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Inhibition of TNF-α gene expression and bioactivity by site-specific transcription factor-binding oligonucleotides

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Ye, Jianping, Liying Wang, Xiaoying Zhang, Vimon Tantishaiyakul, and Yon Rojanasakul. Inhibition of TNF-α gene expression and bioactivity by site-specific transcription factor-binding oligonucleotides. *Am J Physiol Lung Cell Mol Physiol* 284: L386–L394, 2003. First published October 11, 2002; 10.1152/ajplung.00134.2002.—The present study investigated transcriptional inactivation of TNF-α gene by nuclear factor-binding oligonucleotides (ON) and their effects on pulmonary inflammatory responses in mice. PCR-based gene mutation and gel shift assays were used to identify specific *cis*-acting elements necessary for nuclear factor binding and transactivation of TNF-α gene by lipopolysaccharide (LPS). LPS inducibility of TNF-α was shown to require transcriptional activation by NF-κB at multiple binding sites, including the −850 (κ1), −655 (κ2), and −510 (κ3) sites, whereas the −210 (κ4) site had no effect. Maximum inducibility was associated with the activation of κ3 site. The sequence-specific, double-stranded ON targeting this site was most effective in inhibiting TNF-α activity induced by LPS. The inhibitory effect of ON on TNF-α bioactivity was also investigated using a murine lung inflammation model. Pretreatment of mice with ON, but not its mutated sequence, inhibited LPS-induced inflammatory neutrophil influx and TNF-α production by lung cells. Effective inhibition by ON in this model was shown to require a liposomal agent for efficient cellular delivery of the ON. Together, our results indicate that transcriptional inactivation of TNF-α gene can be achieved by using ON that compete for nuclear factor binding to TNF-α gene promoter. This gene inhibition approach may be used as a research tool or as potential therapeutic modality for diseases with etiology dependent on aberrant gene expression.

tumor necrosis factor-α

ABERRANT ACTIVATION AND EXPRESSION of genes are associated with the development of many human diseases. Most genes are quiescent or have minimal activity in affecting physiological processes. However, in certain pathological conditions, these genes are abruptly turned on by a preexisting genetic switch, causing them to overexpress. An activation of the TNF-α gene by nuclear transcription factors is one such example of the uncontrolled genetic switch. Several nuclear transcription factors, including NF-κB, AP1, nuclear factor of activated T cells (NF-AT), Erg-1, cAMP response element binding protein, C/EBPβ, and Ets have been shown to be involved in the transcriptional activation of TNF-α (8, 19, 20, 25, 30, 31, 44). The activation of the TNF-α gene by different transcription factors is dependent on the nature of stimulation (39) and on cell type (19, 38). For example, NF-κB, but not AP1 or AP2, is involved in the activation of TNF-α transcription of LPS-stimulated monocytes (43), whereas NF-AT, not NF-κB, plays a role in phorbol 12-myristate 13-acetate (PMA)-stimulated T cells (38).

Because TNF-α plays an important role in the pathogenesis of a variety of inflammatory and immune diseases, this cytokine has been identified as a key target for pharmacological manipulation (18, 33, 36, 41, 45). TNF-α is produced principally by macrophages and acts on a variety of immune and nonimmune cells to initiate and amplify inflammatory response (36). The expression of TNF-α is regulated at different levels, transcriptional and posttranscriptional (4). At the transcriptional level, TNF-α is regulated primarily by NF-κB, which acts in synergy with other transcription factors such as AP-1 and C/EBP (8, 43). Several high-affinity DNA-binding motifs for NF-κB have been found on the TNF-α promoter (8, 35). Mutational analysis has also shown that these sites are essential for gene induction (9). Such observations provide the basis that blocking the action of NF-κB alone would be sufficient to inhibit TNF-α gene expression.

NF-κB belongs to a superfamily of protein dimers frequently composed of two DNA-binding subunits, NF-κB_1 (p50) and RelA (p65) (1, 2). It is normally kept in an inactive form in the cytoplasm by attachment of the inhibitory subunit IκB. The activation of NF-κB is accomplished by phosphorylation of the IκB by specific IκB kinases, which triggers a complete degradation of the inhibitor (37). The activated NF-κB is then translocated into the nucleus where it binds to the promoter

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region of target gene and activates its transcription. Because the interaction between NF-κB and its gene target is sequence specific, we hypothesize that oligonucleotides (ON) carrying the same base sequences as those of the NF-κB recognition sites may be used to selectively inhibit the transcriptional activation of a target gene. To test this hypothesis, we first identified specific DNA regulatory elements on the TNF-α gene promoter that are involved in NF-κB binding and transcriptional activation of TNF-α. Because previous studies have shown that not all NF-κB binding sites are required for TNF-α activation (8, 35), and because promoter-binding activities may not necessarily reflect the resulting gene expression, we therefore determined the relative contribution of each specific NF-κB-binding domain on TNF-α gene expression. On the basis of the information obtained, we designed specific ON that contain the sequence most critically required for TNF-α gene activation. We tested these ON for their inhibitory effect on TNF-α expression in both in vitro and in vivo murine inflammation lung model.

MATERIALS AND METHODS

Cells and reagents. The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin-streptomycin. Specific antibodies against NF-κB p50 and p65 subunits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used in the supershift assay. The liposomal agent *N*-[1-(2,3-dioleoyl)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP) was obtained from Boehringer Mannheim (Indianapolis, IN), and LPS (*Escherichia coli* 0111:B4, 1 endotoxin unit/μg) was from Sigma Chemical (St. Louis, MO). ON containing different NF-κB-binding sites of the murine TNF-α gene promoter were synthesized according to the underlined DNA sequences shown in Fig. 1*A*. They were named κ1, κ2, κ3, and κ4, respectively, based on their NF-κB binding sequences. Normal phosphodiester and nuclease-resistant phosphorothioate ON containing two repeated sequences of the κ3 motif and their mutated sequences were also synthesized and used in gene inhibition studies (see sequences in Fig. 1*B*). Before use, all ON were annealed with their complementary strands to generate double-stranded DNA. Annealing was achieved by heating the ON to 100°C for 10 min and then cooling to room temperature for 3 h.

