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NF-κB-dependent regulation of tumor necrosis factor-α gene expression by CpG-oligodeoxynucleotides☆

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Abstract

Immunostimulatory activities of synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) have gained attention as potentially useful immunotherapeutics. However, CpG-ODNs induce harmful and lethal shock effects because they greatly enhance the sequence-dependent induction of tumor necrosis factor-α (TNF-α). We have shown that phosphorothioate-modified oligodeoxynucleotides (PS-ODNs) of the CpG-ODN 1826 stimulate TNF-α gene expression, TNF-α promoter activity, IκB degradation, and NF-κB activation at higher levels compared with its phosphodiester ODN (PO-ODN). In contrast to the effects of CpG-ODN 1826, PS-ODN of the CpG-ODN 2006 showed lower stimulatory activities than its PO-ODN. Using transient transfection, it was found that myeloid differentiation protein (MyD88) and tumor necrosis factor receptor-associated factor 6 are commonly required for activation of the TNF-α promoter by various CpG-ODNs with different potencies. These results strongly suggest a possibility to optimally activate the innate immune responses by modulating the potency of CpG-ODNs via sequence rearrangement and phosphorothioate backbone modification.

Keywords: CpG-ODN; Innate immunity; Macrophages; NF-κB; TNF-α

The innate immune system recognizes synthetic oligodeoxynucleotides (ODNs) and bacterial DNA containing unmethylated CpG dinucleotides in the context of particular base sequences (CpG-DNA) [1–3]. Synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODNs) mimic the direct immunostimulatory effects of native bacterial DNA and activate multiple cell types including macrophages, dendritic cells, NK cells, and B lymphocytes [1–6]. Optimally active CpG motifs vary from species to species. In mice, optimal immune responses were activated by the CpG motif in which a central unmethylated CpG dinucleotide is flanked by two 5′ purines and two 3′ pyrimidines [3]. For example, an ODN containing a 'GACGTT' hexameric nucleotide motif stimulates the mouse immune system. However, CpG-ODN, with strong immunostimulatory activity in mice, has relatively low activity on human immune responses [7]. Human peripheral blood mononuclear cells (PBMCs) respond poorly to CpG-ODNs that are optimally active in mice and the cells are potentially activated by ODN containing 'GTCGTT,' TTCGTT,' or 'AACGTT' motifs [8].

CpG-ODNs have gained attention because they have strong innate immune response stimulating effects. However, CpG-ODNs can enhance the sequence-dependent induction of large amounts of TNF-α in mouse macrophages [9–12]. Production of TNF-α in response to CpG-ODNs and to native bacterial DNA, a component of the structural patterns of microbial pathogens, is of particular clinical importance because TNF-α is a mediator of septic shock. The precise mechanism of TNF-α production by CpG-ODNs is not fully understood yet. In this study, we analyzed the molecular mechanism of CpG-ODNs in activating the TNF-α promoter.

☆ *Abbreviations:* ODN, synthetic oligodeoxynucleotide; CpG-ODNs, ODN containing CpG motifs; PO-ODN, phosphodiester ODN; PS-ODN; phosphorothioate-modified ODN.

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Recent studies from several laboratories have employed phosphorothioate-modified oligodeoxynucleotides (PS-ODNs) for clinical applications of CpG-ODNs [13–16]. PS-ODNs have a sulfur substitution for the non-bridging oxygen in the backbone providing nuclease resistance [17] and efficient uptake into cells [18,19]. There have been contrary effects of PS-ODNs and phosphodiester oligodeoxynucleotides (PO-ODNs) containing CpG motifs. To further elucidate the activation mechanisms of innate immune responses stimulated by PS- and PO-ODNs containing CpG motif, we have compared TNF-α gene expression, TNF-α promoter activation, IκBα degradation, and NF-κB activation induced by the actions of PS-ODN and PO-ODN of two sets of identical CpG-ODN sequences in RAW 264.7 cells, a mouse macrophage cell line. CpG-ODNs stimulate cells through the TLR/IL-1R signaling pathway, which results in activation of the transcription factor NF-κB that facilitates transcriptional up-regulation of genes downstream of the κB motif [5,20–22]. In this study, we also examined involvement of myeloid differentiation protein (MyD88) and tumor necrosis factor receptor-associated factor 6 (TRAF6) in CpG-ODN-induced activation of the TNF-α promoter.

