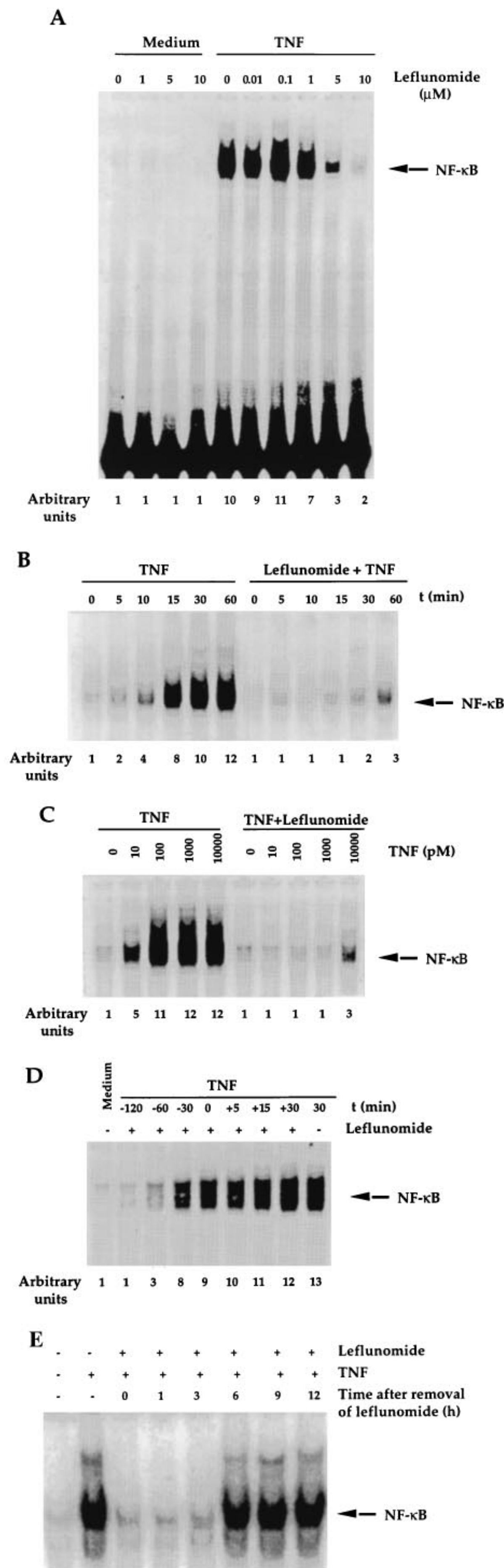

Results

The aim of this study was to examine the effect of leflunomide on the activation of transcription factor NF-κB by different inflammatory stimuli. We used an acute leukemia T cell line (Jurkat) for these studies because leflunomide is known to suppress T cell-mediated responses. The time of incubation and the concentration of drug used in our studies had no effect on cell viability (data not shown). Leflunomide (HWA-486; or *N*-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide) is a prodrug that is rapidly converted in the cell to an active metabolite, *N*-(4-trifluoromethylphenyl-2,2-cyano-3-hydroxycrotoamide), named A77 1726 (see Fig. 1). The initial conversion involves the opening of the isoxazole ring to produce A77 1726, which constitutes >95% of the drug in the circulation. In the present studies we used the A77 1726 compound, which does not require conversion to be active.

Leflunomide inhibits TNF-dependent NF-κB activation

Jurkat cells were preincubated for 2 h with different concentrations of leflunomide and then treated with TNF (100 pM) for 30 min at 37°C. Nuclear extracts were prepared and assayed for NF-κB activation by EMSA. The results presented in Fig. 2*A* indicate that 5–10 μM leflunomide inhibited most of the TNF-induced NF-κB activation and that leflunomide by itself did not activate NF-κB. To determine the effect of leflunomide on the kinetics of NF-κB activation by TNF, both untreated and leflunomide-pretreated cells were incubated with TNF for different times. The activation of NF-κB was detected with increases in incubation time as shown by the increase in band intensity in untreated cells. The leflunomide-pretreated cells showed a dramatic decrease in activation of NF-κB even after up to 60 min of TNF stimulation (Fig. 2*B*).