Animals and bronchoalveolar lavage. Male BALB/c mice, 4–6 wk old, were obtained from Jackson Laboratories (Bar Harbor, ME). They were acclimated in an American Association for Accreditation of Laboratory Animal Care-approved facility for at least 1 wk before use. The mice were fed water and food ad libitum. Intratracheal instillations into mice

A

-985
GAGTGAGGCAGCTTAACTGCCGAGAGACCCAAAGGATGAGCTCAGGGAGATCCATCCAAGGGTGGA
GAGAGATGAGGGTTCTGGGGAGAAGTGACTCCACCGGAGGGTGGGAGAGTGTTTAGGAGTGGGAG
GGTGGGGGA**GGGGAATCCTT**GGAAGACCGGGGAGTCATACGGATTGGGAGAAATCCTGGAAGCAG
-850 κB (κ1)
GGCTGTGGGACCTAAATGTCTGAGTTGATGTACCGCAGTCAAGATATGGCAGAGGCTCCGTGGAAAA
CTCACTTGGGAGCAGGGACCCAAAGCAGCAGCCTGAGCTCATGATCAGAGTGAAAGGAGAAGGCTT
GTGAGGTC**CGTGAATTCCC**AGGGCTGAGTTCATTCCCTCTGGGGCTGCCCCATACTCATCCCATTAC
-655 κB (κ2)
CCCCCCCCACCAGCCCTCCCAAAGCCCATGCACACTTCCCAACTCTCAAGCTGCTCTGCCTTCAGCC
ACTTCCTCCAAGAACTCAAACA**GGGGGCTTTCC**CTCCTCAATATCATGTCTCCCCCCTTATGCACCCA
-510 κB (κ3)
GCTTTCAGAAGCACCCCCCCATGCTAAGTTCTCCCCCATGGATGTCCCATTTAGAAATCAAAAGGAAA
TAGACACAGGCATGGTCTTTCTACAAAGAAACAGACAACGATTAGCTCTGGAGGACAGAGAAGAAATG
GGTTTCAGTTCTCAGGGTCCTATACAACACACACACACACACACACACACACCCTCCTGATTGGCC
CCAGATTGCCACAGAATCCTGGTGGGGACGACGGGGA**GGAGATTCCTT**GATGCCTGGGTGTCCCCA
-210 κB (κ4)
ACTTTCCAAACCCTCTGCCCCCGCGATGGAGAAGAAACCGAGACAGAGGTGTAGGGCCACTACCGCT
TCCTCCACATGAGATATGGTTTTCTCCACCAAGGAAGTTTTCCGAGGGTTGAATGAGAGCTTTTCCCC
GCCCTCTTCCCCAAGGGC**TATAAA**GGCAGCCGTCTGCACAGCCAGCC**A(+1)**GCAGAAGCTCCCTCA
TATA Box
GCGAGGACAGCAAGGGACTAGCCAGGAGGGAGAACAGAAACTCCAGAACATCCTGGAAATAGCTCC
CAGAAAAGCAAGCAGCCAACCAGGCAGGTTCTGTCCCTTTCACTCACTGGCCCAAGGCGCCACATCT
CCCTCCAGAAAAGACAC *+165*

B

κB Site	Sense Sequence
κ1 wt	-863 GTGGGGGA**GGGGAATCCTT**GGAA –840
κ1 mutant	-863 GTGGGGGA**GAGGAATCATT**GGAA -840
κ2 wt	-657 GGTC**CGTGAATTCCC**AGGGC -638
κ2 mutant	-657 GGTC**CGTGAATTCAC**AGGGC -638
κ3 wt	-510 AAACA**GGGGGCTTTCCC**TCCTCA -488
κ3 mutant	-510 AAACA**GGAGGCTTTACC**TCCTCA -488
κ4 wt	-216 GGA**GGAGATTCCTT**GATGCCTG -194
κ4 mutant	-216 GGA**GGAGATTCATT**GATGCCTG -194

Fig. 1. Sequence of murine TNF-α upstream region and PCR primers used to generate the mutated TNF-α promoters. *A*: sequence of TNF-α gene promoter showing the 4 NF-κB elements (underlined) and their binding sequences (bold). *B*: PCR primers used to generate mutated gene promoters. The mutated sites are underlined. wt, Wild type.

were performed according to an established method (10). The protocol was approved by the Animal Care and Use Committee of West Virginia University. Mice were anesthetized with a mixture of ketamine and xylazine (45 and 8 mg/kg ip, respectively) and challenged by aspiration. The animals were placed on a board in a supine position. The animals' tongues were extended with lined forceps, and 50 μl of the test solution were placed on the back of the tongue. At indicated times after treatment, mice were euthanized with an intraperitoneal injection of 0.25 ml of pentobarbital sodium (EUTHA-6; Western Medical Supply, Arcadia, CA), and bronchoalveolar lavage (BAL) was performed. A tracheal cannula was inserted, and the lungs were lavaged through the cannula using ice-cold PBS. Five lavages of 0.8 ml each were collected. BAL cells were isolated by centrifugation at 500 *g* for 10 min, and the supernatants were collected and used for TNF-α measurements. The cell pellets were resuspended in 1 ml of HEPES buffer (10 mM HEPES, 145 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$, and 5.5 mM D-glucose, pH 7.4) and placed on ice. Cell counts and differentials were then determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL).

