Molecular Mechanism of Tumor Necrosis Factor-α Production in 1→3-β-Glucan (Zymosan)-activated Macrophages*

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The molecular details of 1→3-β-glucans, a fungal cell wall component, induced inflammatory responses are not well understood. In the present study, we conducted a systematic analysis of the molecular events leading to tumor necrosis factor (TNF)-α production after glucan stimulation of macrophages. We demonstrated that activation of nuclear factor κB (NF-κB) is essential in zymosan A (a source of 1→3-β-glucans)-induced TNF-α production in macrophages (RAW264.7 cells). Zymosan A-induced TNF-α protein production was associated with an increase in the TNF-α gene promoter activity. Activation of the TNF-α gene promoter was dependent on activation of NF-κB. Time course studies indicated that DNA binding activity of NF-κB preceded TNF-α promoter activity. Inhibition of NF-κB activation led to a dramatic reduction in both TNF-α promoter activity and TNF-α protein production in the response to zymosan A. Mutation of a major NF-κB binding site (κ3) in the gene promoter resulted in a significant decrease in the induction of the gene promoter by zymosan A, while mutation of Egr or CRE sites failed to inhibit the response to zymosan. Together, these results strongly suggest that NF-κB is involved in signal transduction of 1→3-β-glucans-induced TNF-α expression.

The role of fungi or yeast in organic dust toxic syndrome has attracted much attention recently. Zymosan A is a cell wall component of yeast, *Saccharomyces cerevisiae*. Zymosan A contains 50~57% 1→3-β-glucans (1) and was used as a crude preparation for 1→3-β-glucans in this study. Inhalation of zymosan A has been shown to induce an inflammatory response in animal experiments (2). 1→3-β-Glucans are polymers of D-glucose, which comprise a major structural component of fungal cell walls (3). 1→3-β-Glucans have been identified as a major reticuloendothelial-stimulating component in zymosan (4). A broad range of cell types can be activated by zymosan A, such as macrophages (5–7), polymorphonuclear leukocytes (8, 9), and natural killer cells (10). The interaction of zymosan A with macrophages is generally considered as the first step in the initiation of an immune response. Glucan receptors play an important role in mediating binding of zymosan to macrophages (5). The zymosan-induced inflammatory products include cytokines (11, 12), hydrogen peroxide (13), and arachidonic acid (14). Pulmonary exposure to zymosan A leads to the infiltration of polymorphonuclear leukocytes and results in pulmonary inflammation (2).

TNF-α[1] is a pro-inflammatory cytokine released from macrophages or activated T cells in response to microbes or other agents. TNF-α plays a key role in the initiation of inflammation in the lung and other tissues (15). TNF-α acts as a chemotactic agent leading to accumulation of macrophages and polymorphonuclear leukocytes at the inflammatory site (16). It can prime neutrophils by shortening the lag period of the respiratory burst (17). Although expression of TNF-α is controlled at multiple levels, gene transcription is the first and most important step in the control of TNF-α expression. NF-κB is a critical transcription factor in the regulation of TNF-α transcription (18–20). This transcription factor is a heterodimer protein composed of p65 and p50 in most cases. In addition to TNF-α, NF-κB is also involved in the regulation of gene transcription for many other cytokines (21). Nonactivated NF-κB is located in the cytoplasm and is associated with an inhibitory protein, I-κB (22). I-κB is phosphorylated and degraded in response to inflammatory stimuli, leading to the activation of NF-κB. The activated NF-κB translocates from the cytoplasm into the nucleus, where it binds to promoter regions of target genes and regulates their transcription. When target genes are turned on by NF-κB, mRNA synthesis occurs, and protein expression will follow. Although it has been reported that 1→3-β-glucan is able to activate NF-κB (23) and induce TNF-α production (24, 25), details of the relationship between NF-κB and TNF-α transcription in response to 1→3-β-glucan remain to be investigated. This study was designed to explore the details of the mechanisms regulating TNF-α expression induced by zymosan A at both the cellular and molecular levels.

MATERIALS AND METHODS

Cells and Reagents—The mouse macrophage cell line, RAW264.7, was purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, 25 mM HEPES buffer (pH = 7.4), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were maintained in 75-cm^2 tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Specific antibodies against the NF-κB p50 subunit (catalog number SC-114x, Santa Cruz Biotechnology) and p65 subunit (catalog number PC137, Oncogene) were used in the supershift assay. The NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), was purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Chlorophenolred-β-D-galactopyranoside monosodium salt

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[1] The abbreviations used are: TNF, tumor necrosis factor; NF-κB, nuclear factor κB; FBS, fetal bovine serum; CAPE, caffeic acid phenethyl ester; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; IL, interleukin.

Role of NF-κB in Glucan-induced TNF-α Production

was purchased from Roche Molecular Biochemicals. Zymosan A was obtained from Sigma. Lipopolysaccharide (LPS) was supplied by Difco.

The TNF-α luciferase reporter cells were derived from reporter-transfected RAW264.7 cells. The cells were transfected with a TNF-α gene promoter-controlled luciferase plasmid together with a pcDNA3 plasmid that provides a Geneticin resistance gene. The TNF-α promoter (−1260/+60) controls the luciferase gene expression (26). The transfected cells were first screened by G418 and then by luciferase activity. The positive cells were cloned, and one of the clones (clone 6) was used in this study. This clone has been tested for TNF-α production and luciferase activity in response to LPS. The results showed that luciferase activity was associated with TNF-α protein production.

