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Lipopolysaccharide and CpG DNA synergize for tumor necrosis factor-α production through activation of NF-κB

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Abstract

Unmethylated CpG motifs in bacterial DNA (CpG DNA) activate host innate immune responses synergistically with some other microbial products, such as endotoxins, and may contribute to disease pathogenesis through excessive production of proinflammatory cytokines. Because monocyte-derived tumor necrosis factor (TNF)-α is an important mediator of disease, we investigated whether CpG DNA and lipopolysaccharide (LPS) synergize for inducing TNF-α biosynthesis. CpG DNA and LPS synergistically induce TNF-α production in RAW264.7 cells and J774 cells through activation of NF-κB. Furthermore, transient transfection with a super-repressive mutant of IκBα (IκBα-AA) demonstrated that NF-κB plays a critical role in CpG DNA-mediated TNF-α expression. Like NF-κB activation, CpG DNA-induced activation of mitogen-activated protein kinases (MAPK) regulates TNF-α production. Both extracellular receptor kinase (ERK) and p38 can regulate TNF-α gene transcription induced by CpG DNA. Although CpG DNA at the higher concentration slightly enhanced LPS-mediated phosphorylation of ERK, it did not alter the LPS-mediated activation of c-Jun N-terminal kinase and p38. In addition, CpG DNA showed little or no enhancement of LPS-mediated AP-1 activation. These results suggest that CpG DNA- and LPS-mediated signals converge at or above the level of NF-κB and ERK, and that there are distinct, as well as common, signaling pathways which are utilized by both CpG DNA and LPS for activating various transcription factors and MAPK.

Introduction

Innate immune responses are initiated rapidly after infection by host recognition of conserved molecular structures present in microorganisms (e.g. endotoxins and high mannose proteins) and limit pathogen spread. Recent studies have suggested that immune cells recognize unmethylated CpG dinucleotides in particular base contexts ('CpG motifs': GACGTT for murine and GTCGTT for human) in bacterial DNA as one such conserved molecular pattern (1–5). To quickly and efficiently clear infections before they become pathogenic, immune cells recognize and respond to low levels of different innate immune activators in a synergistic manner. However, under certain conditions, this synergistic activation of immune cells by different innate immune activators like CpG DNA and lipopolysaccharide (LPS) can induce the systemic inflammatory response syndrome (SIRS) or inflammatory arthritis (6–8, and Waldschimidt and Krieg, unpublished).

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CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides (CpG DNA) induce B cell proliferation, tumor necrosis factor (TNF)-α, IL-6, IL-10 and Ig secretion, and apoptosis resistance (1,9–17). In addition to its direct effects on B lymphocytes, CpG DNA also directly activates monocytic cells, such as monocytes/macrophages, and dendritic cells to secrete various cytokines, including type I IFN, TNF-α, IL-1, IL-6, IL-10 and IL-12, and to up-regulate expression of co-stimulatory factors (6,9,10,12,13,18–21). In addition, some cytokines, such as IL-12, that are secreted by monocytic cells after CpG DNA stimulation, act on NK cells to induce their lytic activity and IFN-γ production (6,22). Overall, CpG DNA induces a T_h1-like pattern of cytokine production dominated by IL-12 and IFN-γ (18,23–25).

The biochemical mechanisms by which CpG DNA activates immune cells to produce T_h1-type cytokines and protects B cells from apoptosis are yet to be fully understood, although recent studies have provided exciting new insights. The innate immune system detects many infectious agents through a family of proteins known as toll-like receptors (TLR) that function as pattern recognition receptors capable of specifically binding molecular patterns present in microbes but not in vertebrate cells (reviewed in 26). This heterogeneous family of proteins includes TLR-2 (which binds peptidoglycan), TLR-4 (which binds LPS) and the newly described TLR-9 (which is required for the immune stimulatory effects of CpG DNA) (27–30). In addition, CpG DNA signaling is dependent on the MyD88/IRAK pathway which is suggested to be a downstream effector of TLR-9 (31). On the other hand, evidence also exists for a second pathway in which the DNA-activated protein kinase, DNA-PKcs, has been suggested to mediate the response to CpG DNA (32). Prior to cellular activation through these pathways, CpG DNA appears to be endocytosed by leukocytes and acidified in an endosomal compartment, which is coupled to the rapid generation of intracellular reactive oxygen species (ROS) (19). The CpG DNA-induced ROS generation precedes activation of NF-κB, which is required for all downstream events such as proto-oncogene expression, cytokine production, B cell proliferation and apoptosis protection (12,15–17,19). In addition, CpG DNA induces activation of certain mitogen-activated protein kinase (MAPK) kinases (MKK; MKK3, MKK4 and MKK6) and MAPK, including extracellular receptor kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 (33,34, and Yi and Krieg manuscript in preparation). Endosomal acidification of CpG DNA is also required for this MAPK activation (33,34). In turn, CpG DNA-mediated MAPK activation has been reported to be involved in the cytokine production (33–35). However, it is largely unknown how MAPK activated by CpG DNA lead to the production of each cytokine.

The proinflammatory cytokine TNF-α is an important regulator of the early inflammatory immune response and a central mediator of the SIRS (36). As in the LPS response, secretion of TNF-α by leukocytes is an early effect of CpG DNA (6,8,34, and Yi and Krieg, unpublished data). This TNF-α produced by CpG DNA has been shown to play an important role in the CpG DNA-mediated inflammatory arthritis and SIRS (6–8, and Waldschmidt and Krieg, unpublished data). Interestingly, low doses of CpG DNA and LPS can synergistically induce SIRS and inflammatory arthritis which, at least in part, might be due to production of large amounts of TNF-α (6,8). Considering the critical role of TNF-α in CpG DNA-mediated inflammatory arthritis and SIRS, it is important to understand the molecular mechanisms by which CpG DNA mediates TNF-α production, in monocytic cells. Therefore, we investigated whether CpG DNA and LPS synergize for TNF-α production, and how CpG DNA-mediated signals interact with LPS-mediated signals to synergistically induce TNF-α synthesis in murine monocytic cells, RAW264.7 and J774. Our results indicate that CpG DNA-mediated TNF-α production is dependent on the activation of NF-κB, ERK and p38. In addition, our results demonstrated that CpG DNA synergizes with LPS for TNF-α production through activation of NF-κB in RAW264.7 and J774 cells.

Methods

Oligodeoxynucleotides

Nuclease-resistant phosphorothioate oligodeoxynucleotides (S-ODN) were supplied by the Coley Pharmaceutical Group (Wellesley, MA) and had no detectable endotoxins by Limulus assay. The sequences of S-ODN used were 5′-TCCAT-GACGTTCCTGACGTT-3′ (CpG DNA: 1826) and 5′-TCCAG-GACTTCTCTCAGGTT-3′ (non-CpG DNA: 1982). *Escherichia coli* DNA and calf thymus DNA were purchased from Sigma (St Louis, MO), and purified by phenol–chloroform extraction followed by ethanol precipitation using pyrogen-free solutions, and had undetectable endotoxin by Limulus assay (QCL-1000; Biowhittaker, Walkersville, MD) following the manufacturer's protocol. For the sake of consistency, only the results using these two ODN are shown herein. However, essentially the same results have been obtained in experiments with other CpG and control non-CpG DNA, including bacterial DNA.

Cell lines, culture conditions and reagents

The murine monocytic cell line RAW264.7 (ATCC, Rockville, MD) was cultured at 37°C in a 5% CO_2 humidified incubator, and maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 1.5 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. J774 cells (ATCC) were cultured at 37°C in a 5% CO_2 humidified incubator, and maintained in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 1.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). LPS (*Salmonella typhimurium*) was purchased from Sigma (St Louis, MO). SB202190, a p38 kinase inhibitor, and U0126, a MEK inhibitor, were purchased from Calbiochem (La Jolla, CA).

ELISA

RAW264.7 cells (5×10^5 cells/ml) or J774 cells (5×10^6 cells/ml) were treated with CpG or non-CpG DNA (0–30 μg/ml) in the presence or absence of LPS (0–5000 ng/ml) for 6 h. For some experiments, RAW264.7 cells were stimulated with media, CpG DNA or LPS in the presence or absence of U0126 (2.5 μM) or SB202190 (2.5 μM) for 6 h. For IL-6 and IL-12p40 analysis, RAW264.7 cells (2×10^6 cells/ml) or J774 cells (1×10^6 cells/ml) were treated with CpG or non-CpG DNA (0–30 μg/ml) in the

presence or absence of LPS (0–500 ng/ml) for 24 h. Culture supernatants were analyzed by ELISA for TNF-α, IL-6 or IL-12p40 as described previously (10). Antibodies specific for murine TNF-α, IL-6 and IL-12p40, and recombinant murine TNF-α, IL-6 and IL-12 were purchased from PharMingen (San Diego, CA).