Point mutation of TNF-α promoter and gene transfection. PCR-based DNA mutation procedure was used to generate point mutations of the four NF-κB binding sites on the TNF-α gene promoter. The four NF-κB binding sites are indicated in boldface lettering in Fig. 1*A*. PCR primers used for the mutation of κB sites were listed in Fig. 1*B*. The wild-type −863/−18 promoter fragment was first generated and used as a template for subsequent generation of the mutated promoter fragments. The promoter DNAs were inserted into pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA) and ligated by *Hin*dIII/*Xho*I double digestion. The inserts in the right orientation were cloned at the *Hin*dIII/*Xho*I sites in the pGL3-basic luciferase vector (Promega, Madison, WI). These mutated reporter plasmids were named κ1m, κ2m, κ3m, and κ4m, respectively. For gene transfection studies, the plasmids were individually introduced into RAW cells with the aid of the liposomal agent DOTAP. In these experiments, cells were plated on a 12-well plate (10^6/well) and allowed to grow for 24 h before transfection. The plasmid DNA (1 μg/ml) was diluted in DMEM and mixed with DOTAP (10 μg/ml) for 15–20 min. Cells were then incubated in this mixture medium for 4 h at 37°C. After transfection, the medium was replaced with a growth medium containing 10% fetal bovine serum, and the cells were cultured for an additional 48 h before the level of reporter gene expression was determined.

Assays of luciferase activity and TNF-α protein expression. Luciferase activity was measured by enzyme-dependent light production using a luciferase assay kit (Promega). After each experiment, cells were washed and incubated at room temperature for 10 min in 250 μl of lysis buffer (Promega). Ten-microliter samples were then taken and loaded into an automated luminometer (Bio-Rad, Hercules, CA). At the time of measurement, 100 μl of luciferase substrate were automatically injected into each sample, and total luminescence was measured over a 20-s time interval. Output is quantitated as relative light units per microgram of protein of the sample. For analysis of TNF-α protein, cell-free supernatants were used. TNF-α levels were determined using a TNF-α ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Electrophoretic mobility shift assay. To detect NF-κB binding activity, nuclear protein extracts were first prepared as follows. Cells were treated with 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES, 1 mM PMSF, 10 mg/ml leupeptin, 20 μl/ml aprotinin, and 100 mM DTT) on ice for 4 min. Nuclei were pelleted by centrifugation at 14,000 rpm for 1 min and were resuspended in 300 μl of extraction buffer (500 mM KCl, 10% glycerol, 25 mM HEPES, 1 mM PMSF, 1 μl/ml leupeptin, 20 μg/ml aprotinin, and 100 μM DTT). After being centrifuged at 14,000 rpm for 5 min, the supernatant was harvested and stored at −70°C. The protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL).

The DNA-protein binding reaction was conducted in a 24-μl reaction mixture including 3 μg of nuclear protein extract, 1 μg of poly(dI-dC) (Sigma), 3 μg of BSA, and 4×10^4 cpm of ^{32}P-labeled ON probe. The ON probe contained either the NF-κB binding sequence of IL-6 gene promoter (5′-TGGGATTTTCCCATGAGTCT-3′) or, when indicated, the κ3 sequence of TNF-α gene promoter (5′-AAACAGGGGGCTTTCCCTCCTCA-3′). The former was used as a standard NF-κB probe, whereas the latter was used as a specific probe for κ3 binding site. The ON probes were denatured at 80°C for 5 min and annealed with their complementary sequence at room temperature. The double-stranded probes were labeled with [^{32}P]ATP (Amersham, Arlington Heights, IL) using T4 kinase (BRL, Gaithersburg, MD). The reaction mixture was incubated on ice for 10 min with or without antibody in the absence of radiolabeled probe and then for 20 min at room temperature in the presence of radiolabeled probe. In supershift assays, antibody specific to NF-κB p50 or p65 subunit (200 μg) was also added to the reaction mixture. The mixture was resolved on a 5% polyacrylamide gel that had been prerun at 200 V for 30 min with 0.5× Tris-borate-EDTA buffer. The loaded gel was run at 200 V for 90 min, dried, and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

LPS stimulation and ON inhibition studies. For in vitro studies, RAW cells were plated on 96-well plates (10^5 cells/well) and preincubated for 12 h at 37°C with 1 μM ON in serum-free DMEM. After being preincubated, the cells were treated with LPS (1 μg/ml) at 37°C for 6 h. After the treatment, cell culture supernatants were collected and used for TNF-α protein assay. The cell pellets were harvested and used for protein extraction and nuclear factor binding assay. For in vivo studies, mice were treated via an intratracheal instillation with 50 μl of the test solution containing 30 μg of LPS. In studies designed to assess the inhibitory effects of ON, mice were pretreated intratracheally with ON (1–100 μg), either alone or in combination with DOTAP (100 nmol), for 2 h and then challenged with LPS (30 μg). At various times after treatment, mice were killed, and BAL was performed. BAL cells were isolated by centrifugation as earlier described and used for cell counts and differentials. The supernatants were collected and used for TNF-α measurements. For lung histological studies, a separate group of animals was similarly treated but not subjected to BAL. After death, the lungs were inflated with 10% formalin solution instilled through the trachea for 2 h and then fixed with buffered 10% formalin solution for 24 h. After being embedded in paraffin, the samples were sectioned, mounted on glass slides, and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Statistical analysis. Each study group consisted of four experiments. Statistical analysis between study groups was performed with paired two-tailed Student's *t*-test. The level of significance was $P < 0.05$.