Zymosan A Stimulation—For zymosan A stimulation, RAW264.7 cells (0.5×10^6 cells/well) were starved in 1 ml of 0.5% FBS RPMI 1640 medium without phenol red in a 24-well plate for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Before the experiment, the medium was changed to fresh 0.5% FBS RPMI 1640 medium. The stimulation time and zymosan A concentration are indicated in the text.

TNF-α ELISA Assay—After stimulation, the cell cultures were centrifuged at 1700 rpm for 5 min, and the supernatants were collected for determination of TNF-α. An ELISA kit from Endogen (Woburn, MA) was used to determine the TNF-α level in the cell culture supernatant according to the manufacturer's instructions.

Nuclear Extraction—RAW264.7 cells were plated in a 100-mm culture plate at a density of 8×10^6 cells/plate for 24 h. Then, the cells were starved in RPMI 1640, with 0.5% FBS, for an additional 24 h. Before the experiment, the medium was changed to fresh 0.5% FBS RPMI 1640 medium. The cells were treated with zymosan A or LPS for 4 h. At the end of treatment, the cells were harvested and treated with 500 μl of lysis buffer (50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM dithiothreitol) on ice for 4 min. After 1-min centrifugation at 14,000 rpm, the supernatant was saved as a cytoplasmic extract. The nuclear pellet was washed once with the same volume of buffer without Nonidet P-40. The nuclear pellet was then treated with 300 μl of extraction buffer (500 mM KCl, 10% glycerol with the same concentrations of HEPES, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and dithiothreitol as the lysis buffer) and pipetted several times. After centrifugation at 14,000 rpm for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70 °C. The protein concentration was determined using a BCA protein assay reagent (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA assay was carried out as reported previously (27). Briefly, the DNA-protein binding reaction was conducted in a 24 μl of reaction mixture including 1 μg of Poly(dI·dC), 3 μg of nuclear protein extract, 3 μg of bovine serum albumin, 4×10^4 cpm of ^{32}P-labeled oligonucleotide probe, and 12 μl of reaction buffer (24% glycerol, 24 mM HEPES (pH 7.9), 8 mM Tris-HCl (pH 7.9), 2 mM EDTA, 2 mM dithiothreitol). In some cases, the indicated amount of double-stranded oligomer was added as a cold competitor. The reaction mixture was incubated on ice for 10 or 20 min (with an antibody) in the absence of a radiolabeled probe. The double-stranded interleukin (IL)-6 NF-κB probe was labeled with [^{32}P]ATP (Amersham Pharmacia Biotech) using the T4 kinase (Life Technologies, Inc.). It should be noted that although the IL-6 NF-κB probe was utilized for the measurement of NF-κB activation, it is expected that the same results would be obtained using a TNF-α NF-κB probe. After addition of the radiolabeled probe, the mixture was incubated for 20 min at room temperature, then resolved on a 5% acrylamide gel that had been pre-run at 200 V for 30 min with $0.5 \times$ Tri-boric acid EDTA (TBE) buffer. The loaded gel was run at 200 V for 90 min, then dried and placed on Kodak X-Omat film (Eastman Kodak Co.) for autoradiography. The film was developed after an overnight exposure at −70 °C.

Transfection and Luciferase Assay—A set of TNF-α reporter vectors, the TNF-α wild type, κB3-mutated, CRE-mutated, and Egr-mutated, were gifts from Dr. S. T. Fan at the Scripps Research Institute (La Jolla, CA) (28). In the wild type vector, a TNF-α gene promoter fragment (−615/+15) controls the luciferase reporter gene. The mutant vectors were derived from the wild type vector by point mutation of the Egr, CRE, or NF-κB (κB3) sites in the promoter, respectively. The murine macrophage cells (0.5×10^6/well) were plated in a 24-well plate for 16 h, then were transfected with 0.5 μg of reporter DNA/well using LipofectAMINE (Life Technologies, Inc.). After transfection, the cells were washed once in phosphate-buffered saline solution and cultured in 1 ml of the RPMI medium with 0.5% FBS at 37 °C. Zymosan A was added 16 h later, and the cells were harvested at different times for the reporter assay. The luciferase activity was determined using an assay kit from Promega (Madison, WI) in combination with a luminometer (Monolight 3010, Analytical Luminescence Laboratory, Sparks, MD).

For the stable TNF-α reporter cells, 1×10^5 cells/well were used in a 96-well plate. The cells were lysed in 100 μl of lysis buffer, and the luciferase activity was determined with a 96-well luminometer.

β-Galactosidase was used as an internal control for normalizing luciferase activity in transient transfection (29). β-Galactosidase activity was determined by colorimetric reaction that was formed by interaction of 100 μl of reaction buffer (80 mM Na_2HPO_4, 0.5 M $MgCl_2$, and 104 mM 2-mercaptoethanol), 20 μl of chlorophenolred-β-D-galactopyranoside monosodium salt (80 mM), and 80 μl of cell lysate. The absorbance was measured at 574 nM after 20 min of incubation.

Statistical Analysis—Results are given as mean ± S.E. A paired one-tailed *t* test (two sample assuming equal variances) was performed, and the differences were considered statistically significant at $p < 0.05$. For multiple comparison, the one way analysis of variance for comparing several treatment groups with one control was used. Statistical analysis was performed using SigmaStat version 2.0. software (Jandel Corp.).