Previous studies from our laboratory have shown that at high concentrations (10 nM), TNF can activate NF-κB within 5 min and that this induction is higher in its intensity than that obtained with cells using 100-fold lower concentrations of TNF for longer times

FIGURE 2. *A*, Dose response of leflunomide for the inhibition of TNF-dependent NF-κB activation. Jurkat cells (2×10^6/ml) were preincubated at 37°C for 2 h with different concentrations (0–10 μM) of leflunomide followed by 30-min incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF-κB as described in *Materials and Methods*. Arbitrary units at the *bottom* correspond to quantitation of NF-κB bands. *B*, Effect of leflunomide on the kinetics of activation of NF-κB by TNF. Jurkat cells (2×10^6/ml) were incubated at 37°C with 10 μM leflunomide for 2 h, treated with 0.1 nM TNF at 37°C for the times indicated, and then tested for NF-κB activation by EMSA. *C*, Effect of leflunomide on activation of NF-κB induced by different concentrations of TNF. Jurkat cells (2×10^6/ml) were incubated at 37°C with 10 μM leflunomide for 2 h and then tested for NF-κB activation at 37°C for 30 min with the concentrations of TNF indicated. After these treatments nuclear extracts were prepared and then assayed for NF-κB. *D*, Time course of inhibition of TNF-dependent NF-κB by leflunomide. Jurkat cells (2×10^6/ml) were preincubated at 37°C with 10 μM leflunomide for the indicated times and then tested for NF-κB activation at 37°C for 30 min either with or without 0.1 nM TNF. −, the time leflunomide was present before the addition of TNF; 0, coincubation with TNF; +, the time leflunomide was added after TNF. After these treatments nuclear extracts were prepared and assayed for NF-κB. *E*, Reversal of the inhibitory effect of leflunomide on TNF-dependent NF-κB activation. Jurkat cells (2×10^6/ml) were preincubated at 37°C with 10 μM leflunomide; after 2 h leflunomide was washed off, and cells were cultured in drug-free medium for the indicated times and then tested for NF-κB activation after treatment with 0.1 nM TNF at 37°C for 30 min. After these treatments nuclear extracts were prepared and assayed for NF-κB.

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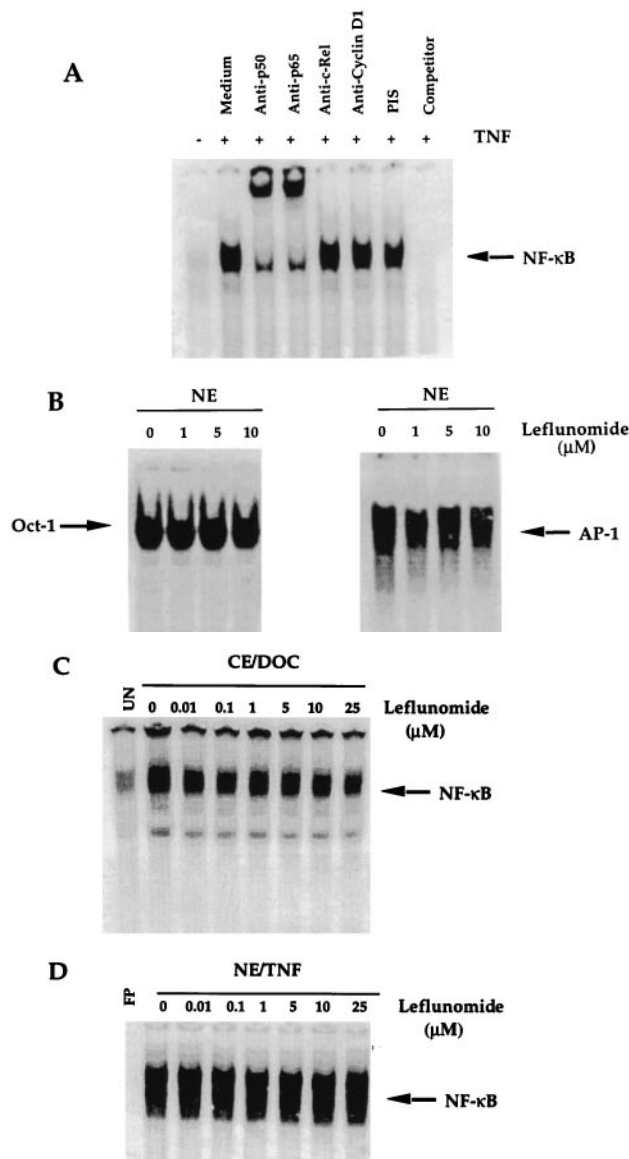