Materials and methods

Cell culture and reagents. RAW 264.7 cells, a mouse macrophage cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Viability was assayed using trypan blue dye exclusion and was typically greater than 95%. Cultures were maintained until passage 20 and then discarded. Anti-NF-κB p65 monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit IκBα polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). PO-ODN and PS-ODN were purchased from GenoTech (Taejon, Korea). The CpG-ODN sequences (Table 1) were phosphodiester(O) or phosphorothioate(S) modified. ODN 1826(S), ODN 2006(S), and ODN 2041(S) are the phosphorothioate version of ODN 1826(O), ODN 2006(O), and ODN 2041(O), respectively. The CpG-ODN 1826 consists of 20 bases containing two CpG motifs. ODN-1826(S-1), 1826(S-2), and 1826(S-3) are derivatives of the CpG-ODN 1826 sequence with one or two of the CpG sequences reversed to GpC (bold letter underlined, Table 1). The non-CpG-ODN 2041 served as a negative control. LPS content of ODN was <1 ng LPS/mg DNA as measured by Limulus amebocyte assay (Whittaker Bioproducts, Walkersville, MD). The expression vector for mutant IκBα protein (IκBα Super Repressor, IκBαSR) that cannot be phosphorylated on serines 32 and 36 was kindly provided by Dr. Harikrishna Nakshatri (Indiana University School of Medicine, Indianapolis, IN). The following plasmids expressing dominant negative mutant proteins were used for transfection: ΔMyD88 [23], ΔTRAF2 [24], and ΔTRAF6 [25].

Reverse-transcription PCR analysis. After cells were treated with LPS (100 ng/ml) or ODNs (3 μM, or as indicated in the individual experiments) for the indicated time periods, total RNA was extracted with MicroRNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Seven micrograms of total RNA was reverse-transcribed in the first-strand buffer containing 6 μg/ml oligo(dT) primer, 50 U StrataScript reverse transcriptase, 2 mM dNTP, and 40 U RNase inhibitor. The reaction was conducted at 42 °C for 1 h. One microliter of the cDNA synthesis was subjected to the standard PCR for 25 cycles by using primers as: mouse TNF-α, 5′TCTCATCAGTTCTATGGCCC3′ (sense) and 5′GGGAGTAGACAAGGTACAAC3′ (anti-sense) (231 bp); mouse GAPDH, 5′ATGGTGAAGGTCGGTGTGAACG3′ (sense), 5′GTTGTCATGGATGATCTTGGCC3′ (anti-sense) (501 bp). PCR products were resolved on a 1% agarose gel and visualized with UV light after stained by ethidium bromide.

Table 1
Oligodeoxynucleotides used in this study

ODN	Sequence	Modification
1826(O)	TCCATGACGTTCCTGACGTT	PO
1826(S)	TCCATGACGTTCCTGACGTT	PS
1826(S-1)	TCCATGA**GC**TTCCTGACGTT	PS
1826(S-2)	TCCATGACGTTCCTGA**GC**TT	PS
1826(S-3)	TCCATGA**GC**TTCCTGA**GC**TT	PS
2006(O)	TCGTCGTTTTGTCGTTTTGTCGTT	PO
2006(S)	TCGTCGTTTTGTCGTTTTGTCGTT	PS
2041(S)	CTGGTCTTTCTGGTTTTTTTCTGG	PS

The CpG motif is underlined; reversal of the CpG dinucleotide to GC dinucleotide is indicated in bold. PS, phosphorothioate backbone modification; PO, phosphodiester.

Construction of luciferase reporter plasmid. The mouse TNF-α promoter fragment −695 to +240 was amplified by polymerase chain reaction using mouse genomic DNA (Clontech, Palo Alto, CA) as a template with the following primer sets: 5′ primer, 5′TGAGCTCATGATCAGAGTGAAAGGAGAA3′; 3′ primer, 5′CCTCGAGTTCTGGAAGCCCCCCATC3′. This fragment was ligated into *Sac*I and *Xho*I sites of the luciferase reporter plasmid pGL3-Basic vector (Promega, Madison, WI), yielding the reporter construct pmTNF-α-*Luc*. The nucleotide sequence of the pmTNF-α-*Luc* construct was verified by DNA sequencing.