RESULTS

Production of TNF-α by Zymosan A-stimulated Macrophages—TNF-α is one of the immediate response gene products from macrophages in the inflammatory response. Therefore, TNF-α was used as an indicator of macrophage response to zymosan A. The RAW264.7 cells were treated with zymosan A at different concentrations and for different times. At the end of treatment, the cell-free supernatant was collected by centrifugation and used for determination of TNF-α production in an ELISA assay. The results show that zymosan A induced a substantial TNF-α production in RAW264.7 cells. This induction was dependent on the concentration of zymosan A (Fig. 1*A*). A significant increase in TNF-α production was observed after 24 h of exposure to 20 μg/ml zymosan A, while a maximum effect (18-fold increase) was noted at 100 μg/ml zymosan A. The zymosan A-induced TNF-α production was also dependent on stimulation time (Fig. 1*B*). The production of TNF-α was significantly increased after a 7-h exposure to 100 μg/ml zymosan A and reached a maximum by 12.5 h of exposure. This maximal level was maintained through 40 h of zymosan A exposure.

Activation of the TNF-α Gene Promoter—Transcriptional activity of the TNF-α gene was investigated by analysis of the gene promoter activity. The TNF-α reporter cells were stimulated with various concentrations of zymosan A for 8 h. As with TNF-α production, TNF-α promoter response exhibited a relationship to zymosan A concentration (Fig. 2*A*). A significant increase of luciferase activity was observed at 10 μg/ml zymosan A. A 5.7-fold increase was observed at 100 μg/ml zymosan A, while an maximum effect (8-fold increase) was noted at 300 μg/ml zymosan A. The zymosan A-induced TNF-α promoter activation was also dependent on stimulation time (Fig. 2*B*). The TNF-α promoter activity was significantly increased at 4 h and reached a peak at 8 h after exposure to 100 μg/ml zymosan A.
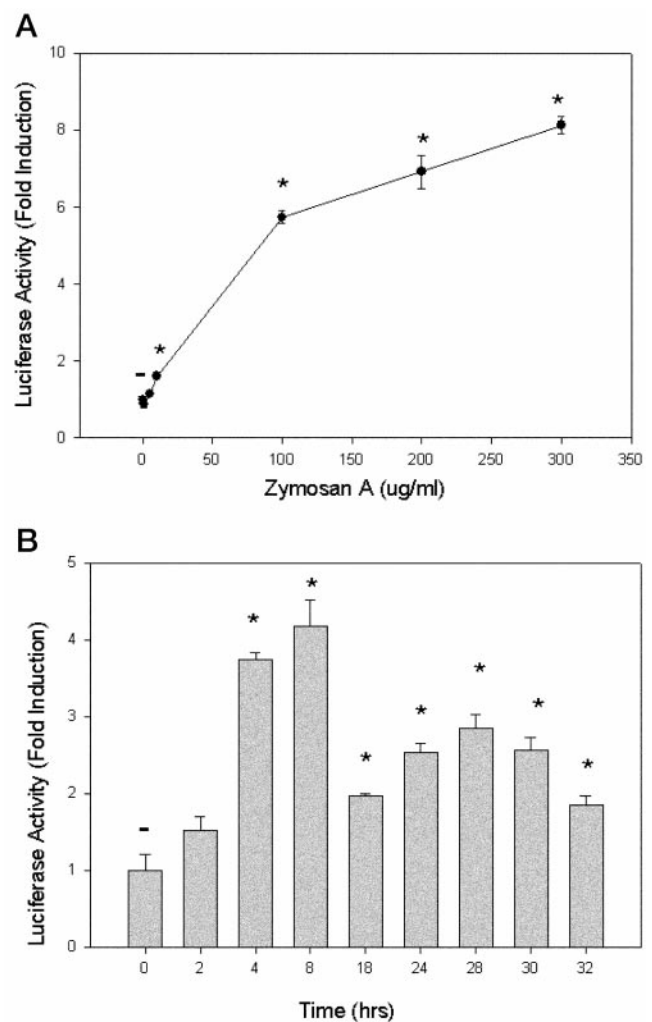
Activation of Transcription Factor NF-κB—NF-κB is a major activator for TNF-α transcription in macrophages. It is not clear if NF-κB regulates TNF-α transcription in response to zymosan A. To investigate the role of NF-κB, the DNA binding activity of NF-κB was examined in the nuclear extract of zymosan A-treated cells. The cells were treated with 100 μg/ml zymosan A for 4 h, and the nuclear protein was extracted as stated under "Materials and Methods." The results show that the nuclear proteins from the control cells formed a typical pattern of NF-κB bands (Fig. 3*A*, *lane 1*), and the binding activities were remarkably increased (2.8-fold) by zymosan A stimulation (Fig. 3*A*, *lane 2*). The nature of the DNA-protein complexes was determined in the supershift assay and the competition assay (Fig. 3*A*, *lanes 3–7*). The supershift result shows that the upper band was formed by a heterodimer of p65 and p50 subunits (Fig. 3*A*, *lane 5*), and the lower band was formed by a homodimer of p50 subunits (Fig. 3*A*, *lane 6*). The
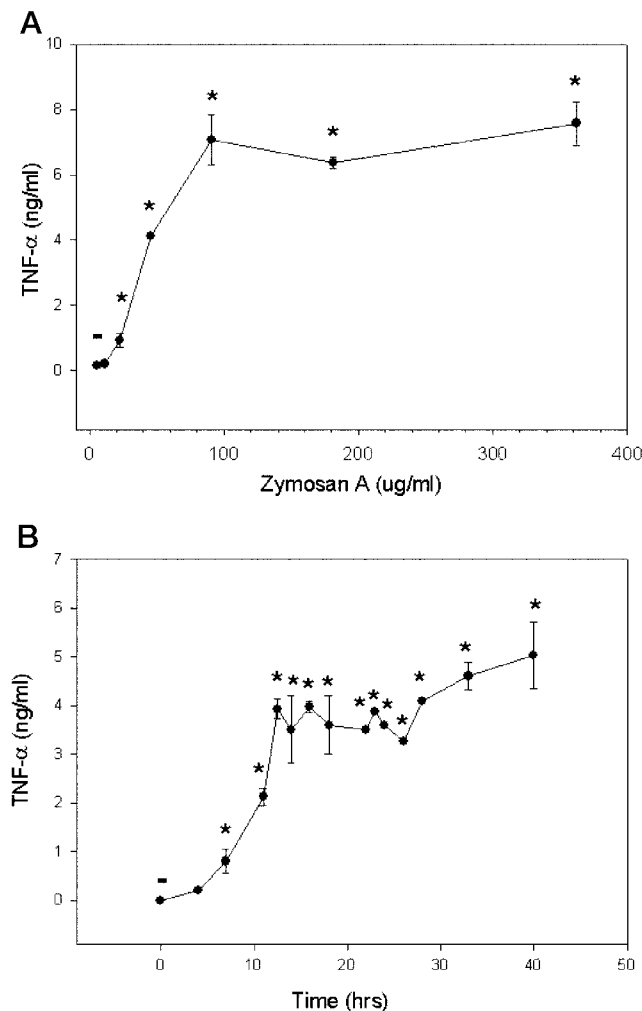
FIG. 1. **Zymosan A-stimulated TNF-α production.** The RAW264.7 cells (0.5 × 10^6 cells/well) were starved for 1 day before addition of zymosan A. The TNF-α level was measured in the supernatant using a TNF-α ELISA kit. Values are means ± S.E. of three experiments, "*" indicates a significant increase from control "-". A, dose-response curve for zymosan A-induced TNF-α production in RAW264.7 cells. RAW264.7cells were treated with various concentrations of zymosan A for 24 h. B, time course of TNF-α production in RAW264.7 cells in response to 100 μg/ml zymosan A.