Transfections, luciferase assay, β-galactosidase assay and chloramphenicol acetyl transferase (CAT) ELISA

RAW264.7 cells or J774 cells (~80% confluent in a 100-mm tissue culture dish) were transfected with AP-1–β-galactosidase (8 μg), 5′ TNF-α–CAT (4 μg) or a mixture of NF-κB–luciferase (4 μg) and pRL-TK–luciferase (1 μg) constructs using LipofectAMINE Plus (Gibco/BRL). Transfected cells were pooled and washed 3 times with culture media. Cells (5×10^5 cells/ml for AP-1–β-galactosidase, 5′ TNF-α–CAT or NF-κB–luciferase and pRL-TK–luciferase) were stimulated with CpG DNA (0–6 μg/ml) in the presence or absence of LPS (0–100 ng/ml) for 12 h. For some experiments, RAW264.7 cells were co-transfected with super-suppressive IκBα mutant construct (IκBα-AA, 15 μg) or control vector (pOPRSVI.mcs1, 15 μg) and 5′ TNF-α–CAT (8 μg) construct, AP-1-β-galactosidase (12 μg), or a mixture of NF-κB–luciferase (8 μg) and pRL-TK–luciferase (2 μg) constructs, and then incubated for 6 h before stimulating with media, CpG DNA (0.6 or 3 μg/ml) and/or LPS (5 or 50 ng/ml) for 12 h. To investigate the role of ERK and p38 in CpG DNA- and LPS-mediated TNF-α transcription, RAW264.7 cells transfected with TNF-α transcriptional promoter linked with gene encoding reporter enzyme CAT (5′ TNF-α–CAT; 4 μg) were stimulated with media, CpG DNA (0.6 or 3 μg/ml) and/or LPS (5 or 50 ng/ml) for 12 h in the presence or absence of DMSO, U0126 (2.5 μM) or SB202190 (2.5 μM). β-Galactosidase and luciferase activities in cell extracts were analyzed according to manufacturer's protocol using the Galacto-Light Plus reporter gene assay for β-galactosidase (Tropix, Bedford, MA) and Dual-Luciferase reporter assay system (Promega, Madison, WI) respectively. For expression of CAT in the transfected cells, CAT protein levels in the cell lysates were analyzed using the CAT-ELISA kit (Boehringer Mannheim, Mannheim, Germany). NF-κB–luciferase activity was normalized using pRL-TK–luciferase activity in each sample. For AP-1–β-galactosidase assay and CAT-ELISA, equal concentrations of cell lysates were used. AP-1–β-galactosidase and NF-κB–luciferase constructs were kindly provided by Dr G. Koretzky (University of Pennsylvania) (37). 5′ TNF-α–CAT construct was provided by Dr B. Beutler (University of Texas Southwestern Medical Center at Dallas) (38) and super-suppressive IκBα mutant construct (IκBα-AA) was provided by Dr. G. Bishop at U. Iowa (39).

Preparation of RNA and quantitation of TNF-α mRNA by real-time PCR analysis

RAW264.7 cells (2×10^6 cells/ml) were treated with various concentrations of CpG DNA (0–0.6 μg/ml) in the presence or absence of LPS (5 ng/ml). Cells were harvested 30 min after LPS and DNA treatments, and total RNA was isolated by using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. To measure the relative amount of TNF-α mRNAs, amplification of sample cDNA was monitored with the fluorescent DNA binding dye SYBR Green in combination with the ABI 5700 sequence detection system (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Forward and reverse primers were designed using primerExpress software (PE Applied Biosystems). GAPDH was used for endogenous control. The PCR primers used in this study were: TNF-α (F: TGGGAGTAGACAAGGTACAACCC; R: AGAGGGAAATCGTGCGTGAC) and GAPDH (F: TTCACCACCATGGAGAAGGC; R: GGCATGGACTGTGGTCATGA).

Preparation of whole-cell lysates and nuclear extracts, Western blot analysis, and electrophoretic mobility shift assay (EMSA)

RAW264.7 cells (2×10^6 cells/ml) were treated with medium, CpG DNA (0–6 μg/ml) and/or LPS (0–5 ng/ml). Cells were harvested at the indicated time points (15 min, 30 min, 1 h and 2 h) and then whole-cell lysates or nuclear extracts were prepared as previously described (15,19). To detect phosphorylated ERK, JNK or p38, equal amounts of whole-cell lysates (50 μg/lane) were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS (SDS–PAGE) and then Western blots were performed as previously described (19) using a specific antibody against the phosphorylated form of each protein. Specific antibodies against the phosphorylated form of ERK, JNK and p38 were purchased from New England BioLabs (Beverly, MA). Specific antibody against p38 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To detect DNA binding activity of the transcription factor AP-1, NF-AT, NFIL-6, or NF-κB, nuclear extracts (3 μg/lane) were analyzed by EMSA as previously described (15) using ^{32}P-labeled double-stranded oligodeoxynucleotides containing the AP-1 (GATCTAGTGATGAGTCAGCCGGATC) (40), NF-AT (TGCCCAAAGAGGAAAATTTGTTTCATACAG) (41), NF-IL-6 (TCGAGACATTGCACAATCTG) (40) or NF-κB (GTAGGGGACTTTCCGAGCTCGAGATCCTATG) (42) binding sequence as a probe.

Results

Synergistic effects of CpG DNA and LPS on TNF-α production in RAW 264.7 and J774 cells

To investigate whether CpG DNA and LPS can synergize for TNF-α production, we used the murine monocytic cell lines, RAW264.7 and J774. RAW264.7 cells or J774 cells were stimulated with various concentrations of CpG DNA or control non-CpG DNA in the presence or absence of various concentrations of LPS for 6 h. The levels of TNF-α in culture supernatants were measured by an ELISA specific for murine TNF-α. As demonstrated in Fig. 1(A), a substimulatory dose of CpG DNA synergistically enhanced LPS-mediated TNF-α production. As anticipated, CpG DNA also induced TNF-α production in a dose-dependent manner and showed synergy with LPS (100 ng/ml) (Fig. 1B). To evaluate whether this synergistic effect of CpG DNA and LPS is unique to TNF-α or has a more general impact on other phenomena, we analyzed effects on IL-6 production. As expected, CpG DNA and LPS synergized for IL-6 production in J774 cells (Fig. 1C and D). As in J774 cells, CpG DNA induced TNF-α production in RAW264.7 cells in a dose-dependent manner (Fig. 2A).