Transfection and luciferase assay. RAW 264.7 cells were placed into six-well plates 1 day before the transfection at a concentration of 5×10^5 cells/well. Cells were used for transfection with FuGene 6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's instructions in DMEM with 10% FBS. Equivalent transfection efficiency was confirmed by cotransfecting the *Renilla* luciferase vector. After transfection, cells were placed in complete medium for 24 h prior to treatment with LPS (100 ng/ml) or ODNs (3 μM) for 8 h or as indicated in the individual experiments. Cells were harvested, washed, and lysed by freeze–thawing three times, and luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturer's specifications. Individual assays were normalized for *Renilla* luciferase and data were presented as the fold-increase in activity relative to control. Data were from two or three independent experiments performed in duplicate or triplicate with similar results. Standard errors are indicated.

Indirect immunofluorescence assays and confocal microscopy. The IκBα degradation and NF-κB p65 nuclear localization were detected by indirect immunofluorescence assays using confocal microscopy as described previously [26]. Raw 264.7 cells (5×10^4) were cultured directly on glass coverslips in 24-well plates. Twenty-four hours later, cells were treated with LPS (100 ng/ml) or ODNs (3 μM). After an additional 40 min, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with a solution of PBS, 15% normal donkey serum (Sigma, St. Louis, MO), and 0.2% Tween 20. Monoclonal antibodies to NF-κB p65 (1 μg/well) were

applied for 1 h followed by 1 h incubation with fluorescein isothiocyanate (FITC) or Texas Red (TR)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). DNA staining (0.5 μg/ml Hoechst No. 33258; Sigma, St. Louis, MO) was used to identify cell nuclei. For double immunofluorescence staining of NF-κB p65 and IκBα, primary rabbit polyclonal antibody that recognizes the IκBα (1 μg/well) was used and detected with goat anti-rabbit IgG antibody linked to FITC. Coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Samples were scanned with a Zeiss LSM 510 laser scanning confocal device attached to an Axiovert 100 microscope using a Plan-Apochromat 100X/Oil DIC objective (Carl Zeiss, Germany).

Results

Induction of TNF-α and activation of TNF-α promoter in CpG-ODN 1826-treated mouse macrophages

To investigate whether ODN derivatives containing CpG motifs stimulate the synthesis of TNF-α, PS- and PO-ODNs of the CpG-ODN 1826 sequence [CpG-ODN 1826(S) and CpG-ODN 1826(O)] were compared for their ability to induce the endogenous TNF-α gene expression in mouse RAW 264.7 cells. Reverse-transcription PCR assay was used to examine the CpG-ODN-induced mouse TNF-α mRNA expression. As shown in Fig. 1A, the TNF-α gene was not expressed in untreated cells. Addition of CpG-ODN 1826(O) induced TNF-α mRNA in 2 h and the levels subsequently declined over the remaining time points. However, the expression level was very low compared with the results obtained in CpG-ODN 1826(S)- and LPS-induced experiments. CpG-ODN 1826(O) induced expression of the TNF-α mRNA in a dose-dependent manner (Fig. 1B). TNF-α mRNA induction reached its maximum level at 3 μM CpG-ODN 1826(O) when the cells were stimulated for 2 h. CpG-ODN 1826(S) induced TNF-α mRNA in 30 min attaining the peak message level by 1 h after stimulation, and the expression was persistent up to 12 h which is in contrast to the case of CpG-ODN 1826(O) stimulation. We also examined TNF-α mRNA expression in LPS-treated cells to compare the expression kinetics. Following LPS treatment, the expression was induced within 30 min, peaked at 2 h, diminished at 4 h, and returned to its basal level by 12 h.