FIG. 2. **Zymosan A-stimulated TNF-α promoter activity in stable transfected RAW264.7 cells.** The stable transfected RAW264.7 cells (1 × 10^5 cells/well) were seeded in a 96-well plate for 1 day before addition of zymosan A. The luciferase activity was measured in the cell lysate solution. A, dose-response curve for zymosan A-induced TNF-α promoter activity in stable transfected RAW264.7 cells. RAW264.7 cells were treated with various concentrations of zymosan A for 8 h. B, time course of TNF-α promoter activity in stable transfected RAW264.7 cells in response to 100 μg/ml of zymosan A. Values are means ± S.E. of four experiments, "*" indicates significantly higher than control "-".

gel shift results in Fig. 3*A* were quantified using a densitometer (Fig. 3*B*). The oligonucleotide competition result demonstrates that NF-κB complexes were formed specifically by interaction between the NF-κB binding probe and nuclear proteins, since DNA binding was inhibited by cold NF-κB but not by cold AP-1 or ATF-1 antibodies. Both the upper band and lower bands were enhanced by zymosan A. The upper band (p65 subunit) is thought to be critical for induction of TNF-α-dependent genes (20).

Time Course of NF-κB Activation—The above results demonstrate that zymosan induced both NF-κB DNA binding and activation of the TNF-α gene promoter. If NF-κB is responsible for activation of the promoter activity in response to zymosan, its activation should precede the promoter activity. To examine this hypothesis, the time course of NF-κB binding activity was investigated in the nuclear proteins from zymosan-treated cells (Fig. 4). The result shows that NF-κB was significantly activated at 2 h after zymosan A stimulation. The NF-κB binding activity continuously increased and reached a peak around 8 h after zymosan A treatment (Fig. 4*A*). The binding intensity was quantified by densitometry, and the results are shown in Fig. 4*B*. As this time course precedes the time course of the gene

promoter activation (Fig. 2*B*), the results indicate that NF-κB might be responsible for activation of the TNF-α gene promoter.