RESULTS

Maximum LPS inducibility of TNF-α promoter requires NF-κB activation at the κ3 site. It has been reported that there are four NF-κB binding sites in the −1 kb region of murine TNF-α promoter (8, 35). These κB binding sites are depicted in Fig. 1*A*. With the use of point mutation assays, we further evaluated the role of specific κB sites on LPS-inducible promoter activity of TNF-α. The four κB sites in the −863 region of TNF-α promoter were individually mutated by PCR. The four mutated and wild-type promoters (−836/−18) were obtained and named κ1m, κ2m, κ3m, κ4m, and −863WT, respectively. These promoters were cloned into the T/A cloning vector and then subcloned into the pGL-3 basic vector at *Hin*dIII/*Xho*I sites. The promoter activity was determined by luciferase assay using transiently transfected macrophage RAW 264.7 cells. The results showed that the plasmid containing wild-type promoter had a strongly LPS-inducible promoter activity, whereas those containing κ1m, κ2m, or κ3m had reduced promoter responsiveness (Fig. 2*A*). It was noted that mutation of the κ3 site led to a very strong reduction in both the basic and inducible promoter activities, whereas mutation of the κ4 site had no effect on the promoter activity.

To study the DNA-binding activity of NF-κB to these specific sites, four ON containing the corresponding κB sequences (κ1, κ2, κ3, and κ4) were synthesized and used to examine the NF-κB binding activities by electrophoretic mobility shift assay (EMSA). In this assay, a standard NF-κB probe was separately synthesized, radiolabeled, and used together with LPS-treated RAW cell nuclear protein to generate a standard NF-κB complex (Fig. 2*B*, *lane 1*). The four κB ON were then used as competitors for NF-κB binding. The results showed that the κ3 ON competed most efficiently with the radiolabeled probe, whereas the κ1 and κ2 ON exhibited a weak competition. The κ4 ON did not give any appreciable competition. These results are in good agreement with our gene mutation assay (Fig. 2*A*), which indicate that the κ3 site has the strongest NF-κB binding activity and that this site is required for maximum activation of the TNF-α promoter by LPS.

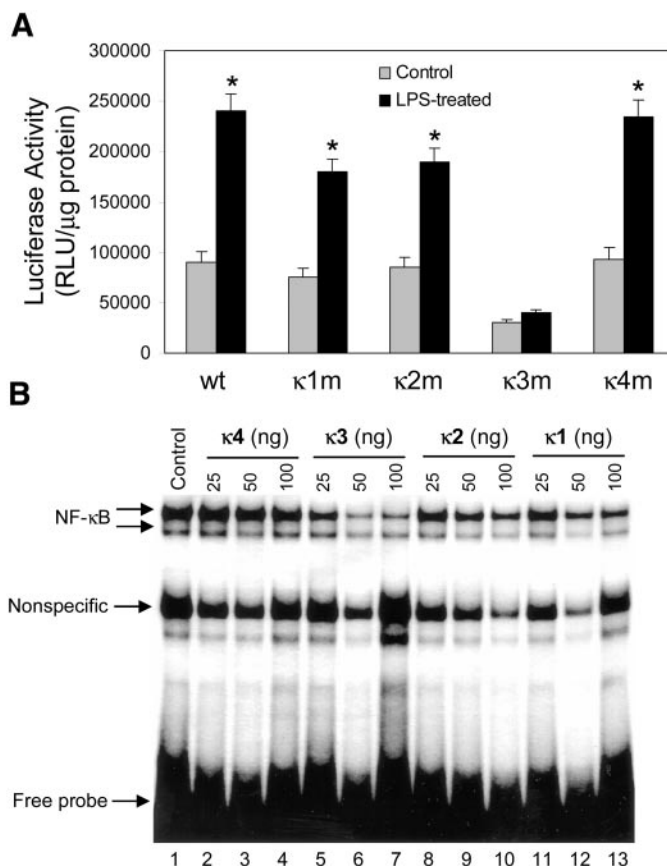

Fig. 2. Analysis of TNF-α gene promoter and NF-κB binding activity. *A*: promoter activities of PCR-generated plasmids containing the mutated or wild-type TNF-α promoter linked to a luciferase reporter gene. RAW cells were transfected with the mutated or wild-type reporter plasmids and were exposed to LPS (0.1 μg/ml) at 37°C for 6 h. After being treated, cells were assayed for luciferase activity. Each data point represents the mean ± SE of quadruplicate samples, and the data are normalized to protein content. *Significant difference from nontreated control ($P < 0.05$). *B*: NF-κB binding and oligonucleotide (ON) competition studies. Radiolabeled probe containing the NF-κB binding site of IL-6 was used to form standard NF-κB complexes with the nuclear extract obtained from LPS-stimulated RAW cells (1 μg/ml, 1 h). Varying amounts of the NF-κB binding ON (κ1, κ2, κ3, and κ4) were used as competitors in this assay. RLU, relative light units.

Inhibition of TNF-α expression in RAW cells by site-specific ON. The identification of the κ3 site as the most critical site for LPS induction of TNF-α suggests the potential utility of κ3-containing ON as an effective and specific inhibitor of TNF-α expression in cell systems. To test this possibility, two ON, each containing two repeated κ3 sequences (to increase the NF-κB binding capability) but with different chemical modifications, were synthesized. The first ON contains a naturally occurring phosphodiester backbone (PD), whereas the second ON contains a nuclease-resistant phosphorothioate backbone (PT). The two ON were tested for their inhibitory effect on LPS-induced TNF-α expression in RAW cells. Figure 3*A* showed that both PD and PT were effective in inhibiting TNF-α protein expression, whereas their mutated sequences (mPD and mPT) had no effects, thus suggesting the specificity of the inhibitory effect. The observation that PD was as effective as PT also suggests the relative stability of this ON to nuclease digestion under the experimental conditions.