To define the contribution of CpG-ODNs to the activation of TNF-α promoter, we employed the mouse TNF-α promoter–reporter construct containing 935 bp 5′ of the transcription start site, linked to a luciferase gene. This construct was transiently transfected into RAW 264.7 cells, which were treated with CpG-ODNs (3 μM) or LPS (100 ng/ml). CpG-ODN 1826(S) was able to activate the mouse TNF-α promoter in a time-dependent manner (Fig. 1C). Peak expression level of the luciferase was reached at 8 h after CpG-ODN 1826(S) stimulation, and the luciferase activity returned almost to its level of unstimulated cells at 16 h. In contrast, the CpG-ODN 1826(O) sequence influenced TNF-α promoter activity at a much lower level compared with the activity in the cells stimulated by CpG-ODN 1826(S).

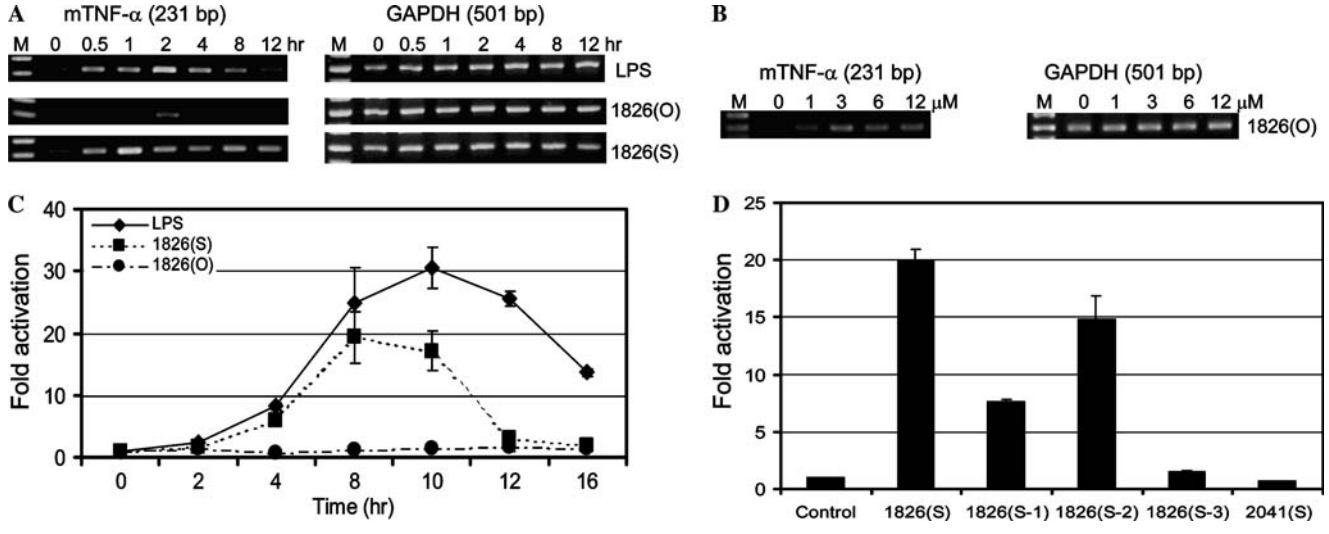

Fig. 1. CpG-ODN 1826 stimulates induction of TNF-α and activation of TNF-α promoter in RAW 264.7 cells. (A,B) Expression of mouse TNF-α in CpG-ODN 1826(S)- and LPS-treated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) or CpG-ODNs (3 μM) for the indicated time periods (A) or increasing amounts of CpG-ODN 1826(O) for 2 h (B), lysed, and total RNA was extracted. Expression of TNF-α mRNA (left panel, 231 bp) was analyzed by PCR following reverse transcription. GAPDH expression level was used as a control (right panel, 501 bp). The PCRs were performed for 25 cycles. M, DNA standard markers. (C,D) Effect of LPS (100 ng/ml), CpG-ODN 1826, and 1826 derivatives on transcriptional activity of the mouse TNF-α promoter. RAW 264.7 cells were transiently transfected with TNF-α promoter–luciferase construct for 24 h. The cells were then stimulated with LPS (100 ng/ml), or CpG-ODNs (3 μM) for different periods of time (C). Eight hours before harvest, cells were treated with 3 μM of CpG-ODNs (D). Luciferase assays were performed as described under "Materials and methods." Experiments (C and D) are one representative of three independent experiments with similar qualitative results.