*Inhibition of TNF-α Promoter by the NF-κB Inhibitor CAPE*—To explore the role of NF-κB in activation of TNF-α transcription further, the NF-κB inhibitor CAPE was used in the study. This NF-κB inhibitor has been reported to prevent the translocation of the p65 subunit of NF-κB from the cytoplasm to the nucleus (30), resulting in a decrease in DNA binding activity of NF-κB. The inhibition is specific for NF-κB and does not affect the DNA binding activities of other transcription factors, including AP-1, Oct-1, and TFIID (30). The cells were pre-treated with CAPE for 1 h, then followed by 4-h zymosan A exposure. DNA binding activity of NF-κB was examined in EMSA. The results show that CAPE significantly suppressed the zymosan A-induced activation of NF-κB (Fig. 5, *A* and *B*). The upper band (p65/p50 heterodimer), which is critical for induction of TNF-α-dependent genes, was completely inhibited by CAPE (Fig. 5*A*, *lane 3*). The RAW264.7 cells show a normal NF-κB induction when using a positive control LPS (Fig. 5*A*, *lane 4*). TNF-α promoter response was
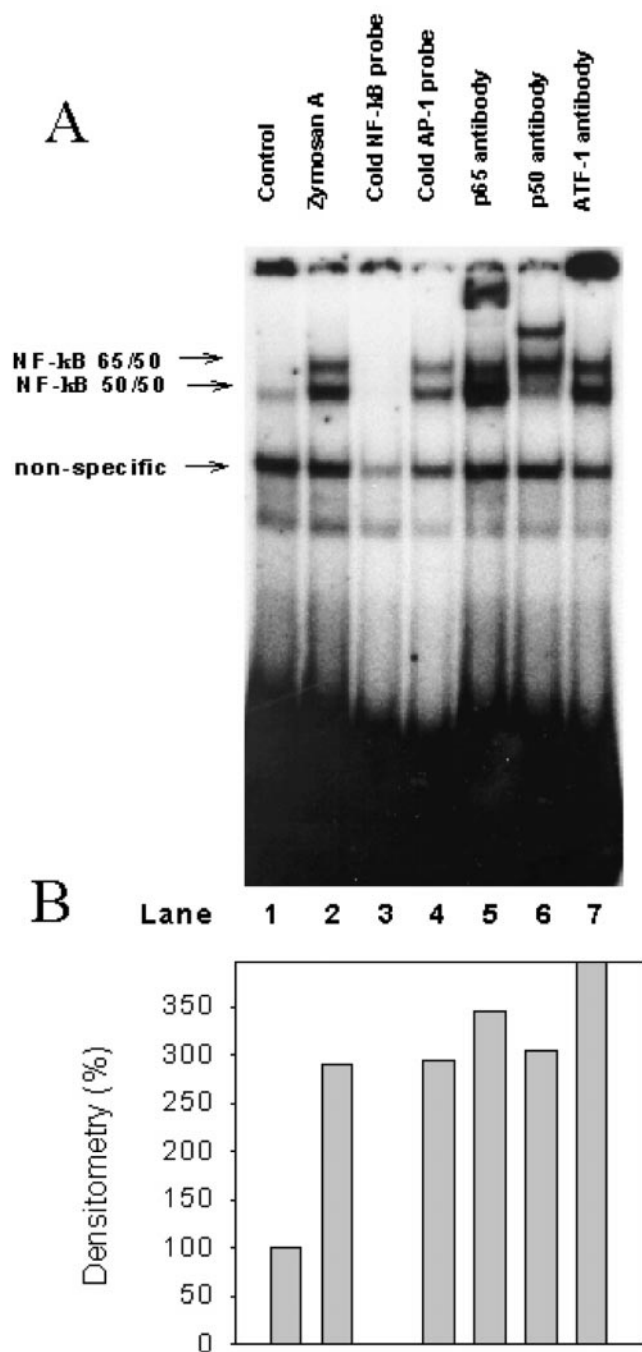
FIG. 3. **DNA binding activity of transcription factor NF-κB in the nuclear extract of RAW264.7 cells.** The DNA binding activity of NF-κB in the nuclear extract was determined using the EMSA gel shift assay (*A*) and the densities of the NF-κB bands measured and normalized for gel loading (*B*). The normalization was done by the sum of density of NF-κB p65/p50 and p50/p50 bands divided by the nonspecific band. *A*, characterization of NF-κB complexes in the oligonucleotide competition and antibody supershift assays. *Lane 1* served as a control for the oligonucleotide competition assay. *Lanes 2–7* are nuclear proteins from cells treated with zymosan A (100 μg/ml) for 4 h. The unlabeled NF-κB probe (0.2 μg) was used in *lane 3* as a specific competitor. The same amount of unlabeled AP-1 probe was used in *lane 4* for nonspecific competition. The antibodies against the p65 or p50 subunit of NF-κB protein were added in *lane 5* or *6* to confirm the nature of DNA-protein complexes. *Lane 7* contained 1 μg of ATF-1 antibody to serve as a nonspecific antibody.

also assayed after the CAPE treatment. The TNF-α luciferase cells were used to test inhibitory effect of CAPE. The results demonstrate that CAPE significantly reduced (by 80%) zymosan A-induced promoter activity (Fig. 5*C*). In line with this
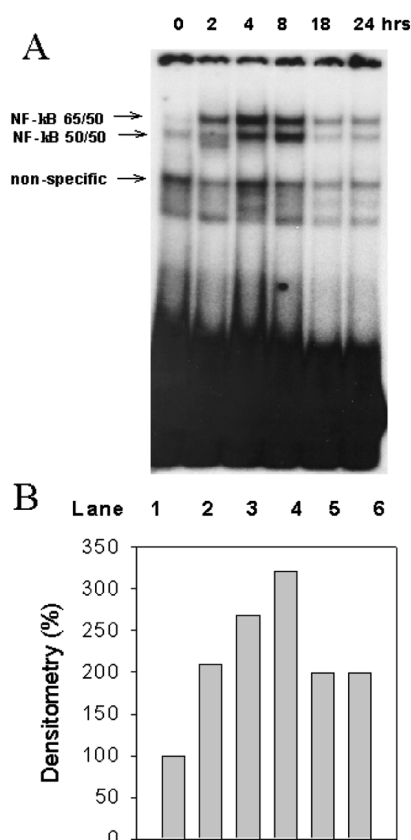

FIG. 4. **Time course of DNA binding activity of transcription factor NF-κB in the nuclear extract of RAW264.7 cells.** Macrophages were treated with 100 μg/ml zymosan A for various times, and the DNA binding activity of NF-κB in the nuclear extract was determined using the EMSA gel shift assay (*A*), and the densities of the NF-κB bands were normalized (*B*). The normalization was done by the sum of the density of NF-κB p65/p50 and p50/p50 bands divided by the nonspecific band.

inhibition, the zymosan-induced TNF-α expression was also completely abolished by CAPE (Fig. 5*D*). These results further support a role of NF-κB in zymosan-induced TNF-α production.