FIGURE 3. *A*, Supershift and specificity of the NF-κB. Nuclear extracts were prepared from untreated or TNF (0.1 nM)-treated Jurkat cells (2 × 10^6/ml), incubated for 15 min with different Abs and cold NF-κB oligo probe, and then assayed for NF-κB as described. *B*, Effect of leflunomide on transcription factors AP-1 and Oct-1. Cells were treated with the indicated concentrations of leflunomide for 120 min at 37°C, and nuclear extracts (NE) were prepared and then used for EMSA of AP-1 and Oct-1 as described in *Materials and Methods*. *C* and *D*, In vitro effect of leflunomide on DNA binding of NF-κB protein. *C*, Cytoplasmic extracts (CE) from untreated Jurkat cells (10 μg protein/sample) were treated with 0.8% DOC for 15 min at room temperature, incubated with different concentrations of leflunomide for 2 h at room temperature, and then assayed for DNA binding by EMSA. *D*, Nuclear extracts were prepared from 0.1 nM TNF-treated Jurkat cells; 5 μg/sample nuclear extract protein was treated with the indicated concentrations of leflunomide for 2 h at room temperature and then assayed for DNA binding by EMSA.

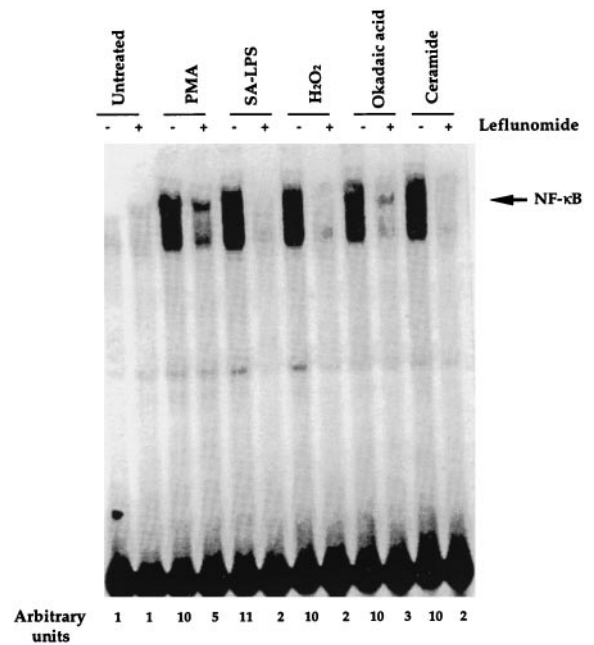

FIGURE 4. Effects of leflunomide on different activators (PMA, serum-activated LPS (SA-LPS), H_2O_2, okadaic acid, and ceramide) of NF-κB. Jurkat cells (2 × 10^6/ml) were preincubated for 120 min at 37°C with leflunomide (10 μM) followed by PMA (25 ng/ml), serum-activated LPS (10 μg/ml), H_2O_2 (250 μM), okadaic acid (500 nM), or ceramide (10 μM) for 30 min and then tested for NF-κB activation as described. Arbitrary units at the *bottom* correspond to quantitation of NF-κB bands.

(20). To determine the effect of leflunomide on NF-κB activation at higher concentrations, both untreated and leflunomide-pretreated cells were incubated with various concentrations of TNF (0–10,000 pM) for 30 min, and then NF-κB was assayed by EMSA (Fig. 2*C*). Although the activation of NF-κB by 10 nM TNF was strong, leflunomide almost completely inhibited it as efficiently as it did at 0.01 nM. These results show that leflunomide is a very potent inhibitor of TNF-induced NF-κB activation.

We next tested the length of leflunomide incubation on NF-κB activation by TNF. For this, cells were incubated with leflunomide for 120, 60, and 30 min before the addition of TNF; at the same time as the addition of TNF; or 5, 15, and 30 min after the addition of TNF. The cells were treated with TNF for 30 min. Only when the cells were pretreated for 120 min with leflunomide was NF-κB activation almost completely inhibited, and the inhibition decreased gradually with decreased preincubation time. Cotreatment or posttreatment with leflunomide was not effective in the inhibition of NF-κB activation by TNF (Fig. 2*D*).

We also investigated whether the effect of leflunomide on the TNF-dependent NF-κB activation was reversible. To determine this, Jurkat cells were treated with 10 μM leflunomide for 2 h, then the drug was removed, and the cells were incubated for different times in the drug-free medium before checking for TNF-induced NF-κB activation. As shown in Fig. 2*E*, suppression of NF-κB activation is reversible; it remained suppressed up to 3 h after the removal of the drug and then reversed at 6 h. These results show that the effects of leflunomide are reversible.