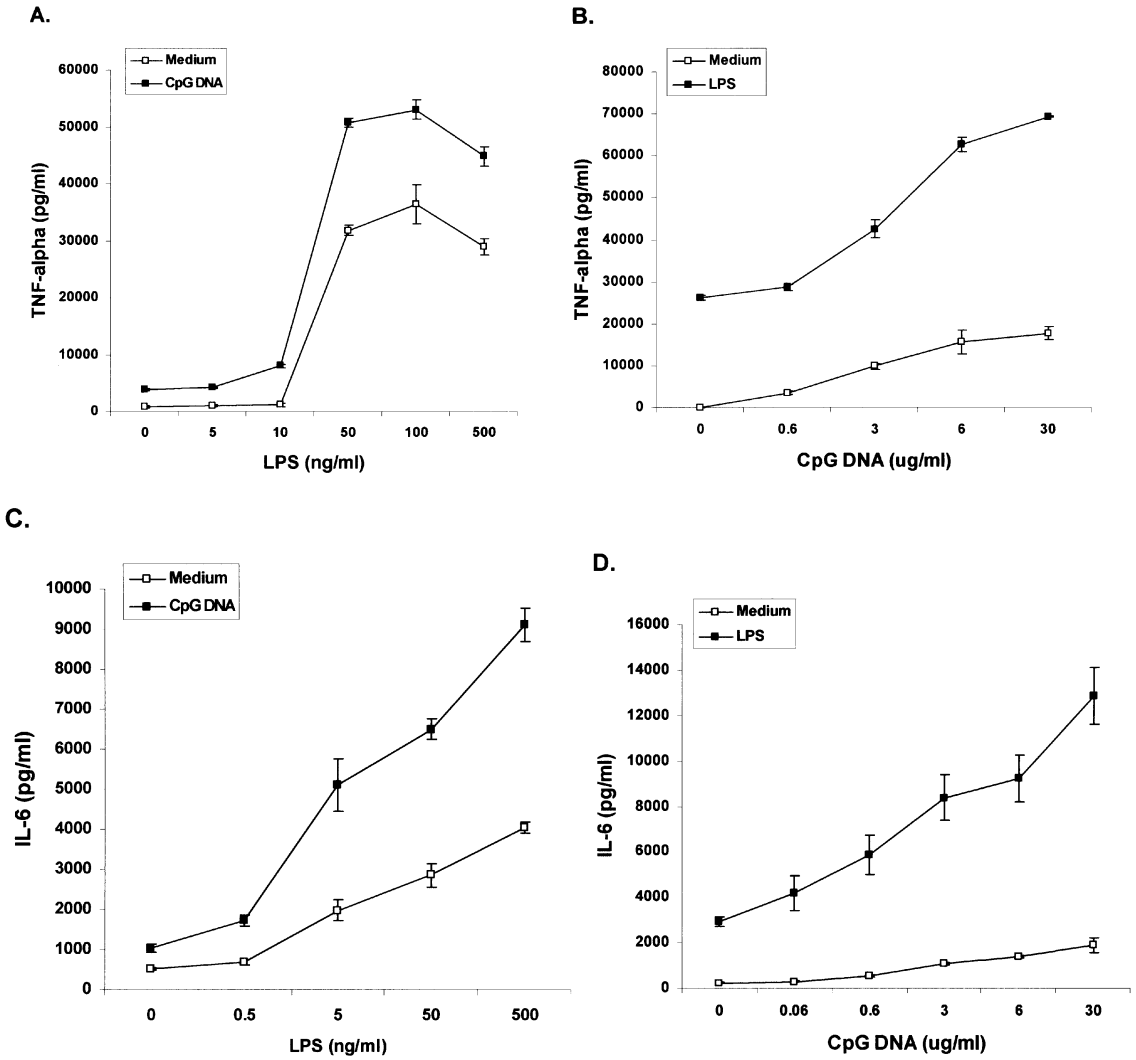

Fig. 1. Synergistic production of TNF-α and IL-6 by CpG DNA and LPS in J774 cells. (A) J774 cells (5×10^6 cells/ml) were stimulated with various concentrations of LPS (0–500 ng/ml) in the presence (■) or absence (□) of 0.6 μg/ml of CpG DNA for 6 h. (B) J774 cells (5×10^6 cells/ml) were stimulated with various concentrations of CpG DNA (0–30 μg/ml) in the presence (■) or absence (□) of 100 ng/ml of LPS for 6 h. (C) J774 cells (1×10^6 cells/ml) were stimulated with various concentrations of LPS (0–500 ng/ml) in the presence (■) or absence (□) of 0.6 μg/ml of CpG DNA for 24 h. (D) J774 cells (1×10^6 cells/ml) were stimulated with various concentrations of CpG DNA (0–30 μg/ml) in the presence (■) or absence (□) of 100 ng/ml of LPS for 24 h. The levels of TNF-α (A and B) and IL-6 (C and D) in culture supernatants were determined by ELISA. Data present the mean ± SD of triplicates. The experiment was performed 3 times with similar results.

Moreover, a suboptimal concentration of LPS (5 ng/ml) synergistically enhanced CpG DNA-induced TNF-α production. This synergistic effect of CpG DNA and LPS for TNF-α production was observed not only at low concentrations of CpG DNA, but also with the concentration which induced maximal TNF-α production (Fig. 2A). As anticipated, LPS also induced TNF-α production in a dose-dependent manner and showed synergy with a suboptimal dose of CpG DNA (0.06 μg/ml) (data not shown). In contrast, control non-CpG DNA neither induced TNF-α production by itself nor enhanced LPS-mediated TNF-α production in RAW264.7 cells (Fig. 2B and data not shown). In addition to synergistic production of TNF-α, CpG DNA and LPS also synergistically induced production of later cytokines including IL-6 and IL-12 in RAW264.7 cells (Fig. 2C and D). These results indicate that CpG DNA and LPS are able to synergize for production of TNF-α as well as other cytokines in murine monocytic cells, and that in addition to the common shared pathway of MyD88/TRFA6, CpG DNA and LPS might also have a capability to transduce signals through different pathways that converge for TNF-α production.

Agonistic effects of CpG DNA and LPS for activation of TNF-α promoter and expression of TNF-α mRNA in RAW264.7 cells

It has been demonstrated that the biosynthesis of TNF-α is regulated at both the transcriptional and translational levels

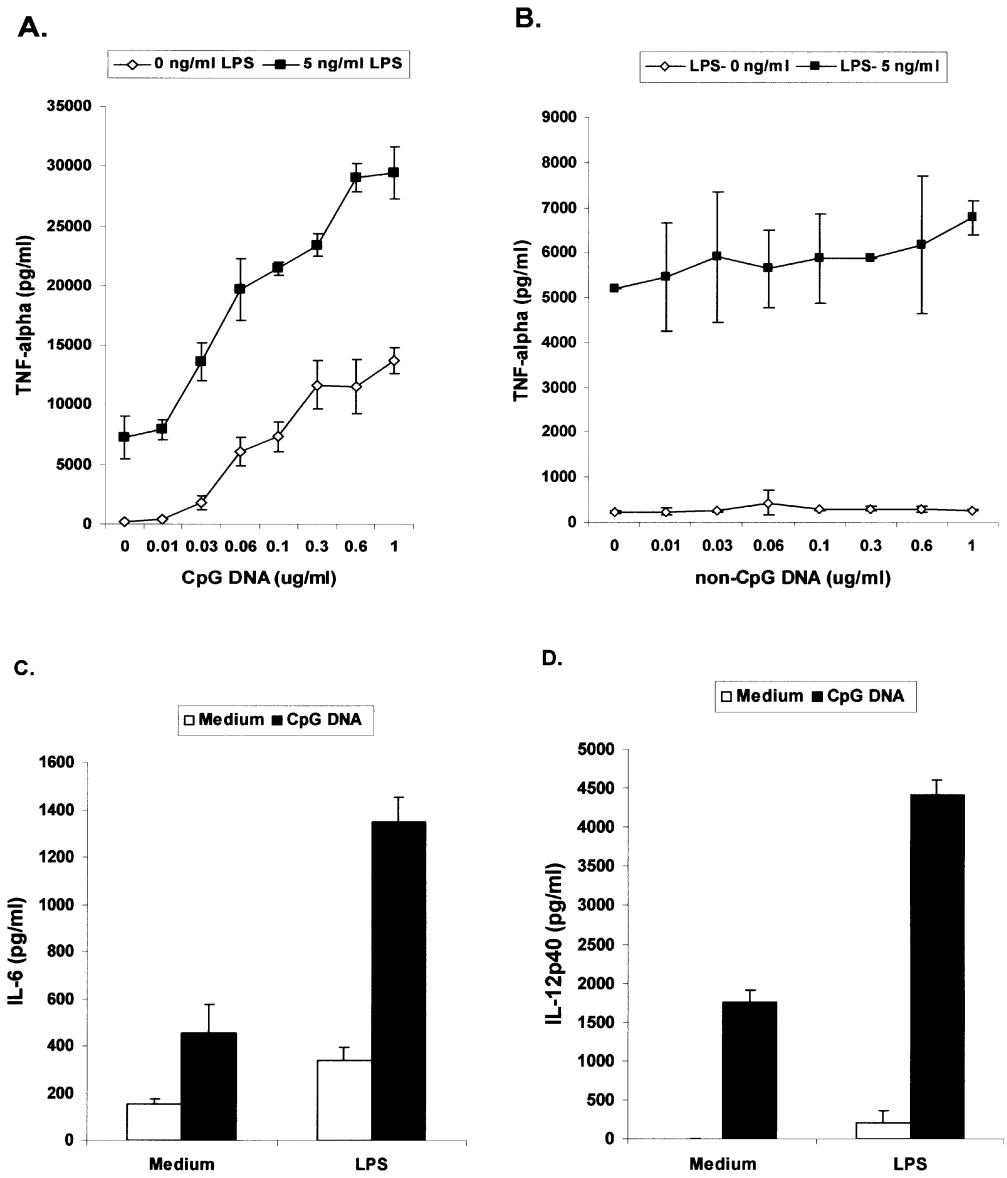

Fig. 2. Synergistic production of TNF-α, IL-6 and IL-12 by CpG DNA and LPS in RAW264.7 cells. (A) RAW264.7 cells (5×10^5 cells/ml) were stimulated with various concentrations of CpG DNA in the presence (■) or absence (◇) of 5 ng/ml of LPS for 6 h. (B) RAW264.7 cells were stimulated with various concentrations of control non-CpG DNA in the presence (■) or absence (◇) of 5 ng/ml of LPS for 6 h. (C and D) RAW264.7 cells (2×10^6 cells/ml) were stimulated with medium or LPS (1 ng/ml) in the presence (solid bar) or absence (empty bar) of 0.03 μg/ml (~0.005 μM) of CpG DNA for 24 h. The levels of TNF-α, IL-6 and IL-12p40 in culture supernatants were determined by ELISA. Data present the mean ± SD of triplicates. The experiment was performed 3 times with similar results.