To test whether the observed inhibitory effect of ON occurred at the transcriptional level, RAW cells were transfected with a −1,000/+200 TNF-luciferase plasmid, and their transcription activities were determined by luciferase assay. The reporter cells were treated with the ON inhibitors and then challenged with LPS. The results showed that both PD and PT could reduce LPS-induced luciferase activity, whereas the mutated mPD and mPS had no effect (Fig. 3*B*). The inhibitory effect of PD and PT was due to competitive inhibition of NF-κB binding, as demonstrated by our EMSA study. In this study, a radiolabeled probe containing the κ3 sequence was used to detect the NF-κB complexes. PD

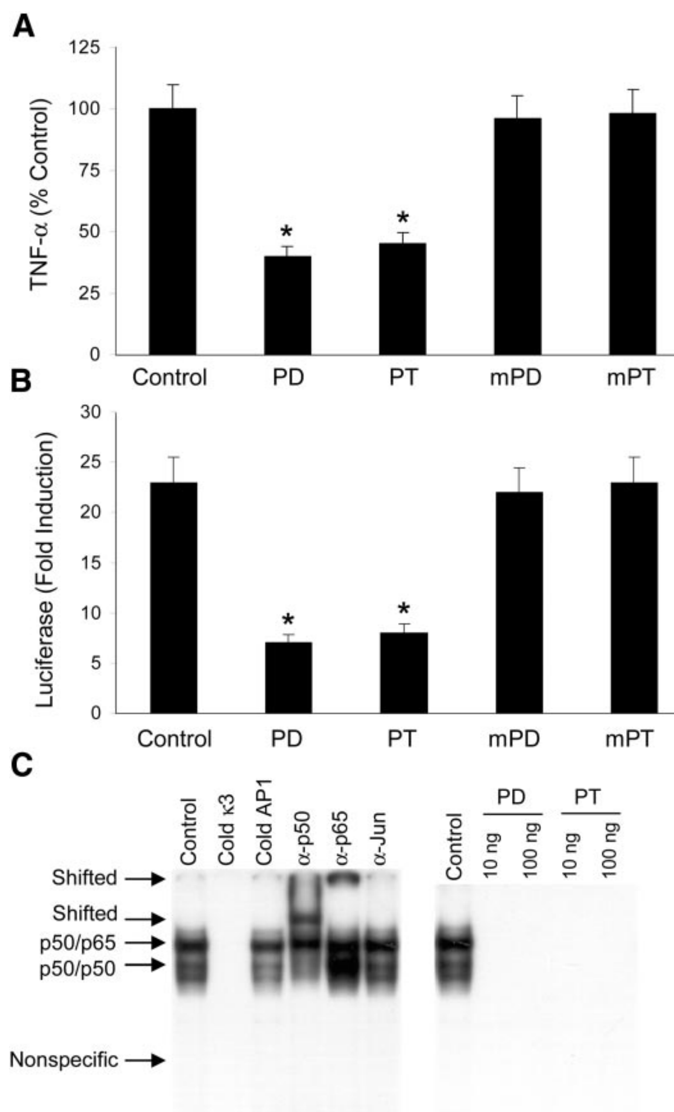

Fig. 3. Inhibition of TNF-α by κ3 ON. *A*: inhibition of LPS-induced TNF-α protein expression by phosphodiester (PD) and phosphorothioate (PT) κ3 ON and their mutated sequences (mPD and mPT). RAW cells (10^5/well) were incubated with 1 μM ON for 12 h and then treated with 1 μg/ml of LPS for 6 h at 37°C. The cell supernatants were collected and analyzed for TNF-α protein levels using ELISA. *B*: inhibition of TNF-α promoter activity. RAW cells were transfected with wild-type TNF-luciferase plasmid. The transfected cells were treated with ON and LPS as described in *A*. *C*: inhibition of NF-κB binding activity. Radiolabeled κ3 probe (~0.5 ng) was used to form NF-κB complexes with nuclear extracts from LPS-treated cells (1 μg/ml, 1 h). Bars indicate SE of the mean, $n = 4$. *Significant difference from control ($P < 0.05$).

and PT effectively competed with the κ3 probe for NF-κB binding (Fig. 3*C*), whereas the mutated mPD and mPS had no effect (not shown). Furthermore, a nonlabeled κ3 probe but not nonspecific AP1 probe was able to compete for this binding, thus indicating the specificity of NF-κB binding in this assay. Supershift assays using antibodies specific to the p50 and p65 subunits of NF-κB showed a band shift of the NF-κB complexes. In contrast, antibody specific to Jun had no shifting effect. Thus these results strongly indicated the DNA binding specificity of NF-κB and the formation of p65/p50 and p50/p50 complexes under the experimental conditions. The lack of nonspecific NF-κB band observed in this study compared with the earlier EMSA study (Fig. 2*B*) also indicated an improved specificity of the NF-κB binding to κ3 ON over the standard NF-κB ON.

Inhibition of LPS-induced pulmonary inflammation in mice by ON inhibitors. LPS-induced pulmonary inflammation is associated with an increased production of TNF-α and sequestered pulmonary neutrophils (6, 10). In the present study, mice were treated with LPS intratracheally (30 μg/mouse), and the levels of TNF-α and infiltrating neutrophils in BAL fluids were determined. Figure 4*A* shows that LPS treatment caused a rapid increase in TNF-α level with a peak response at 6 h. Neutrophil cell count also increased with a peak response at 24 h. Treatment of mice with saline control had no significant effects on both TNF-α and neutrophil cell count at all times (results not shown). To test the effect of ON inhibitors on lung inflammatory response, mice were pretreated intratracheally with varying amounts of ON inhibitors or their mutated sequences (1–10 μg/mouse) and then challenged with LPS (30 μg/mouse). Figure 5, *A* and *B*, shows that the ON inhibitors PD and PT, when used alone, had relatively minor effects on LPS-induced TNF-α production and neutrophil influx. No inhibitory effects were ob-