Activated NF-κB inhibited by leflunomide consists of p50 and p65 subunits

Various combinations of Rel/NF-κB proteins can constitute an active NF-κB heterodimer that binds to specific sequences in DNA. To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extract from TNF-activated cells with Abs to either p50 (NF-κBI) or p65 (Rel A) subunits and then conducted EMSA. Abs to either subunit of NF-κB shifted the band to a higher m.w. (Fig. 3*A*), thus suggesting that the TNF-activated complex consists of p50 and p65 subunits. When a higher concentration of the Ab or the combination of p50 and p65 Abs were used, a complete supershift of the band was

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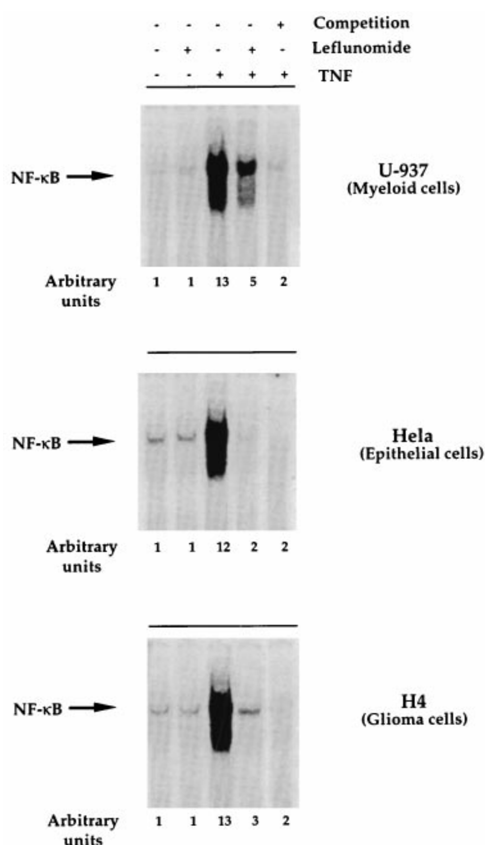

FIGURE 5. Effect of leflunomide on TNF-induced NF-κB in different cell types. U-937, HeLa, or H4 cells (2 × 10^6/ml) were preincubated for 2 h with or without leflunomide (10 μM), followed by TNF (100 pM) for 30 min. Nuclear extracts were prepared and tested for NF-κB activation as described in *Materials and Methods*. The specificity of NF-κB binding was determined by performing EMSA in the presence of a 25-fold molar excess of cold NF-κB oligonucleotide.

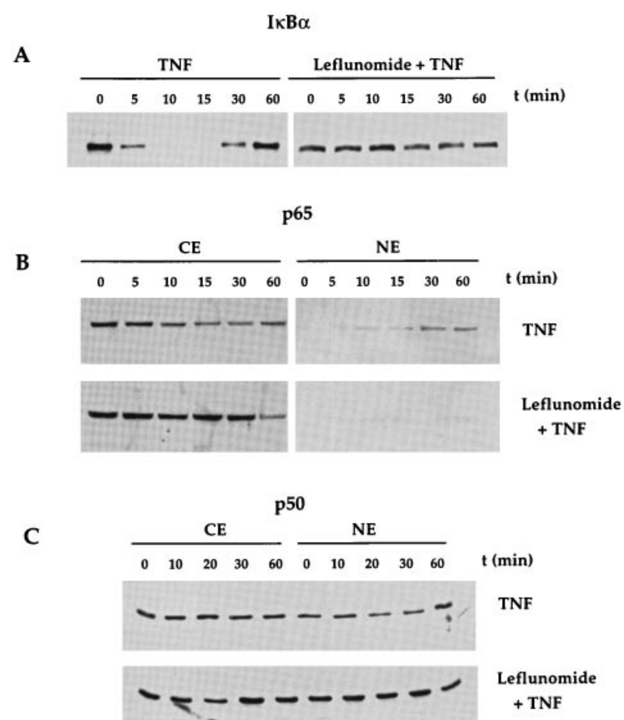

FIGURE 6. Effect of leflunomide on TNF-induced degradation of IκBα and on the levels of p65 and p50. Jurkat cells (2 × 10^6/ml) either untreated or pretreated for 120 min with 10 μM leflunomide at 37°C were incubated for different times with TNF (0.1 nM) and then assayed for IκBα in cytosolic fractions by Western blot analysis (*A*) and for both p65 and p50 from cytoplasmic as well as nuclear extracts by Western blot analysis (*B* and *C*).

observed (data not shown). Neither preimmune serum nor irrelevant Abs such as anti-c-Rel or anti-cyclin DI had any effect on the mobility of NF-κB. This is consistent with a recent report from our laboratory (21).