(38,43). Therefore we investigated whether CpG DNA induces production of TNF-α by activating TNF-α promoter activities and whether it can synergize with LPS at the level of TNF-α transcription. To investigate these possibilities, we transiently transfected RAW264.7 cells with the TNF-α transcriptional promoter reporter plasmids which encoding the 5′ TNF-α promoter linked with CAT as a reporter gene (5′ TNF-α–CAT) (38) and then stimulated with various concentrations of CpG DNA (0–3 μg/ml) in the presence or absence of a low concentration of LPS (1 ng/ml) for 12 h. Levels of CAT proteins in the cell lysates were measured by CAT-ELISA. As demonstrated in Fig. 3(A), CpG DNA induced TNF-α promoter activation in a dose-dependent manner. In addition, a low concentration of LPS greatly enhanced CpG DNA-mediated TNF-α promoter activation (Fig. 3A). To investigate whether agonistic effects of CpG DNA and LPS for activation of the TNF-α promoter actually correlate with the levels of TNF-α mRNA expression, we stimulated RAW264.7 cells with low concentrations of CpG DNA (0–0.6 μg/ml) in the presence or absence of LPS (5 ng/ml) for 30 min and then measured levels of TNF-α mRNA by real-time PCR. As shown in Fig. 3(B), LPS alone at the concentration of 5 ng/ml induced a minimal increase of TNF-α mRNA. The levels of TNF-α mRNA in RAW264.7 cells increased upon CpG DNA stimulation in a

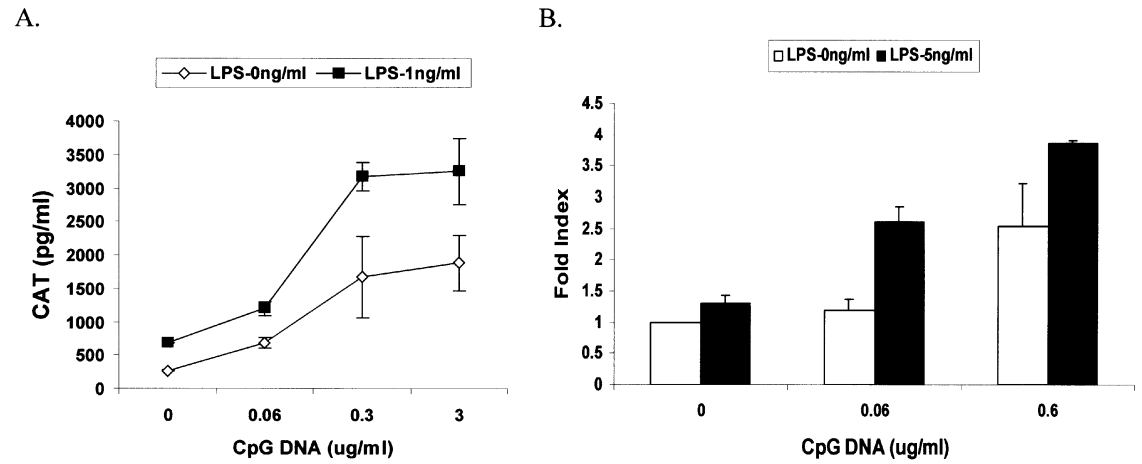

Fig. 3. Synergistic effects of CpG DNA and LPS for activation of TNF-α promoter and mRNA expression. (A) Synergistic activation of TNF-α promoter by CpG DNA and LPS. RAW264.7 cells were transiently transfected with a murine TNF-α promoter transcriptional reporter (5′ TNF-α–CAT) construct. Transfected cells were pooled and washed 3 times with culture media and then stimulated with various concentrations of CpG DNA (0–3 μg/ml) in the presence (■) or absence (◇) of 1 ng/ml of LPS for 12 h. Levels of CAT proteins in the cell lysates (50 μg/sample) were determined by CAT-ELISA. Data present the mean ± SD of triplicates. The experiment was performed 4 times with similar results. (B) Synergistic increases in the TNF-α mRNA levels by CpG DNA and LPS. RAW264.7 cells were stimulated with various concentrations of CpG DNA (0–0.6 μg/ml) in the presence (solid bar) or absence (empty bar) of 5 ng/ml of LPS for 30 min. Total RNA was isolated using the RNeasy mini kit (Qiagen), and the presence of TNF-α mRNA and GAPDH mRNA in each sample was detected using real-time PCR using SYBR green. GAPDH was used for endogenous control. Data present the mean (fold induction from unstimulated control) ± SD of triplicates. Of note, similar experiments were performed >3 times using a RNase protection assay, and radioactivity in the TNF-α mRNA band in each sample was counted using a phospholmager and then normalized to the amount of L32 mRNA in each sample. Results from the RNase protection assay were comparable to that of real-time PCR.

dose-dependent manner. Furthermore, the levels of TNF-α mRNA were greatly increased when CpG DNA and LPS were added simultaneously (Fig. 3B). These results indicated that synergistic production of TNF-α by CpG DNA and LPS might be controlled, at least in part, at the transcriptional level.

CpG DNA and LPS synergize for activation of NF-κB but not AP-1, NF-IL-6 or NF-AT

Since CpG DNA and LPS synergize for TNF-α promoter activity, we investigated whether CpG DNA and LPS activate different transcription factors and/or can synergize for activation of a particular transcription factor. RAW264.7 cells were stimulated with CpG DNA (0.06 or 6 μg/ml) and/or LPS (1, 5 or 50 ng/ml) for 1–2 h, and nuclear extracts were analyzed for DNA binding activities of several transcription factors. At these concentrations, CpG DNA and LPS synergistically induced DNA binding activity of p65/p50 NF-κB heterodimers, but not AP-1, NF-AT or NF-IL-6 (Fig. 4A and 4B). To investigate whether this synergistic effect of CpG DNA and LPS on the DNA binding activity of NF-κB correlates with its transcriptional ability, RAW264.7 cells or J774 cells were transiently transfected with a reporter construct encoding NF-κB–luciferase and then stimulated with various concentrations of CpG DNA in the absence or presence of various concentrations of LPS for 12 h. As demonstrated in Fig. 5(A), addition of CpG DNA synergistically enhanced LPS-mediated transcriptional activation of NF-κB in J774 cells. In addition, CpG DNA- or *E. coli* DNA-mediated activation of NF-κB was greatly enhanced by addition of a low concentration of LPS in RAW264.7 cells (Fig. 5B). As shown in Fig. 5(C), CpG DNA induced transcriptional activities of NF-κB in a dose-dependent manner and the simultaneous addition of LPS synergistically enhanced CpG DNA-mediated NF-κB activation in RAW264.7 cells. As expected, LPS alone also induced transcriptional activities of NF-κB in a dose-dependent manner and addition of CpG DNA synergistically enhanced this (data not shown). As for NF-κB, either CpG DNA alone or LPS alone induced the transcriptional activity of AP-1 in a dose-dependent manner (Fig. 5D and data not shown). However, neither CpG DNA nor LPS showed synergistic enhancing effects on the transcriptional activity of AP-1 induced by each other in RAW264.7 and J774 cells (Fig. 5D and data not shown). Thus, CpG DNA and LPS synergize for both DNA binding activity and transcriptional activity of NF-κB that may contribute to the synergistic biosynthesis of TNF-α at the transcriptional level.

Transcriptional activation of TNF-α synthesis induced by CpG DNA is dependent upon NF-κB activation

Because our data demonstrated that CpG DNA and LPS synergize for TNF-α biosynthesis, at least in part, at the transcriptional level (Fig. 3) and activation of a transcription factor NF-κB is synergistically induced by both signals (Figs 4 and 5), we investigated whether this synergistic activation of NF-κB has a functional role in the synergistic activation of TNF-α transcription. To directly determine the role of NF-κB in the CpG DNA-mediated TNF-α expression, RAW264.7 cells were co-transfected with constructs encoding super-repressive IκBα mutant (IκBα-AA) or control empty vector and 5′ TNF-α–CAT construct, AP-1-β-galactosidase construct or NF-κB–luciferase and pRL-TK–luciferase constructs. As shown in Fig. 6(A), super-suppressive IκBα mutant, IκBα-AA,

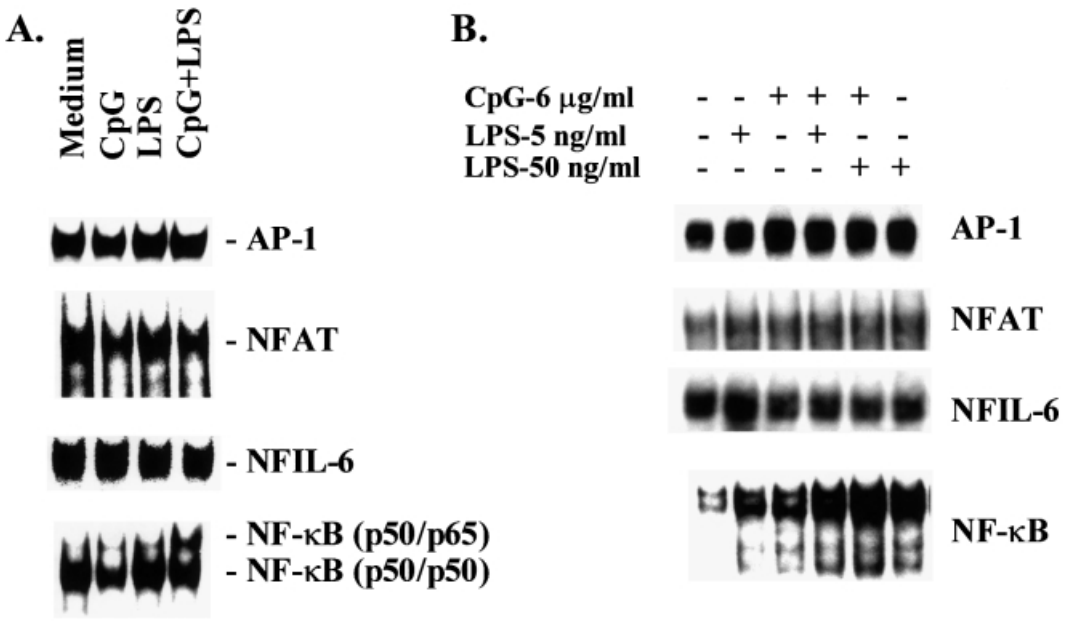

Fig. 4. CpG DNA and LPS synergize for NF-κB but not for AP-1, NF-AT or NF-IL-6 DNA binding activities. (A) RAW264.7 cells were stimulated with medium, CpG DNA (0.06 μg/ml), LPS (1 ng/ml) or the combination of CpG DNA and LPS for 2 h, and then nuclear extracts were prepared. (B) RAW264.7 cells were stimulated with medium, CpG DNA (6 μg/ml), LPS (5 or 50 ng/ml) or the combination of CpG DNA and LPS for 1 h, and then nuclear extracts were prepared. DNA binding activities of transcription factor NF-κB, AP-1, NF-AT or NF-IL-6 in equal amounts of nuclear extracts (3 μg/lane) were analyzed by EMSA using ^{32}P-labeled double-stranded oligodeoxynucleotides containing the AP-1, NF-AT, NF-IL-6 or NF-κB consensus DNA binding sequence as a probe. Specificity of bands was determined by cold-competition and super-shift EMSA. The experiment was performed 2–3 times with similar results.