Mutation Analysis for NF-κB Binding Site—If NF-κB is required for TNF-α transcription, mutation of the NF-κB binding site in the gene promoter should lead to loss of the promoter response to zymosan A. To test this hypothesis, mutated TNF-α promoters were employed in the transient transfection assay. The result shows that zymosan A generated a 4-fold induction with the wild type TNF-α promoter (Fig. 6*A*), while only a 1.5-fold induction was observed with the κB3-mutated TNF-α promoter in which the major NF-κB binding site (κ3) was mutated to inhibit the NF-κB binding activity (Fig. 6*B*). In contrast, when the CRE or Egr site was mutated in the TNF-α promoter, no reduction in zymosan A-induced TNF-α promoter activity was observed (Fig. 6, *C* and *D*). These results demonstrate that the NF-κB binding site (κB3) is critical for activation of the TNF-α promoter by zymosan A.

DISCUSSION

Zymosan A is a particulate 1→3-β-glucan that induces immune responses by activating macrophages. Macrophages play an essential role in orchestrating the inflammatory response by the selective production of cytokines. *In vitro* studies have demonstrated that 1→3-β-glucans induce secretion of both IL-1 and TNF-α in mouse peritoneal macrophages (31) and in human monocytes (32, 33). Northern blot analysis showed that TNF-α mRNA was increased within 30 min, peaked at 2 h, and
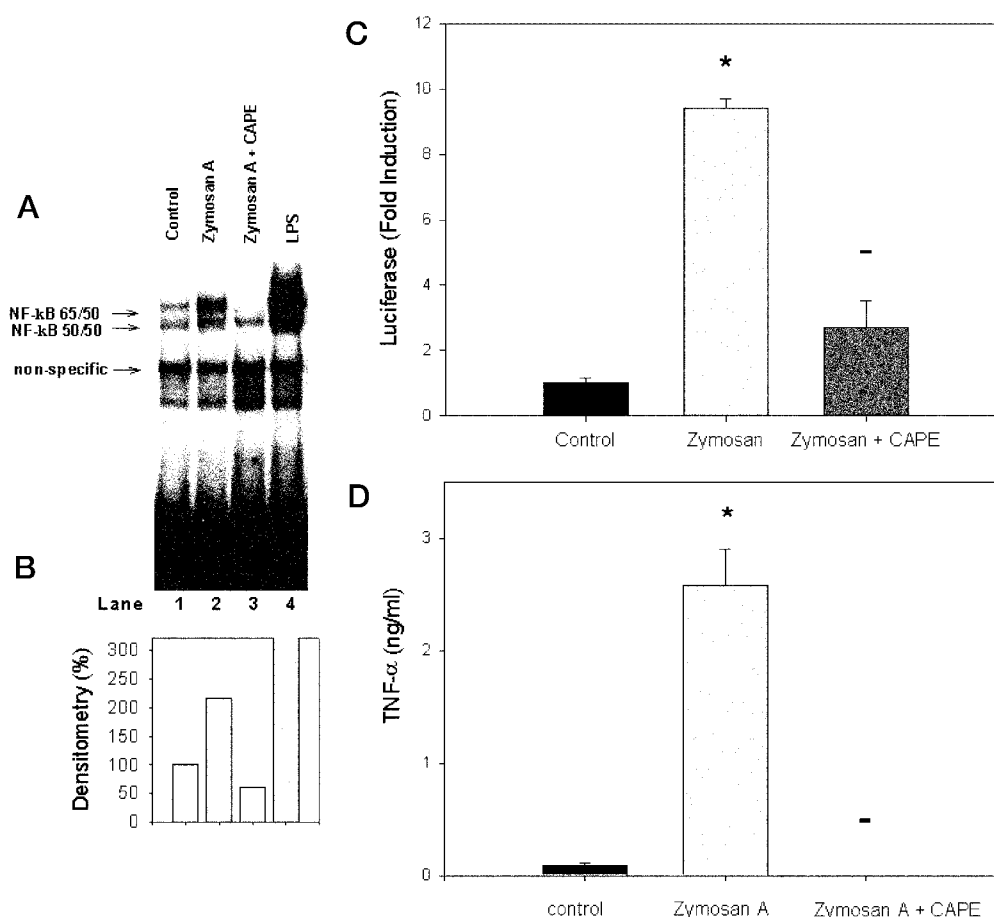
FIG. 5. **NF-κB inhibitor, CAPE, decreased DNA binding activity of NF-κB, TNF-α promoter activation, and TNF-α expression.** *A* and *B*, CAPE inhibits DNA binding activity of NF-κB. *A*: *lane 1*, untreated RAW264.7 cells; *lane 2*, cells treated for 4 h with 100 μg/ml zymosan *A*, which show enhanced NF-κB binding activity to the DNA; *lane 3*, cells pre-treated with CAPE (10 μg/ml) for 1 h, then exposed to zymosan for 4 h; *lane 4*, the positive control LPS (10 μg/ml). *C*, CAPE inhibits TNF-α promoter activity. The TNF-α reporter cells were pre-treated with CAPE (10 μg/ml) for 1 h before addition of 100 μg/ml zymosan A into the culture medium. The luciferase activity was measured in the cell lysate solution at 8 h after adding zymosan A. Values are means ± S.E. of four experiments. "*" indicates a significant increase from the control. "-" indicates a significant decrease from the zymosan A-induced level. *D*, CAPE inhibits TNF-α production. RAW264.7 cells were pre-treated with CAPE (10 μg/ml) for 1 h before addition of 100 μg/ml zymosan A into the culture medium. The TNF-α production was measured 24 h after zymosan A stimulation. Values are means ± S.E. of three experiments. "*" indicates a significant increase from control. "-" indicates a significant decrease from the zymosan A-induced level.