Effect of the sequence of CpG-ODN 1826(S) on TNF-α promoter activity and NF-κB activation

To better evaluate the individual contribution of the CpG dinucleotide sequence to the activity of the mouse TNF-α promoter, we synthesized PS-ODNs of CpG-ODNs 1826(S-1), 1826(S-2), and 1826(S-3) which differ only in having reversal of one or two CpG dinucleotides to GpC dinucleotides (bold letter, underlined, Table 1) of the CpG-ODN 1826(S) sequence. As shown in Fig. 1D, luciferase activity was decreased by approximately 25% when the cells were treated with CpG-ODN 1826(S-2) compared to the activity in CpG-ODN 1826(S)-treated cells. CpG-ODN 1826(S-1) induced a much lower level of the luciferase activity (~60% reduction) than CpG-ODN 1826(S). CpG-ODN 1826(S-3), having reversal of the two CpG dinucleotides to GpC dinucleotides (bold letter, underlined, Table 1) of the CpG-ODN 1826(S) sequence, was not able to activate TNF-α promoter. Basal luciferase activity was detected in the cells stimulated by non-CpG PS-ODN 2041(S). These results indicate that the CpG-ODN 1826(S) sequence having two CpG motifs induced maximal TNF-α promoter activation in RAW 264.7 cells. Furthermore, the particular CpG-ODN 1826(S) sequence is capable of activating the mouse macrophage cell line in a CpG sequence-dependent manner.

Since CpG-ODN 1826(S) and CpG dinucleotide-modified PS-ODNs [1826(S-1) and 1826(S-2)] activated TNF-α promoter, we examined whether nuclear localization of NF-κB p65 is modulated by these CpG-ODNs. In the absence of any cell stimulation, NF-κB p65 is present in the cytoplasm as an inactive, IκBα-bound complex (Table 2 and Fig. 3). After stimulation of the cells with CpG-ODN 1826(S) for 40 min, activation of NF-κB p65 is mediated by signal-induced degradation of IκBα, which allows released NF-κB p65 to translocate to the nucleus (Table 2 and Fig. 3). Treatment with CpG-ODN 1826(S-1) and 1826(S-2) led to the partial nuclear accumulation of NF-κB p65 (Table 2) in some populations of cells. CpG-ODN 1826(S-1) treatment induces NF-κB p65 nuclear accumulation in much smaller populations of the cells than in the case of CpG-ODN 1826(S-2). Both PS-ODN 1826(S-3) having reversal of the two CpG dinucleotides to GpC dinucleotides (bold letter, underlined, Table 1) of the CpG-ODN 1826(S) sequence and PS-ODN 2041(S) containing non-CpG motif had no effect on the nuclear localization of NF-κB p65. Consistent with the ability of CpG-ODN 1826(S) sequence to induce maximal TNF-α promoter activation, CpG-ODN 1826(S) led to maximal nuclear localization of NF-κB p65 in RAW 264.7 cells. These results suggest that CpG-ODN 1826(S) containing two CpG motifs stimulates NF-κB nuclear localization and TNF-α promoter activation of mouse macrophages in a CpG sequence-dependent manner.

Table 2
Nuclear localization of NF-κB p65 in CpG-ODN- and LPS-treated RAW 264.7 cells

Stimulations	% Cells
PBS	0
LPS	100
1826(O)	8
1826(S)	100
1826(S-1)	66.7
1826(S-2)	83.3
1826(S-3)	0
2006(O)	34.7
2006(S)	23.1
2041(S)	0

Cells were treated with CpG-ODNs (3 μM) or LPS (100 ng/ml) for 40 min and all cultures were fixed with 4% paraformaldehyde. NF-κB p65 localization was visualized with a confocal laser scanning microscope by indirect immunofluorescence using NF-κB p65 mouse monoclonal antibodies. The percentages of cells in which nuclear NF-κB p65 staining was stronger than cytoplasmic NF-κB p65 staining are shown in each condition. 55–86 cells were examined in each condition. Experiments were performed three times with similar results.