completely abolished CpG DNA- or LPS-mediated NF-κB activation in RAW264.7 cells indicating the functional inhibitory activities of IκBα-AA for NF-κB. CpG DNA and LPS synergized for NF-κB activation (Fig. 5 and 6B), which was completely abolished by over-expression of IκBα-AA (Fig. 6B). Interestingly, both CpG DNA- and LPS-induced TNF-α promoter activity was greatly suppressed in the presence of IκBα-AA in these cells, indicating the critical role of NF-κB in CpG DNA-mediated TNF-α expression (Fig. 6C). Furthermore, synergistic effects of CpG DNA and LPS on the induction of TNF-α promoter activity were also abolished (Fig. 6D). Of note, neither CpG DNA- nor LPS-mediated AP-1 activation is suppressed by over-expression of IκBα-AA, demonstrating the specific functional role of IκBα-AA for suppression of NF-κB (Fig. 6E). These results demonstrate that as in LPS-mediated biosynthesis of TNF-α (44), NF-κB plays a critical role in the CpG DNA-induced TNF-α expression in RAW264.7 cells, and suggest that synergistic activation of NF-κB by CpG DNA and LPS may be the major contributor for the synergistic induction of TNF-α promoter activity by these stimuli.

CpG DNA slightly enhances LPS-induced phosphorylation of ERK but not JNK or p38 in RAW264.7 cells

Activation of one or more members of MAPK is one of the early signaling events in the CpG DNA-mediated leukocyte activation (33,34). It has previously been indicated that CpG DNA-mediated production of various cytokines is regulated by one or more activated MAPK in murine monocytic and B cells (33–35). In addition, inhibition of ERK or p38 by pharmacological inhibitors greatly suppresses TNF-α production induced by CpG DNA (33–35). Therefore, we investigated whether CpG DNA-mediated MAPK contributes to biosynthesis of TNF-α at the transcriptional level, and whether CpG DNA and LPS also synergize for activation of one or more MAPK in RAW264.7 cells. To evaluate whether ERK or p38 has a functional role in CpG DNA-induced TNF-α transcription, RAW264.7 cells were transiently transfected with TNF-α transcriptional promoter (5′ TNF-α–CAT) and then stimulated with CpG DNA (3 μg/ml) in the presence or absence of DMSO, MEK inhibitor, U0126 (2.5 μM), or p38 inhibitor, SB202190 (2.5 μM), for 12 h. Levels of CAT protein in the cell lysates were measured by CAT-ELISA. Specific inhibitory effects of U0126 and SB202190 on MEK1/2 and p38 respectively at the concentration used for this study were verified by phospho-specific Western blot and *in vitro* kinase assay (data not shown). As shown in Fig. 7, both CpG DNA- and LPS-induced TNF-α promoter activities were partially impaired in the presence of MEK inhibitor or p38 inhibitor, indicating regulatory effects of ERK and p38 in TNF-α synthesis at the transcriptional level. Our results indicate that both ERK and p38 have a regulatory function in CpG DNA-induced TNF-α synthesis at the transcriptional level, and that CpG DNA and LPS synergize for TNF-α synthesis at the transcriptional level. Therefore we further investigated whether CpG DNA and LPS also synergize for activation of one or more MAPK, and whether MEK inhibitor or p38 inhibitor abolishes synergistic effects of CpG DNA and LPS on the TNF-α promoter activation. RAW264.7 cells or J774 cells were stimulated with low concentrations of CpG DNA (0–0.1 μg/ml) in the presence

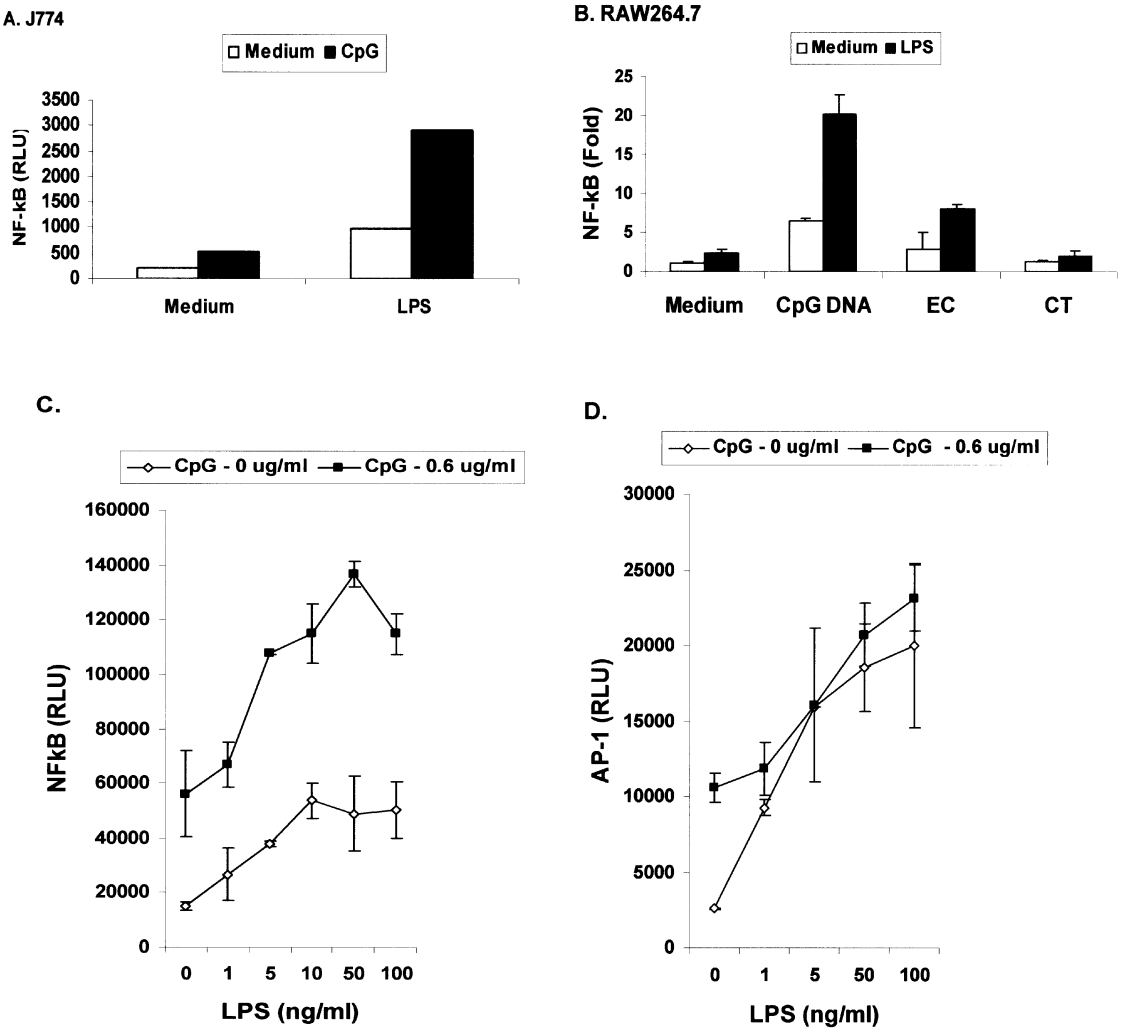

Fig. 5. CpG DNA and LPS synergize for NF-κB but not for AP-1 transcriptional activities. (A) J774 cells were transiently transfected with NF-κB–luciferase constructs (2 μg) using LipofectAMINE Plus (Gibco/BRL). Transfected cells were pooled and washed 3 times with culture media. Cells (10^6 cells/2 ml/well) were stimulated with medium, or LPS (100 ng/ml) in the presence (solid bar) or absence (empty bar) of 0.6 μg/ml of CpG DNA for 12 h. NF-κB–luciferase activities in cell extracts were analyzed by luciferase assay. The experiment was performed 3 times with similar results. (B) RAW264.7 cells were transiently transfected with NF-κB–luciferase + pRL-TK–luciferase constructs using LipofectAMINE Plus. Transfected cells were pooled and washed 3 times with culture media. Cells (2×10^5 viable cells) were stimulated with medium, CpG DNA (0.6 μg/ml), *E. coli* DNA (EC; 0.1 μg/ml) or calf thymus DNA (CT; 0.1 μg/ml) in the presence (solid bar) or absence (empty bar) of LPS (0.1 ng/ml) for 12 h. NF-κB–luciferase activities in cell extracts were analyzed by the Dual-Luciferase reporter assay system and normalized using pRL-TK–luciferase activity in each sample. Data present the mean ± SD of triplicates. The experiment was performed 3 times with similar results. (C and D) RAW264.7 cells were transiently transfected with NF-κB–luciferase + pRL-TK–luciferase (C) or AP-1–β-galactosidase (D) constructs using LipofectAMINE Plus. Transfected cells were pooled and washed 3 times with culture media. Cells (10^5 cells/200 μl/well) were stimulated with various concentrations of LPS in the presence (■) or absence (◇) of 0.6 μg/ml of CpG DNA for 12 h. NF-κB–luciferase activities in cell extracts were analyzed by Dual-Luciferase reporter assay system and normalized using pRL-TK–luciferase activity in each sample. AP-1–β-galactosidase activities in cell extracts were analyzed using Galacto-Light Plus reporter gene assay for β-galactosidase and normalized by equal concentrations of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was performed 3 times with similar results.