Leflunomide does not interfere with DNA binding of AP-1 and Oct-1

Whether leflunomide affects DNA binding of other transcription factors was investigated. Leflunomide had no effect on the Oct-1 transcription factor (Fig. 3*B*, *left panel*). DNA binding of AP-1 transcription factors (22), however, was down-modulated only at the highest concentration (25 μM) of leflunomide. Up to a 10-μM concentration of leflunomide, which inhibits most of the NF-κB activation, had no effect on the AP-1 transcription factors (Fig. 3*B*, *right panel*).

Leflunomide does not interfere with the DNA-binding ability of NF-κB proteins

It has been shown that both TPCK, a serine protease inhibitor, and herbimycin A, a protein tyrosine kinase inhibitor, down-regulate NF-κB activation by chemical modification of the NF-κB subunits, thus preventing its binding to DNA (23, 24). To determine whether leflunomide also directly modifies NF-κB proteins, we incubated cytoplasmic extracts from untreated cells and those treated with deoxycholate (DOC; 0.8%) for 15 min at room temperature or incubated nuclear extracts from TNF-triggered cells and then treated them with various concentrations of leflunomide. Then DNA binding activity was detected using EMSA. The DOC treatment has been shown to dissociate the IκBα subunit, thus releasing NF-κB for binding to the DNA. Our results in Fig. 3, *C* and *D*, show that leflunomide did not modify the DNA-binding ability of NF-κB proteins prepared by treatment with either DOC or TNF. Therefore, leflunomide inhibits NF-κB activation through a mechanism different from that of TPCK or herbimycin A.

Leflunomide blocks phorbol ester-, LPS-, okadaic acid-, ceramide-, and H_2O_2-mediated activation of NF-κB

Besides TNF, NF-κB is also activated by phorbol ester, H_2O_2, LPS, okadaic acid, and ceramide (25). However, the signal transduction pathways induced by these agents differ. We therefore examined the effect of leflunomide on the activation of transcription factor by these various agents. The results shown in Fig. 4 indicate that leflunomide completely blocked the activation of NF-κB induced by all five agents. These results suggest that leflunomide may act at a step where all these agents converge in the signal transduction pathway leading to NF-κB activation.

Inhibition of NF-κB activation by leflunomide is not cell type specific

All the effects of leflunomide described above were found using Jurkat T cells. Whether leflunomide affects other cell types was also investigated. We examined the ability of leflunomide to block TNF-induced NF-κB activation in myeloid (U-937), epithelial (HeLa), and glioma (H4) cells. The results of these experiments (Fig. 5) indicate that leflunomide inhibited NF-κB in all three cell types. Almost complete inhibition was observed with epithelial

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and glioma cells, and partial inhibition was found with myeloid cells, thus suggesting that this effect of leflunomide is not cell type specific. The NF-κB binding in all cells was abrogated by a 25-fold molar excess of unlabeled oligonucleotide.

DTT does not reverse the effect of leflunomide

It has been shown that while agents such as TPCK, which modify the sulfhydryl group in NF-κB, inhibit NF-κB activation, this inhibition can be reversed by DTT (23). To determine whether the inhibitory effect of leflunomide on NF-κB could be reversed by this reducing agent, Jurkat cells were treated with leflunomide in the presence or the absence of DTT and then examined for the activation of NF-κB by TNF. DTT did not reverse the inhibition caused by leflunomide (data not shown), suggesting that leflunomide did not suppress NF-κB activation by blocking sulfhydryl groups.

Leflunomide inhibits TNF-dependent degradation of IκBα and hence nuclear translocation of the p65 subunit

The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα (26). To determine whether the inhibitory action of leflunomide was due to its effect on IκBα degradation, the cytoplasmic levels of IκBα protein were examined by Western blot analysis. An IκBα decrease appeared within 5 min of TNF treatment of Jurkat cells, and it completely disappeared by 15 min. The IκBα was fully resynthesized by 60 min, as indicated by the reappearance of the band (Fig. 6*A*). The presence of leflunomide completely blocked the TNF-induced disappearance of the band. Thus, these results strongly suggest that leflunomide blocks TNF-mediated degradation of IκBα.