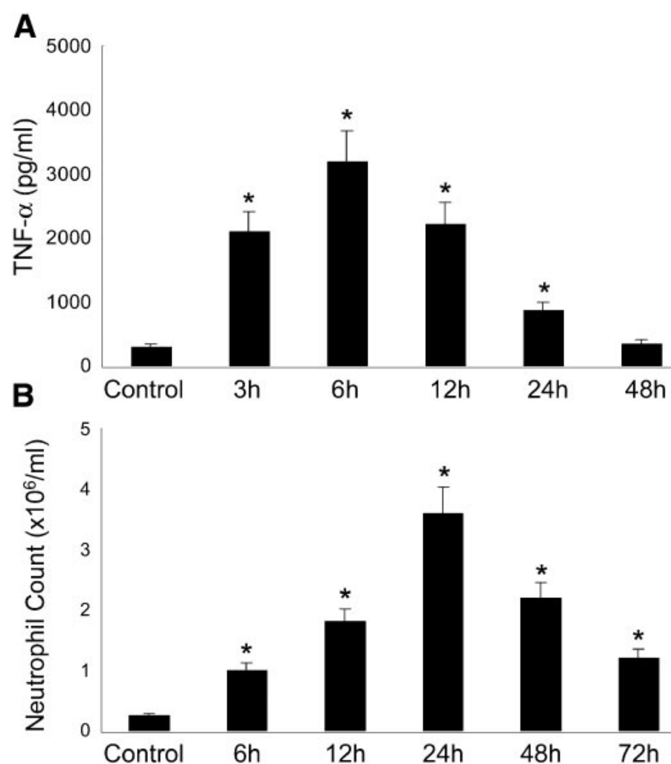

Fig. 4. LPS-induced pulmonary inflammatory responses. Mice were treated with LPS (30 μg/mouse) or saline via intratracheal instillations. Bronchoalveolar lavage (BAL) was performed at indicated times and analyzed for TNF-α level (*A*) and neutrophil cell count (*B*). Data are shown as the means ± SE, $n = 4$ mice/group. *Significant difference from control group ($P < 0.05$).

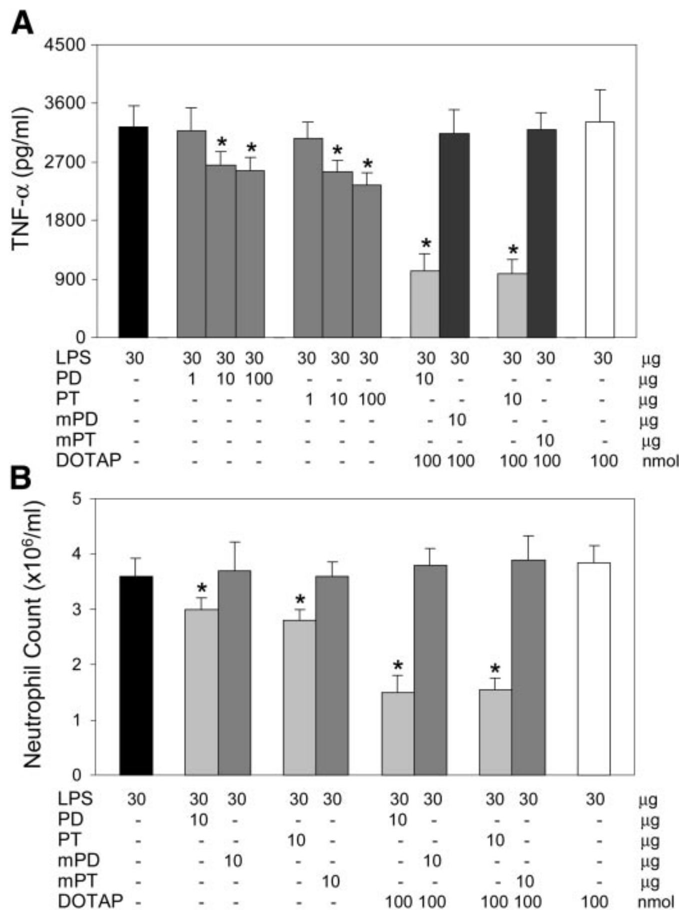

Fig. 5. Inhibitory effects of κ3 ON on TNF-α production and neutrophil influx. Mice were treated with ON (1–100 μg) with or without *N*-[1-(2,3-dioleoyl)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; 100 nmol), and 2 h later they were treated with LPS (30 μg) intratracheally. BAL was performed and analyzed for TNF-α production (*A*) and neutrophil cell count (*B*) at 6 h and 24 h after LPS treatment, respectively. Values are means ± SE, $n = 4$ mice/group. *Significant difference from control group treated with LPS alone ($P < 0.05$).

served with the control mPD or mPT. Increasing the amount of ON inhibitors beyond 100 μg/mouse did not result in improved inhibitory effects. Because ON are known to be taken up poorly by cells due to their hydrophilic nature and are relatively unstable due to nuclease digestion (32, 34), it is, therefore, possible that poor cellular uptake or enzymatic instability of these compounds, coupled with rapid clearance from the lung (22, 28), may be responsible for the in vivo inefficiency. Because the results of this study showed that the nuclease-resistant PT did not give better inhibitory effects compared with the nuclease-sensitive PD, it is therefore more likely that poor cellular uptake and/or rapid clearance may be the key contributor(s) of ON inefficiency.