or absence of suboptimal concentrations of LPS (1–5 ng/ml) for 15, 30 or 60 min. As shown in Fig. 8, low concentrations of CpG DNA or LPS alone activated JNK, ERK and p38 very weakly in RAW264.7 cells. CpG DNA slightly enhanced LPS-mediated ERK phosphorylation at higher doses, but not at lower doses (see CpG DNA at 0.06 and 0.1 μg/ml in the presence of 5 ng/ml of LPS in Fig. 8). In addition, CpG DNA failed to enhance phosphorylation of JNK and p38 induced by LPS in RAW264.7 cells (Fig. 8). Furthermore, CpG DNA did not alter the LPS-mediated ERK, JNK or p38 activation in J774 cells (data not shown). To further verify that inhibition of either ERK or p38 does not affect the agonistic effects of CpG DNA and LPS on the induction of TNF-α transcriptional promoter activation, RAW264.7 cells were transiently transfected with 5′ TNF-α–CAT, and then stimulated with low doses of CpG DNA and/or LPS in the presence or absence of

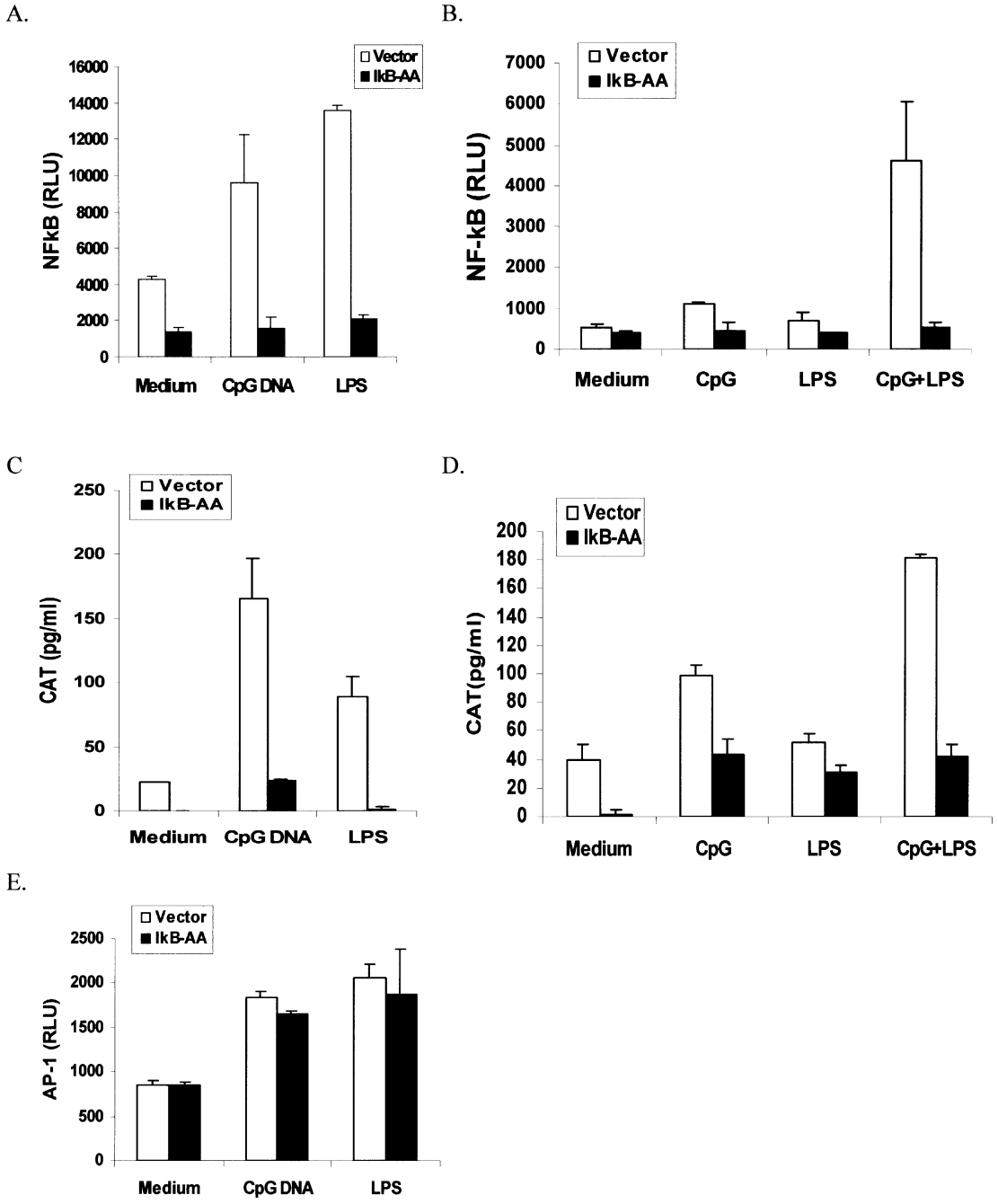

Fig. 6. Overexpression of super-suppressive mutant IκBα (IκBα-AA) inhibits CpG DNA- and LPS-induced NF-κB and TNF-α promoter activities. (A and B) RAW264.7 cells were transiently co-transfected with NF-κB–luciferase + pRL-TK–luciferase constructs and IκBα-AA (solid bar) or control empty vector (empty bar). (C and D) RAW264.7 cells were transiently co-transfected with 5′ TNF-α–CAT constructs, RSV–luciferase and IκBα-AA (solid bar) or control empty vector (empty bar). (E) RAW264.7 cells were transiently co-transfected with AP-1–β-galactosidase constructs and IκBα-AA (solid bar) or control empty vector (empty bar). Transfected cells were pooled and washed 3 times with culture media, and then incubated for 6 h. For (A), (C) and (E), cells were stimulated with medium, CpG DNA (3 μg/ml) or LPS (50 ng/ml) for 12 h. For (B) and (D), cells were stimulated with medium, CpG DNA (0.6 μg/ml), LPS (5 ng/ml) or CpG DNA (0.6 μg/ml) + LPS (5 ng/ml) for 12 h. NF-κB–luciferase activity in cell extracts was analyzed by the Dual-Luciferase reporter assay system and normalized using pRL-TK–luciferase activity in each sample. For 5′ TNF-α–CAT, the levels of CAT protein in cell extracts was analyzed using CAT-ELISA and parts of cell extracts were used for luciferase assay to detect luciferase activities in each sample. The levels of CAT protein were normalized based on luciferase activities in each sample. In some replicated experiments, the levels of CAT protein in the cell extracts were normalized to equal concentration of total protein in each sample and gave virtually the same results as normalized using luciferase activities in the cell extracts. AP-1–β-galactosidase activities in cell extracts were analyzed using Galacto-Light Plus reporter gene assay for β-galactosidase and normalized by equal concentrations of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was performed 2–4 times with similar results.

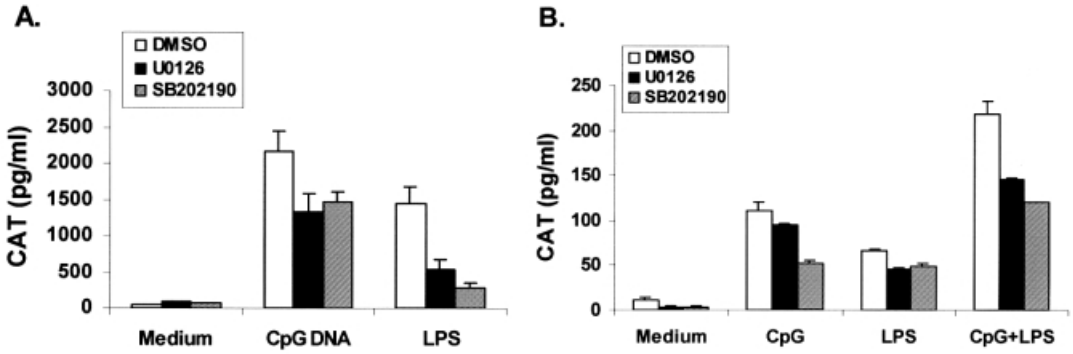

Fig. 7. CpG DNA-mediated TNF-α transcription is partly regulated by ERK and p38. RAW264.7 cells were transiently transfected with the murine TNF-α–CAT construct. Transfected cells were pooled and washed 3 times with culture media. For (A), cells were stimulated with medium, CpG DNA (3 μg/ml) or LPS (50 ng/ml) for 12 h. For (B), cells were stimulated with medium, CpG DNA (0.6 μg/ml), LPS (5 ng/ml) or CpG DNA (0.6 μg/ml) + LPS (5 ng/ml) for 12 h. The levels of CAT protein in cell extracts were analyzed using CAT-ELISA and normalized by equal concentrations (50 μg/sample) of cell lysates used in each sample. Data present the mean ± SD of triplicates. The experiment was performed 2–4 times with similar results.