Because NF-κB activation also requires nuclear translocation of the p65 subunit of NF-κB, we measured the level of p65 in the cytoplasm and in the nucleus. As expected, upon TNF treatment the level of p65 declined in the cytoplasm with a concurrent increase in the nucleus (Fig. 6*B*). Treatment of the cells with leflunomide abolished the TNF-dependent change in the nuclear and cytoplasmic p65 pools. These results show that leflunomide inhibits the TNF-induced translocation of p65 to the nucleus, which is consistent with the inhibition of TNF-dependent degradation of IκBα. Besides p65, the effect of leflunomide was also examined on the cytoplasmic and nuclear pools of other members of the Rel family of proteins. The results shown in Fig. 6*C* indicate that neither TNF by itself nor in combination with leflunomide had any effect on the level of p50.

Leflunomide blocks the TNF-induced $p56^{lck}$ and MAPKK activation

Previously it has been shown that leflunomide is a potent inhibitor of $p56^{lck}$, a member of the Src family of protein tyrosine kinases (8, 19). There is also recent report that shows that pervanadate-induced NF-κB activation requires the activation of $p56^{lck}$ (27). Whether TNF activates $p56^{lck}$ and if this activation is blocked by leflunomide are not known. The results shown in Fig. 7*A* indicate that TNF activates $p56^{lck}$ in a dose-dependent manner in Jurkat cells and that this activation is blocked quite effectively by leflunomide (Fig. 7*B*). The inhibitory effect could be noted with as little as 1 μM leflunomide.

TNF is also a potent activator of a MAPK pathway consisting of MAPK kinase kinase (MEKK, a serine/threonine kinase), which activates MAPKK (MEK, a dual-specificity kinase), which, in turn, activates MAPK (a serine/threonine kinase). A role for MEKK1 and its novel homologue NF-κB-inducing kinase in TNF-induced NF-κB activation has been implicated (28, 29). Whether leflunomide inhibits TNF-induced NF-κB activation by blocking this pathway was also investigated. For this, Jurkat cells were pretreated with leflunomide and then activated with TNF; cytoplasmic extracts were prepared and then assayed for activation of MEK by Western blot analysis. The results presented in Fig. 7*C* show that TNF activated MEK in a dose-dependent manner. Treatment of cells with leflunomide completely suppressed the TNF-induced activation of MEK.

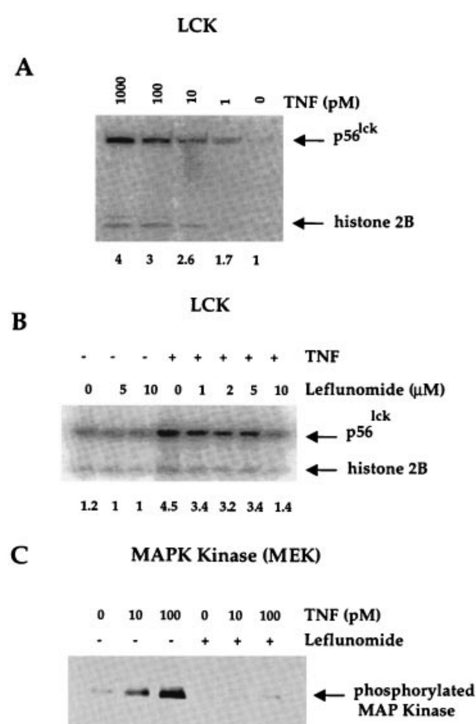

FIGURE 7. Activation of $p56^{lck}$ and MEK by TNF and their inhibition by leflunomide. *A*, Jurkat cells (5×10^6/ml) were treated with different concentrations of TNF for 30 min at 37°C, cell extracts were prepared and immunoprecipitated with anti-$p56^{lck}$ Ab, and the kinase reaction was performed and then analyzed by SDS-PAGE. *B*, Cells pretreated with different concentrations of leflunomide for 2 h at 37°C were activated with TNF (0.1 nM) for 30 min and then analyzed for $p56^{lck}$ as described in *A*. *C*, Cells pretreated or untreated with leflunomide (10 μM) for 2 h at 37°C were activated with TNF (either 10 or 100 pM) for 30 min and then analyzed for MEK by Western blot analysis as described in *Materials and Methods*.