remained elevated for at least 8 h after exposure of human monocytes to 1→3-β-glucans (32). Zymosan A activity was mediated by β-glucan receptors (32). *In vivo* study shows that 1→3-β-glucans are able to induce a transient increase of both IL-1 and IL-6 in mouse blood (34). In addition to IL-1, TNF-α, and IL-6, 1→3-β-glucans also induce expression of IL-8 (11). These cytokines may share a common mechanism of induction in the response to 1→3-β-glucans. It has been shown in the literature that many human cytokines are regulated by NF-κB (21), which includes IL-1, IL-6, IL-8, and TNF-α. NF-κB binding sites have been identified in the promoter regions of these cytokine genes. Therefore, we hypothesize that NF-κB might be one of the major mediators of zymosan A signals for induction of inflammatory responses.

Conflicting results as to whether or not zymosan activates macrophages NF-κB signal transduction pathway have been reported. Tran-Thi *et al.* (35) reported that zymosan is incapable of activating NF-κB in rat liver macrophages. However, 1→3-β-glucans have been shown to activate NF-κB in the human promonocytic cell line U937 (23). This agrees with results reported in the present study using RAW264.7 mouse peritoneal macrophages. In addition, our laboratory has observed NF-κB activation in response to 1→3-β-glucans with NR8383

cells, an alveolar macrophage cell line from the rat.[2] Furthermore, the Tran-Thi *et al.* (35) study was focused on LPS-induced NF-κB and AP-1 activation rather than zymosan-induced responses. Therefore, the dose and exposure time may not have been optimized for zymosan. Last, 1→3-β-glucans activation of rat liver macrophage has been reported in the literature. Indeed, Adachi *et al.* (36) described that 1→3-β-glucans could enhance the production of cytokines and nitric oxide in these macrophages. Although they did not study the activation of NF-κB, it is quite likely that NF-κB was activated during 1→3-β-glucans exposure, since NF-κB is an upstream signal for these inflammation responses.

The NF-κB signaling system has been considered an evolutionarily conserved system that can operate in divergent genes in many different species (37). NF-κB is inducible. When cells receive an inflammatory stimulation, NF-κB will be activated and up-regulate the transcription of cytokine production. Transcriptional regulation is a major mechanism controlling cytokine expression. Initiation of transcription is determined by the promoter region in a cytokine gene. When the κB motif in the

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[2] S.-H. Young, J. Ye, D. G. Frazer, X. Shi, and V. Castranova, unpublished result.

Role of NF-κB in Glucan-induced TNF-α Production

FIG. 6. **Zymosan A-induced promoter activity of TNF-α in the transient transfection assay and the dependence of TNF-α gene expression on κB, Egr, and CRE sites in the promoter.** The wild type or κB-, Egr-, or CRE-mutated TNF-α luciferase reporters were transfected into the cells. The cells were treated with zymosan A (100 μg/ml) for 4 h. The reporter activity in the cell lysate was determined using a luminometer, and the reading was normalized by β-galactosidase activity. Values are means ± S.E. of three experiments. "+" indicates a significant increase from the control. "-" indicates a value significantly less than the wild type.
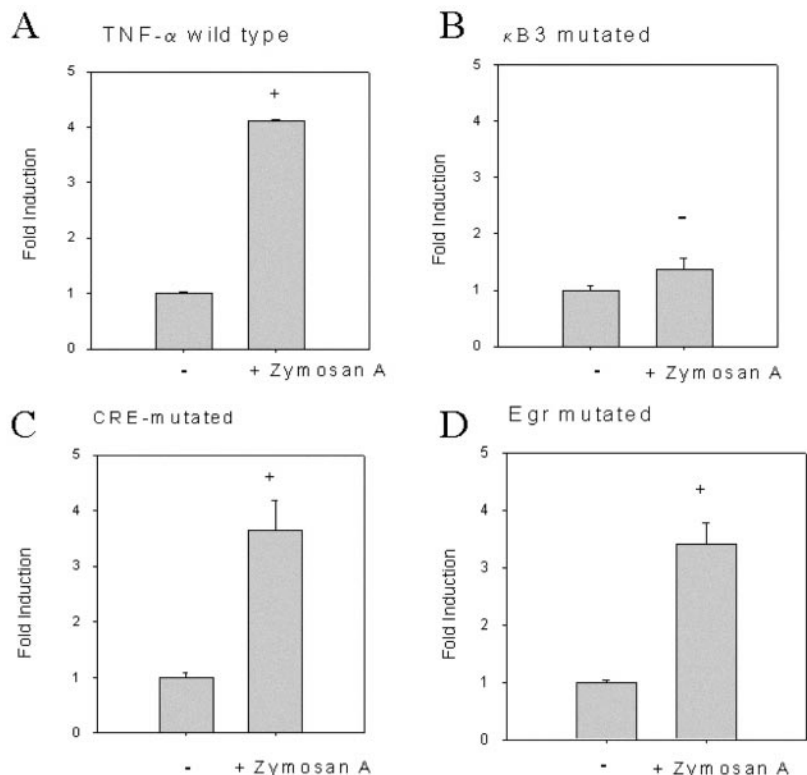
promoter DNA is occupied by NF-κB, there is initiation of transcription. The consensus DNA sequence for the NF-κB motif is, 5′-GGGRNNYYCC-3′. The DNA sequence of NF-κB binding site in IL-6 is 5′-GGGATTTTCC-3′ and in TNF-α is 5′-GGGGCTTTCC-3′, 5′-GGGAAAGCCC-3′, and 5′-GGGAAT-TCAC-3′ (38). The present study examines the relationship between zymosan-stimulated NF-κB activation and TNF-α production. The results support a model for transcriptional regulation of cytokines induced by 1→3-β-glucans.