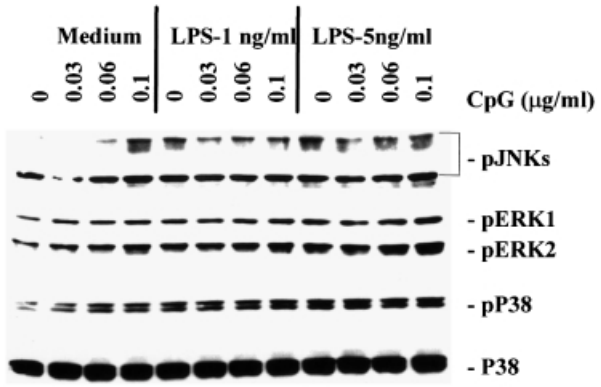

Fig. 8. CpG DNA slightly enhances LPS-mediated ERK but not JNK or p38 phosphorylation. RAW264.7 cells (2×10^6 cells/ml) were stimulated with medium, CpG or non-CpG DNA (0–0.1 μg/ml) in the presence (1 or 5 ng/ml) or absence of LPS for 30 min. Equal amounts of whole-cell lysates (50 μg/lane) were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS (SDS–PAGE) and then Western blots were performed using a specific antibody against the phosphorylated form of JNK (pJNK), ERK (pERK1 and pERK2) or p38 (pP38). Total p38 (P38) in each sample was used as the equal loading control. The sample blot was used for each different antibody after stripping of the previous antibody. The experiment was performed 3 times with similar results.

DMSO, U0126 or SB202190. As anticipated, CpG DNA and LPS induced activation of TNF-α transcriptional promoter, 5′ TNF-α–CAT, and U0126 and SB202190 partially inhibited activation of 5′ TNF-α–CAT induced by low doses of CpG DNA plus LPS (Fig. 7B). However, CpG DNA- and LPS-mediated agonistic effects on the TNF-α transcriptional promoter activation were not inhibited (Fig. 7B). These results indicated that even though they are involved in the regulation of TNF-α biosynthesis induced by either CpG DNA or LPS alone (33–35,45), MAPK may have little or no contribution to the synergistic effects of CpG DNA and LPS for production of TNF-α at the transcriptional level. Our results also suggested that, at least in part, CpG DNA and LPS might utilize common pathways to activate MAPK in RAW 264.7 and J774 cells.

Discussion

Over the past few years, CpG motifs in bacterial DNA have received tremendous attention for their strong innate immune stimulatory effects. Because of their abilities to induce T_h1-type responses, and to strongly activate immune cells such as B lymphocytes, NK cells and monocytic cells, the utility of CpG DNA as a therapeutic agent has been vigorously studied and well documented (reviewed in 46,47). However, CpG DNA could have detrimental effects in the body under certain pathologic circumstances such as overwhelming bacterial infection. Recent studies have demonstrated that high doses of CpG DNA can induce SIRS or inflammatory arthritis under certain experimental conditions (6–8,48, and Waldschmidt and Krieg, unpublished data). In addition, low concentrations of CpG DNA enhance LPS-mediated SIRS and septic arthritis (6,8). Furthermore, CpG DNA and LPS also act in synergy for nitric oxide production, which might play an important role in SIRS (49). The proinflammatory cytokine TNF-α has also been known to up-regulate nitric oxide production (50). Interestingly, TNF-α produced by monocytic cells has been demonstrated to play a critical role in CpG DNA-induced SIRS and inflammatory arthritis (6,8,48). Moreover, previously we showed that priming with bacterial DNA dramatically enhances LPS-induced secretion of several cytokines, including TNF-α and IL-6, and can promote the SIRS (6). These results suggest that synergistic induction of SIRS and inflammatory arthritis by low doses of CpG DNA and LPS might be due to the production of large amounts of TNF-α by both innate stimulators through separate and/or shared pathways. In the present study, the murine monocytic cell lines RAW264.7 and J774 were used to investigate some aspects of the molecular mechanisms mediating the synergistic effects of CpG DNA and LPS on TNF-α biosynthesis.

TNF-α is the first cytokine produced by leukocytes upon stimulation by CpG DNA (Yi and Krieg, unpublished data). The levels of TNF-α mRNA in the spleen were dramatically increased within 30 min, and the serum levels of TNF-α protein were detected within 30 min and peaked within 1 h after the injection of CpG DNA in BALB/c mice (Yi and Krieg, unpublished data). As seen *in vivo*, CpG DNA is a strong TNF-α inducer in RAW264.7 cells. Like LPS, CpG DNA induced TNF-α secretion in RAW264.7 cells in a dose-dependent manner at concentrations as low as 0.03 μg/ml (~0.005 μM) (Fig. 1A). In addition, a suboptimal concentration (5 ng/ml) of LPS synergizes with a broad concentration range (0.03–1 μg/ml) of CpG DNA for TNF-α production. Likewise, a suboptimal concentration (0.06 μg/ml) of CpG DNA synergizes with a broad dose range of LPS (0.1–100 ng/ml) for TNF-α production (data not shown). This synergistic effect of CpG DNA and LPS for TNF-α production was also seen in another murine monocytic cell J774 (Fig. 1). Of note, synergy between CpG DNA and LPS was seen not only for TNF-α production, but also for production of many other cytokines, including IL-6, IL-10, IL-12 and IFN-γ, nitric oxide production, and B cell proliferation and Ig secretion (Figs 1 and 2) (49, and Yi and Krieg, unpublished data). Taken together, these data suggest the possibility that the innate immune response might have evolved synergistic activation to low LPS and CpG DNA concentrations to increase sensitivity to small infections. At higher concentrations of each agent, the magnitude of the synergy is reduced, which may help to reduce the risk of inducing SIRS. These synergistic effects at low concentrations also indicate that CpG DNA and LPS act through, at least in part, distinct intracellular biochemical signaling pathways.

Synergistic effects by simultaneous exposure to different bacterial components is not unique for CpG DNA and LPS. Several other reports demonstrated that bacterial products which are recognized by TLR-2, such as bacterial lipopeptides and lipoproteins, also synergize with LPS for inflammatory cytokine production (51–53). In addition to these synergistic actions, lipopeptides and LPS induce cross-tolerance (53). Similar to this, pretreatment of CpG DNA also induces tolerance to later LPS-induced iNOS and TNF-α production (49,54). However, LPS pretreatment is less effective at induction of tolerance to CpG DNA (49,54). Molecular mechanisms of synergy and cross-tolerance between CpG DNA and LPS are poorly understood at the present time and of great interest.

Biosynthesis of TNF-α requires two types of signals. One signal induces transcription of its promoter and the second signal releases the translational repression imposed on the AU-rich region in the 3′-untranslated region (3′-UTR) of its mRNA (43,45,55). Previous studies have demonstrated that LPS-mediated biosynthesis of TNF-α is regulated at both the transcriptional and translational levels through activation of the transcription factor NF-κB, and MAPK including ERK and p38 (49,56). Like LPS, CpG DNA has been shown to activate NF-κB and MAPK within 30 min after stimulation in leukocytes (13,19,33,34,57). CpG DNA induces activation of both the TNF-α transcriptional reporter (5′ TNF-α–CAT) and the functional reporter, which is 3′-UTR of TNF-α linked to the 5′ TNF-α–CAT reporter gene (5′ TNF-α–CAT–3′-UTR) (Fig. 3 and data not shown) indicating the ability of CpG DNA to induce TNF-α biosynthesis at both the transcriptional and the translational levels. In addition, our data demonstrated that low concentrations of CpG DNA and LPS additively induce TNF-α mRNA expression and activation of the TNF-α transcriptional reporter, 5′ TNF-α–CAT, indicating that at least part of the synergistic increase in production of TNF-α by these stimuli takes place at the transcriptional level (Fig. 3). While we were revising this manuscript, Morrison and colleagues reported that bacterial DNA and LPS synergize for TNF-α production at the post-transcriptional level but not at the transcriptional level (54). However, this conclusion was based on indirect evidence from quantitating TNF-α mRNA levels by RT-PCR which is relatively insensitive to small differences, and they did not include studies to more directly assess the activity of the TNF-α promoter, as in our present study. In addition, those authors did not examine the effects of CpG and LPS on transcription factor function. While other differences between our experimental systems could also contribute to our different results, our data nevertheless support the conclusion of at least additive effects of CpG DNA and LPS on the TNF-α promoter.