Leflunomide represses the MDR-NF-κB-CAT gene expression

To determine the effect of leflunomide on TNF-induced NF-κB-dependent gene expression, the promoter of the rat *mdr1b* gene containing the NF-κB binding site linked to the CAT reporter gene was used. Jurkat cells were transiently transfected with the CAT reporter construct and then stimulated with TNF in either the presence or the absence of leflunomide. Almost threefold increase in CAT activity was obtained upon stimulation with TNF (Fig. 8). However, TNF-induced CAT activity was reduced significantly when the cells transfected with the wild-type NF-κB sequence were pretreated with leflunomide for 2 h before TNF treatment. Transfection with the MDR gene containing mutated NF-κB binding site did not result in induction of CAT by TNF. These results demonstrate that leflunomide represses gene expression induced by TNF.

Discussion

In the present report we demonstrate that treatment of human T cells with leflunomide blocks TNF-mediated NF-κB activation.

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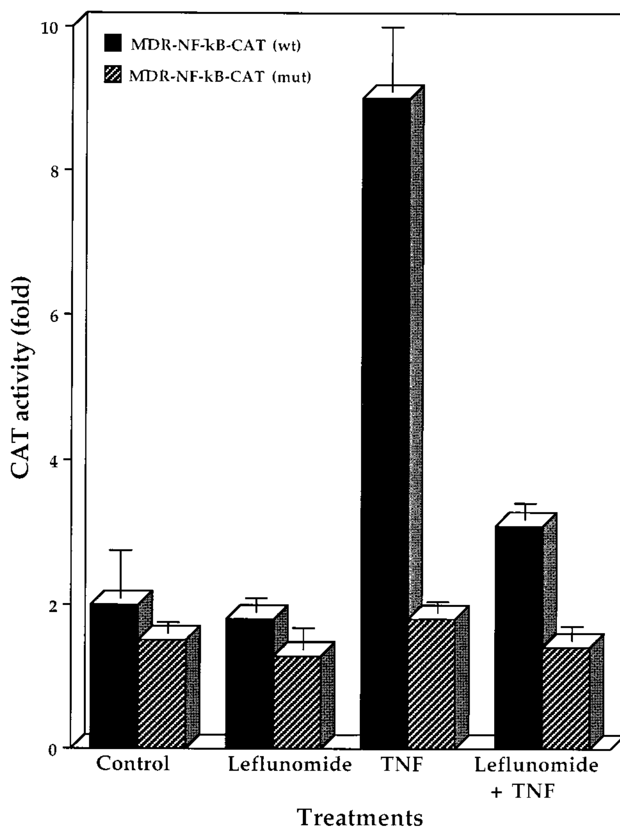

FIGURE 8. Effect of leflunomide on the expression of CAT gene linked to NF-κB containing mdr1b promoter. Cells were transiently transfected with MDR-NF-κB-CAT (-243RMICAT), both the wild-type and the mutant gene; treated with 10 μM leflunomide for 2 h; exposed to 0.1 nM TNF for 1 h; and then assayed for CAT activity as described in *Materials and Methods*. Results are expressed as fold activity over that in the nontransfected control.

Inhibition was not restricted to TNF, as NF-κB activation induced by other inflammatory agents was also blocked. The inhibitory effect of leflunomide was not cell type specific, as NF-κB activation was inhibited in myeloid and epithelial cells as well as in T cells. Leflunomide blocked the phosphorylation and degradation of IκBα and subsequent nuclear translocation of p65 subunit, steps essential for NF-κB activation. Leflunomide also blocked the activation of a tyrosine kinase $p56^{lck}$ and of a dual specificity kinase MEK. Leflunomide completely suppressed the TNF-induced gene expression dependent on NF-κB activation. Thus, these results may help to explain why leflunomide is a potent immunosuppressive and anti-inflammatory agent.

How leflunomide inhibited NF-κB activation is not clear at present. Since NF-κB activation induced by highly diverse stimuli, including TNF, H_2O_2, LPS, PMA, okadaic acid, and ceramide, was inhibited, leflunomide must block activation at a step common to all these activators. In response to most of these stimuli, IκBα undergoes phosphorylation at serines 32 and 36 by activation of kinase complex consisting of IKK-α and IKK-β (for references, see Refs. 30 and 31), which leads to ubiquitination at lysines 21 and 22 and then degradation (1, 2). Since leflunomide blocked this entire cascade, it must act upstream of IκBα phosphorylation. Previously, it has been reported that leflunomide can inhibit several protein tyrosine kinases, including those of the Src family ($p59^{fyn}$ and $p56^{lck}$) (8, 19), the Janus kinase family (JAK1 and JAK3) (32), and epidermal growth factor receptor kinase (33). More recent studies have shown that inhibition of tyrosine phosphorylation of JAK3 and STAT6 by leflunomide causes inhibition of IgG1 secretion (34). The concentration of leflunomide required to inhibit these PTKs, however, is much higher than that used in our studies. For instance, in vitro leflunomide blocks the autophosphorylation and histone 2B phosphorylation by $p59^{fyn}$ with IC_{50} values of 125–175 μM and 22–40 μM, respectively (8). Similarly, $p56^{lck}$ is inhibited with IC_{50} values of 160 and 65 μM for autophosphorylation and histone, respectively (8, 19). A complete inhibition of NF-κB activation, however, occurs at 5–10 μM leflunomide. We found that this concentration of leflunomide was sufficient to inhibit TNF-induced activation of $p56^{lck}$. This is the first demonstration that TNF can activate $p56^{lck}$. The role of $p56^{lck}$ in pervanadate-induced NF-κB activation has been reported (27). Our results show that $p56^{lck}$ may also play a role in the pathway leading to NF-κB activation by TNF and perhaps other agents.