The activation of NF-κB is commonly associated with the degradation of I-κB. The I-κB family are NF-κB inhibitory units that contain ankyrin repeat domains that bind to NF-κB and mask the nuclear localization signal (21). Following 1→3-β-glucans stimulation, I-κB is phosphorylated and degraded, unmasking nuclear localization signals and allowing NF-κB to be transported to the cell nucleus. NF-κB then binds to promoter regions of target genes and regulates their transcription. Whether or not zymosan activation of NF-κB is done through the degradation of I-κB is unclear. A decrease of I-κB level was associated with Betafectin®-induced stimulation of mouse BMC2.3 macrophage cells (39). However, Bondeson *et al.* (40) reported that overexpression of I-κBα by adenoviral gene transfection had no effect on zymosan-induced IL-8, IL-1β, or TNF-α levels. This may imply that zymosan activates NF-κB through a pathway other than I-κBα degradation (41). However, the lack of direct evidence on I-κB activity after zymosan exposure suggests that further study is needed to verify the above hypothesis.

In the present study, we have demonstrated that activation of NF-κB is essential in zymosan A (a source of 1→3-β-glucans)-induced TNF-α production by RAW264.7 macrophages. Zymosan A increased TNF-α production in a time- and concentration-dependent manner (Fig. 1, *A* and *B*). The optimum dose for induction was about 100 μg/ml. This dose was approximately the optimal dose for TNF-α promoter activation (Fig. 2*A*). We then established the time course for TNF-α production and TNF-α promoter activation (Figs. 1*B* and 2*B*). The results show that TNF-α promoter activation was significantly

increased 4 h after zymosan A exposure and peaked at 8 h. TNF-α promoter activation preceded TNF-α production (significant increase at 7 h with a peak at 12.5 h). TNF-α promoter activation was, in turn, preceded by NF-κB/DNA binding, which was significantly elevated 2 h after zymosan A treatment and peaked at 8 h. Pre-treatment of macrophage cells with a NF-κB inhibitor led to a decrease in DNA binding activity of the NF-κB p65/p50 heterodimer (Fig. 5, *A* and *B*), which led to a subsequent decrease in TNF-α promoter activity (Fig. 5*C*) and a suppression of TNF-α production (Fig. 5*D*). These results suggest that zymosan A is able to induce TNF-α expression by NF-κB-dependent activation of gene transcription.

This study provides substantial data for the role of NF-κB in the transcriptional activation of TNF-α by zymosan A. 1) Zymosan A induced the activation of NF-κB. The DNA binding activity of NF-κB in the nuclear extract was enhanced (2.8-fold) after zymosan A treatment. 2) The time course of DNA binding activity of NF-κB preceded the promoter activation of TNF-α. This suggests that the dynamic change of the TNF-α promoter activity results from a change in the DNA binding activity of NF-κB. 3) Inhibition of NF-κB activation decreased zymosan A-stimulated TNF-α promoter activity by 80%. 4) Removal of the NF-κB binding site led to inhibition of the TNF-α promoter activation. The importance of NF-κB in TNF-α transcription was investigated with mutation of the major NF-κB binding site (κB3). Mutation studies show that the promoter response to zymosan A was dramatically reduced (decreased by 80%) after mutation, while mutation of the Egr or CRE site had no effect on promoter activation (Fig. 6). Taken together, these data strongly support the hypothesis that NF-κB mediates zymosan A-induced TNF-α transcription and TNF-α production in macrophages.

In summary, transcriptional regulation is a major mechanism controlling cytokine expression. It has been suggested that gene transcription was activated quickly following 1→3-β-glucans exposure (32). Initiation of transcription is determined by the promoter region in a cytokine gene. NF-κB is an

activator protein for many cytokine gene promoters, including IL-1, IL-6, IL-8, and TNF-α (22). We hypothesize that NF-κB is a major mediator of zymosan A signals for induction of these cytokines. The present study used the TNF-α gene as a model for analysis of the transcriptional regulation of 1→3-β-glucans response cytokines. The results demonstrate that the activation of NF-κB is essential for zymosan A-induced TNF-α production in macrophages.

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Molecular Mechanism of Tumor Necrosis Factor-α Production in 1→3-β-Glucan (Zymosan)-activated Macrophages

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