Additive or synergistic activation of TNF-α transcription induced by low concentrations of CpG DNA and LPS could be due to activation of different transcription factors which up-regulate TNF-α expression and/or due to synergistic activation of one transcription factor by both CpG DNA and LPS. Both CpG DNA and LPS induce activation of the transcription factors AP-1, NF-κB and NF-IL-6 in murine and human leukocytes (13,19,34,48,57–59, and Yi and Krieg, unpublished data). Consensus binding sites for the transcription factors AP-1, NF-AT, NF-κB and NFIL-6 are present in the TNF-α promoter (60–62). Both CpG DNA and LPS at optimal concentrations induce nuclear DNA binding activity of AP-1 and NF-κB (p50/p65 heterodimers) in RAW264.7 cells (data not shown). Neither CpG DNA nor LPS induced the transcriptional activity of NF-AT in RAW264.7 cells in our experimental conditions (data not shown). At a substimulatory concentration, CpG DNA and LPS synergistically induced DNA binding activity of p65/p50 NF-κB heterodimers but not AP-1, NF-AT or NF-IL-6 (Fig. 4A). This synergistic induction of DNA binding activity of nuclear NF-κB was also observed with high concentrations of CpG DNA and LPS treatments (Fig. 4B). In addition to synergistically inducing the DNA binding activity of nuclear NF-κB, various concentrations of CpG DNA and LPS also synergistically induced the transcriptional activity of NF-κB (Fig. 5). However, as for DNA binding activity, there was little or no significant enhancement in the AP-1 transcription activity by the same concentrations of CpG DNA and LPS (Figs 4 and 5). This synergistic NF-κB activation by CpG DNA and LPS suggests the presence of signaling pathways that are unique for each stimulus in addition to the known shared signaling pathway through MyD88 (31). Interestingly, recent studies demonstrated that CpG DNA, but not LPS, activates NF-κB through a wortmannin-sensitive DNA-PKcs-dependent pathway (32). Furthermore, wortmannin greatly suppresses TNF-α production induced by CpG DNA while it shows no effects on LPS-induced TNF-α production in RAW264.7 cells (Yi and Choi, unpublished data), indicating the presence of additional CpG

DNA-triggered unique signaling pathways for NF-κB activation and subsequent TNF-α production.

It has been demonstrated that NF-κB activation plays a critical role in the TNF-α production induced by many stimuli including LPS (44,63). In addition, it has been speculated that CpG DNA-induced TNF-α production is due to its ability to activate NF-κB (57). Previous studies by us using pharmacological inhibitors of NF-κB also suggested a correlation between CpG DNA-induced NF-κB activation and TNF-α production (19). We now demonstrate directly that overexpression of a super-supressive IκBα mutant in RAW264.7 cells greatly, but not completely, inhibits the TNF-α promoter activity induced by CpG DNA (Fig. 6C). These demonstrate the critical role of NF-κB activation in TNF-α biosynthesis at the transcriptional level. Furthermore, our study shows that CpG DNA and LPS-mediated synergistic activations of NF-κB and TNF-α promoter are also abolished by over-expression of IκBα-AA (Fig. 6B and D). These results are compatible with the hypothesis that cooperative induction of TNF-α promoter activity by CpG DNA and LPS might be due to the synergistic activation of NF-κB by these stimuli.

Of note, CpG DNA-mediated transcriptional activity of NF-κB was completely inhibited by over-expression of IκBα-AA (Fig. 6A), while AP-1 activation is unaffected (Fig. 6E), demonstrating the functional and specific inhibitory activities of IκBα-AA. Compared to this, there is always residual CpG DNA-induced TNF-α promoter activity that is not inhibited by over-expression of IκBα-AA under the same conditions (Fig. 6C), indicating NF-κB may be the major but not the sole transcription factor involved in the CpG DNA-induced TNF-α transcription in RAW264.7 cells. Recent studies have demonstrated regulatory effects of MAPK on the production of various cytokines through an activation of transcription factors, such as AP-1 and NF-κB, and/or a signal which releases the translational repression factor which acts on the AU-rich region in the 3′-UTR of cytokine mRNA (49,56,64). LPS-mediated ERK and p38 activation have been demonstrated to be involved in TNF-α biosynthesis at both the transcriptional and translational levels (49,56). Recently, it has been reported that pharmacological inhibitors of MEK, which is an upstream kinase of ERK, or of p38 suppress CpG DNA-induced TNF-α production, indicating the important role of MAPK in CpG DNA-mediated signaling pathways (33–35). As in the LPS response, U0126, a MEK inhibitor, or SB202190, a p38 inhibitor, partially suppress CpG DNA-induced TNF-α promoter activation (Fig. 7), suggesting the functional role of ERK and p38 in the TNF-α transcription induced by CpG DNA. However, in contrast to the present study, Hacker *et al.* (35) showed no significant inhibition of the CpG DNA-induced TNF-α transcription by PD98059, another MEK inhibitor, in RAW264.7 cells. This conflicting result between Hacker *et al.*'s and our study may be due to slightly different experimental conditions, different methods for analysis and/or different effects of inhibitors which block MEK activities through different mechanisms. The target transcription factors of p38 or ERK that mediate TNF-α transcription induced by CpG DNA are currently unknown. In our hands, U0126 at 2.5 μM, a submaximal concentration without affect on cell viability, slightly inhibits CpG DNA-induced transcriptional activities of NF-κB, while it greatly suppresses CpG DNA-induced transcriptional activities of AP-1. In contrast, SB202190 at 2.5 μM slightly suppresses CpG DNA-induced transcriptional activities of both NF-κB and AP-1. However, neither U0126 nor SB202190 inhibits DNA binding activities of nuclear NF-κB induced by CpG DNA in RAW264.7 cells (Yi and Krieg, manuscript in preparation). These studies suggest CpG DNA-induced NF-κB DNA binding activity is independent of either MEK or p38, but CpG DNA-induced NF-κB transcriptional activity is minimally dependent on p38 and MEK activation. As pointed out previously, addition of CpG DNA slightly, but not significantly, enhances LPS-induced AP-1 activation (Fig. 5D), which might be correlated with the slight enhancement of ERK phosphorylation by LPS and CpG DNA in RAW264.7 cells. Currently, the role of AP-1 in the CpG DNA-mediated transcriptional activation of TNF-α is not yet fully understood.

Although the present study is focused on the synergistic regulation of TNF-α transcription via signals generated upon exposure to low concentrations of CpG DNA and LPS, the possibility of regulation of TNF-α biosynthesis at the translational level by ERK and p38 cannot be ruled out. Of note, CpG DNA does not activate ERK in J774 cells but does activate ERK in RAW264.7 cells (34). This lack of ERK activation by CpG DNA in J774 cells might be related to the lower level of TNF-α production in J774 cells compared to that in RAW264.7 cells. This also indicates that ERK activation may play a critical role in the CpG DNA-mediated TNF-α production at the post-transcriptional level in RAW264.7 cells. Furthermore, it is also possible that LPS-mediated ERK activation in J774 cells may contribute to the synergistic production of TNF-α by CpG DNA and LPS at the post-transcriptional level. Therefore, further investigation of the synergistic effects of CpG DNA and LPS on TNF-α production at the post-transcriptional level through ERK activation is of great interest.

Related to the observations that addition of LPS slightly enhances CpG DNA-mediated ERK phosphorylation without affecting phosphorylation of JNK and p38 in RAW264.7 cells (Fig. 8), CpG DNA failed to alter LPS-mediated ERK, JNK and p38 activation in J774 cells (data not shown). In addition, unlike LPS which strongly activated ERK, CpG DNA does not activate ERK in J774 cells (34). Of note, CpG DNA- and LPS-mediated synergistic effects of the TNF-α transcriptional reporter activation were not suppressed by inhibition of either ERK or p38 (Fig. 7B). Taken together, these observations suggest that CpG DNA and LPS might utilize some common pathways to activate MAPK, which is in agreement with recent reports using MyD88 gene-deficient mouse (31). However, these observations also suggest the presence of different signaling pathways for ERK activation by CpG DNA and LPS which are yet to be fully unrevealed.

In summary, the present study demonstrates that low concentrations of CpG DNA and LPS synergize for TNF-α production in the murine monocytic cell lines RAW264.7 and J774. This synergistic induction of TNF-α biosynthesis by CpG DNA and LPS is, at least partly, mediated at the transcriptional level though a synergistic activation of NF-κB which is demonstrated to play a critical role in the regulation of TNF-α transcription. In contrast to the synergistic activation of NF-κB, combinations of CpG DNA and LPS do not synergistically activate other upstream signaling modulators,

including JNK and p38, which activate AP-1. These results suggest the presence of distinct as well as shared common pathways for the activation of NF-κB and MAPK by LPS and CpG DNA.

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Abbreviations

CAT	chloramphenicol acetyl transferase
EMSA	electrophoretic mobility shift assay
ERK	extracellular receptor kinase
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MKK	mitogen-activated protein kinase kinase
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
TLR	toll-like receptor
TNF	tumor necrosis factor
UTR	untranslated region

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