To improve the cellular uptake of ON, the liposomal delivery agent DOTAP was used. DOTAP has been shown to aid the cellular delivery of plasmid DNA in the lung (26, 27). Pulmonary administration of DOTAP was reported to cause no toxic effects to lung cells in mice (10). Therefore, this compound was chosen in this study. When codelivered with the ON, DOTAP was able to promote the inhibitory effects of PD and PT on LPS-induced TNF-α production and neutrophil influx (Fig. 5, *A* and *B*). However, when given with mPD or mPT, DOTAP did not exhibit any inhibitory effects. Previous studies by our group indicated that DOTAP, in the absence of LPS, did not induce lung inflammation when used at the same concentration reported in this study (10, 11). The inhibition of neutrophilic inflammatory response by PD/DOTAP treatment was also examined microscopically using H&E-stained lung sections (Fig. 6). Increased cellularity is evident after LPS treatment (Fig. 6, *A* and *B*). Careful examination of the alveolar air spaces revealed the presence of polymorphonuclear neutrophils (Fig. 6*C*), which are substantially reduced in the lung sections of mice pretreated with PD and DOTAP (Fig. 6, *E* and *F*). Pretreatment of LPS-exposed mice with PD alone had negligible effect on neutrophil emigration (Fig. 6*D*). These results support our earlier observations on neutrophil cell count (Fig. 5) and indicate the requirement of liposomal agent for effective inhibition of neutrophilic inflammatory response by the ON.

DISCUSSION

The use of sequence-specific ON as inhibitors of gene expression provides a powerful tool for elucidating the role of a particular gene and allows specific therapeutic intervention when that gene is overexpressed (32, 34). The strong binding affinity of ON to their targets makes these compounds potentially effective and specific against pathological gene expression. Inhibition of gene expression by double-stranded transcription factor-binding ON (also called "decoy" ON) has previously been reported (5) and has increasingly been investigated as a new therapeutic strategy for the treatment of various diseases (see Ref. 24 for review). In general, the use of ON-based therapeutics requires that two conditions be met: the identification of an appropriate target and the use of an efficient and specific means for inhibition. In several inflammatory and immune disorders, an overexpression of the early response cytokine TNF-α has been shown to play a pivotal role in the induction and progression of the disease (23). Therefore, suppression of this cytokine represents a logical therapeutic approach for treating disease.

We have shown in this study that it is possible to inhibit TNF-α gene expression by utilizing ON that bind specifically and competitively to the regulatory protein NF-κB. Our approach was based on the identification of specific target sequences on the TNF-α promoter that are required for NF-κB binding and transactivation of the TNF-α gene. Several NF-κB binding sites with various transcriptional activities were identified on the TNF-α promoter. The κ3 (−510) site was the most critical site for LPS inducibility of TNF-α expression. This conclusion was supported by our gene mutation and EMSA studies, which indicated that mutation of the κ3 site abolished LPS-induced

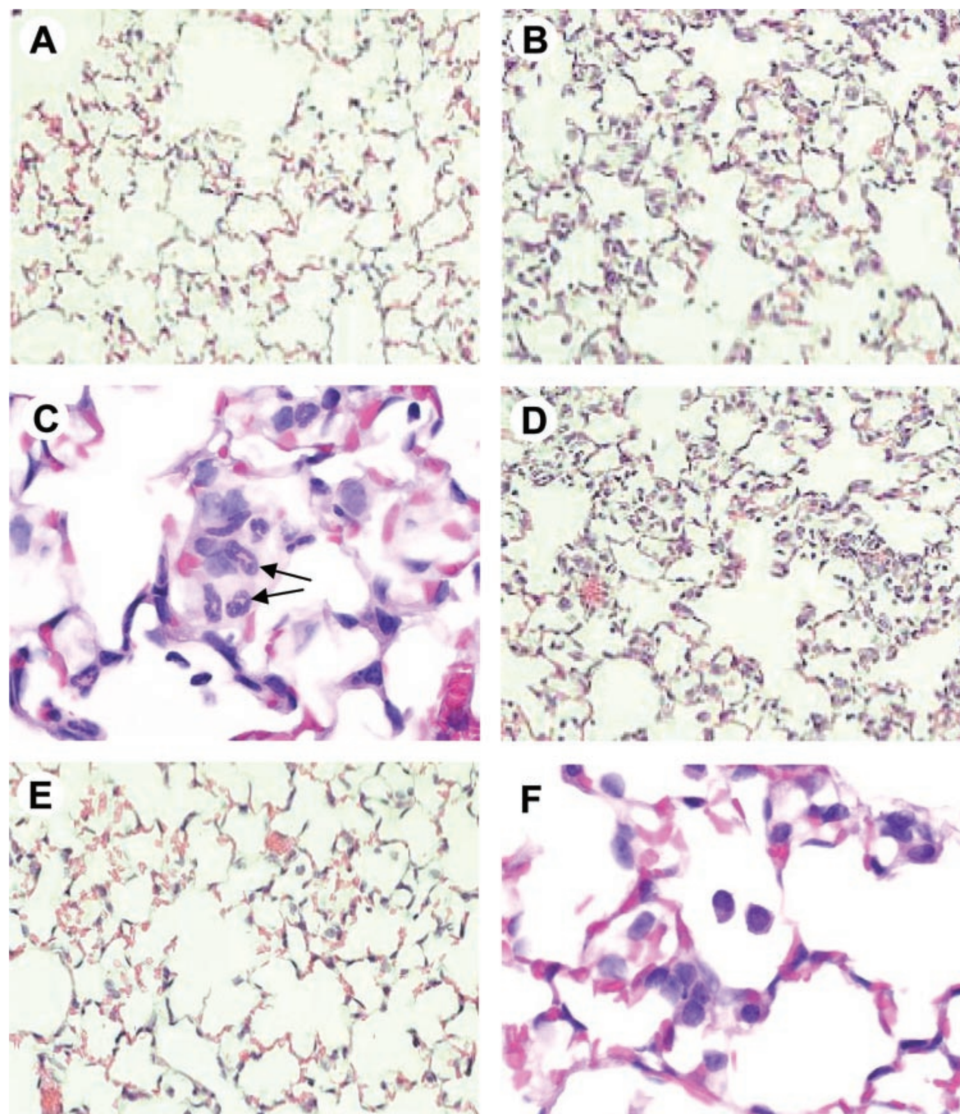

Fig. 6. Histological assessment of mouse lung. Mice were treated with saline (*A*) or LPS (*B*, 30 μg), and 1 day later they were killed and the lung tissues were processed and stained with hematoxylin and eosin (×400 magnification). *C*: same as *B* but at a higher magnification (×1,000) showing infiltration of polymorphonuclear neutrophils (arrows). *D*: mice were treated with PD (10 μg) alone or with DOTAP (100 nmol) (*E*), and 2 h later they were challenged with LPS as described in *B* (×400). *F*: high magnification of *E* (×1,000) showing some mononuclear macrophages and reduced number of polymorphonuclear neutrophils in the air spaces.