Our results indicate that leflunomide inhibits NF-κB activation not only in T cells but also in myeloid and epithelial cells. The $p56^{lck}$ is a T cell-specific protein tyrosine kinase. Thus, how leflunomide inhibits NF-κB activation in other cell types is not clear. It is possible that there are other kinases that are more ubiquitously expressed, and their inhibition by leflunomide blocks NF-κB activation. MEKK1 is one such kinase; it is expressed in all different cell types and has been shown to play a role in NF-κB activation (28). The target of this kinase is MEK, a dual-specific kinase, the activation of which leads to phosphorylation of MAPK at threonine and tyrosine residues. Another structural homologue of MEKK1, NF-κB-inducing kinase, has been identified, which is involved in TNF-induced NF-κB activation (29). We found that leflunomide also completely suppressed the TNF-induced activation of MEK. Therefore, it is possible that leflunomide blocks NF-κB activation in all different cell types by suppression of the MAPK pathway activated by TNF (see Fig. 7*C*).

Several reports indicate that leflunomide can block the proliferation of T and B cells (4–6) and inhibit dihydroorotate dehydrogenase (DHODH) (19, 35–37), a rate-limiting enzyme in the biosynthesis pathway of pyrimidine, a pathway critical for the proliferation of these cells (4–6). The cell proliferation block was due to the inhibition of DHODH by leflunomide, given that the antiproliferative effects could be reversed by addition of uridine (19, 37). In vitro the K_i of inhibition of DHODH by leflunomide ranges from 179 nM to 2.7 μM (35, 36), which is 10–500 times lower than that for PTK. While leflunomide suppresses the proliferation of cells by inhibiting DHODH, PTK inhibition was implicated in its ability to suppress autoimmune and lymphoproliferative disorders (38). It is unlikely that inhibition of NF-κB activation by leflunomide is due to inhibition of DHODH, because NF-κB activation occurs very rapidly at a low concentration of leflunomide and does not require any new protein synthesis (1, 2).

Identifying how leflunomide blocks the activation of NF-κB requires an understanding of the mechanism by which various inducers activate this important transcription factor. TNF is one of the most potent activators of NF-κB, but the mechanism by which the activation occurs is not understood. Roles for ceramide, superoxide radicals, proteases, protein serine kinases, and protein tyrosine phosphatases upstream of IκBα phosphorylation have been suggested (1, 15, 39–41). Whether these signals are generated by TNF sequentially or independently of each other, however, is not known. Such NF-κB inducers as phorbol ester, H_2O_2, and TNF are known to produce reactive oxygen intermediates (ROI). The inhibitors of mitochondrial electron transport have been shown to impair the TNF-induced activation of NF-κB (42), thus also suggesting a role for ROI. Therefore, it is possible that leflunomide blocks NF-κB activation by quenching ROI production. However,

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there is no published report to suggest that leflunomide has antioxidant properties.

Our results indicate that leflunomide also blocks NF-κB-dependent gene expression. Transcription of a number of genes, including those involved in inflammation, transplant rejection, tumor promotion, tumor metastasis, cell proliferation, and autoimmunity, requires NF-κB activation. It is quite likely that the roles of leflunomide in the suppression of transplant rejection (43–45), adjuvant arthritis (3), proliferation of B and T cells and smooth muscle cells (8, 32, 43), and IL-2R expression (8) are due to the inhibition of NF-κB activation. Overall, we conclude that because of its very low pharmacological toxicity (46) and its ability to modulate activation of NF-κB by various agents, leflunomide has a high potential for use as an immunosuppressive and growth modulatory agent.

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