TNF-α promoter activity (Fig. 2*A*) and that ON containing the κ3 sequence was most effective in inhibiting NF-κB binding activity (Fig. 2*B*). These results are consistent with previous gene deletion experiments that demonstrated that the promoter region spanning the nucleotide −655 to −427 was required for maximum induction of mouse TNF-α gene (35). In a separate study, however, Drouet et al. (12) reported that all four κB sites of the TNF-α gene promoter were roughly equal in importance regarding their LPS inducibility as determined by gene mutation assay. The basis for this discrepancy is not clear but may be due to differences in specific point mutations of the κB motifs, cellular sources, and treatment conditions in the two studies. It is important to note that individual κB sites normally act in concert with other κB sites as well as other protein binding regions; therefore, their relative activity and contribution are generally interdependent.

Although the role of NF-κB in the regulation of mouse TNF-α gene has been established, its role in human TNF-α gene remains a subject of controversy, partly because the high-affinity κ3 (−510) site in the mouse promoter is absent in the human gene (21). Previous studies of the inducibility of human TNF-α gene promoter by PMA failed to indicate a role for NF-κB (13, 16). However, subsequent studies showed that both NF-κB and non-NF-κB nuclear proteins are required for maximum induction of the human TNF-α gene by LPS and to a lesser extent by PMA (14, 21). Comparative studies of the similarities and differences between human and mouse TNF-α promoters and their responses to LPS have been reported (21).

With the use of a supershift assay, we further demonstrated in this study that the DNA-NF-κB complexes induced by LPS in mouse RAW cells consisted of the p65/p50 heterodimer and the p50/p50 homodimer. The p65 has previously been shown to provide a *trans*-acting domain for NF-κB activation, whereas the p50 acts as a repressor in the transcription (7). The ability of the κ3 ON (PD and PT) to inhibit the NF-κB complex formation (Fig. 3*C*) supports our findings of the inhibitory effect of κ3 ON on TNF-α expression (Fig. 3*A*).

With the use of a murine lung inflammation model, we also demonstrated that the κ3 ON could inhibit LPS-induced TNF-α production and inflammatory neutrophil influx (Fig. 5). This inhibition was sequence specific because ON carrying mutated κ3 sequence (mPD and mPT) had no effects. Although the inhibitory effect of κ3 ON on lung inflammation can be attributed to the blockage of TNF-α, other possible mechanisms, such as blockage of other NF-κB-dependent genes, may also be involved. In this study, the inhibitory effects of κ3 ON were shown to require a liposomal delivery agent, DOTAP, for efficient inhibition. Previous studies have shown that DNA, when given alone via pulmonary administration, is rapidly cleared from the lung (22, 28). However, when codelivered with liposomes, the DNA remains in the lung for an extended period and to a greater level before being washed out of the capillary bed by normal blood flow (22, 28). Thus it appears that the retention time of the DNA or other drug molecules in the lung is likely to play a critical role in determining therapeutic efficacy. With regard to ON, it has been reported that ON, due to their polyanionic nature, poorly permeate the cells to reach their intracellular target sites (17, 42). Several research groups (3, 29, 33, 40) also observed that in the absence of appropriate delivery systems, ON exhibited weak or no biological activity, whereas in the presence of delivery systems, e.g., liposomes, ON showed strong activity. In agreement with these findings, our results showed that coadministration of the ON with the liposomal agent DOTAP greatly enhanced the inhibitory activities of ON. The DOTAP itself exhibited no inhibitory effects, indicating that this agent has no direct effect on lung cells but likely acts by increasing the cellular uptake and/or retention time of ON in the lung. Furthermore, both PD and PT ON were similarly effective when codelivered with DOTAP, suggesting a stability-enhancing effect of DOTAP on the PD ON. It is interesting to note that unlike the in vivo inhibitory effect, the effect of ON in vitro did not require the liposomal delivery agent. However, such an effect required a prolonged incubation of the cells with ON, i.e., 12 h before LPS stimulation, in serum-free medium to minimize degradation (15). A short-term incubation with ON, i.e., <2 h, did not result in any significant reduction in the cellular TNF-α response (results not shown). Likewise, a long-term pretreatment of mice with ON (12 h, without liposome) before LPS stimulation did not result in an improved inhibitory effect of the ON, presumably due to their rapid lung clearance and slow cellular uptake. These results suggest that to be biologically active in vivo, the ON must be delivered by appropriate means to enhance their residence time and cellular uptake characteristics.

In summary, we demonstrated that the κ3 (−510) site of TNF-α gene promoter was required for maximum LPS inducibility in macrophage RAW cells. Mutation of this site caused a major reduction in LPS inducibility of the TNF-α gene. EMSA studies showed that ON carrying the κ3 sequence were able to compete for NF-κB binding. Supershift assays revealed that the NF-κB complexes were composed of the p65/p50 heterodimer and the p50/p50 homodimer. The κ3 ON was effective in inhibiting LPS-induced TNF-α gene expression and neutrophil infiltration in a murine lung inflammation model. These findings have a direct implication on the therapeutic utilization of this compound in inflammatory and immune diseases. A similar gene inhibition approach may be employed to aid the study of other gene functions and their roles in disease pathogenesis.

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