Effect of CpG-ODN 2006 on induction of TNF-α, TNF-α promoter activity, and NF-κB activation

To compare the functional properties of CpG-ODNs which are active in human or mouse, we synthesized PO-ODN and PS-ODN of CpG-ODN 2006 containing three CpG motifs (GTCGTT). As indicated in Fig. 1 and Table 2, CpG-ODN 1826(S) exhibits higher induction of TNF-α promoter and NF-κB activity than CpG-ODN 1826(O) does. However, it was revealed in Fig. 2 that PS-ODN of the CpG-ODN 2006 sequence influenced TNF-α promoter activity, TNF-α mRNA induction, and NF-κB p65 activation at lower levels than those of PO-ODN of the CpG-ODN 2006. As shown in Fig. 2A, addition of CpG-ODN 2006(O) induced TNF-α mRNA in 1 h and the expression was persistent up to 12 h. In CpG-ODN 2006(S)-treated RAW 264.7 cells, peak TNF-α expression level was observed about 2 h after the stimulation and the expression levels subsequently declined over the remaining time points. CpG-ODN 2006(S) induced expression of the TNF-α mRNA in a dose-dependent manner (Fig. 2B). TNF-α mRNA induction reached its maximum level at 3 μM CpG-ODN 2006(S) when the cells were stimulated for 2 h.

To define the contribution of PO- and PS-derivatives of the CpG-ODN 2006 sequence to the activation of the mouse TNF-α promoter, the TNF-α promoter–reporter construct was transiently transfected into RAW 264.7 cells and the luciferase activity was monitored. As shown in Fig. 2C, treatment of CpG-ODN 2006(O) at a concentration of 3 μM conferred about 5-fold activation over the basal transcription level. In the case of CpG-ODN 2006(S) addition, the luciferase activity was lower than

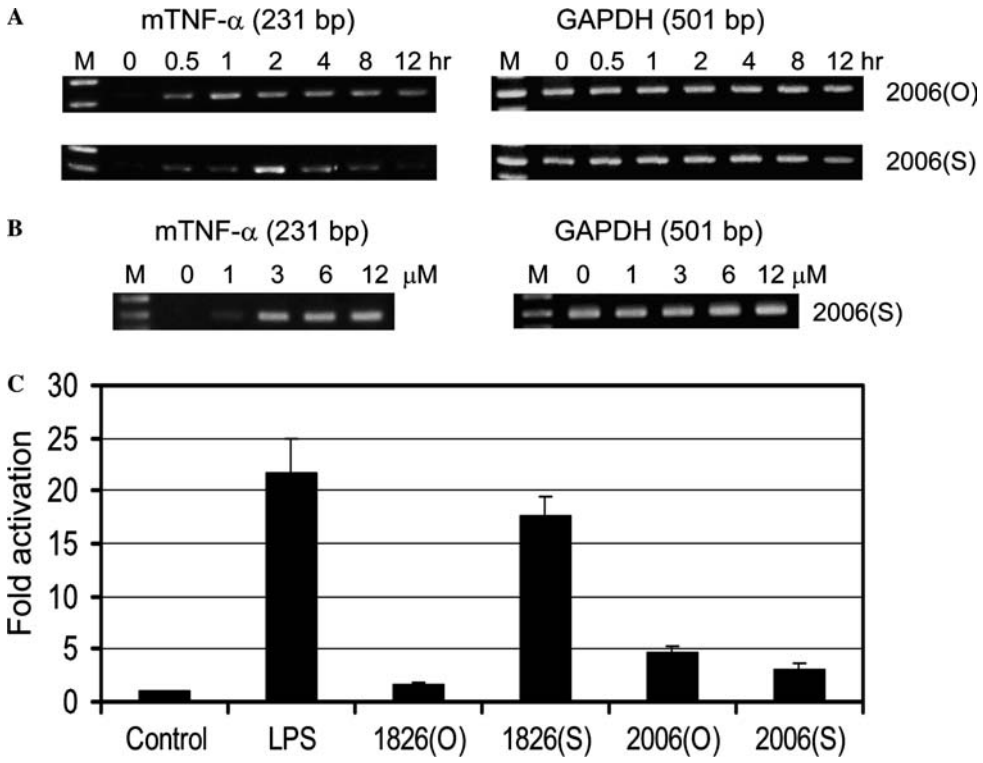

Fig. 2. Induction of TNF-α and activation of TNF-α promoter in CpG-ODNs-treated RAW 264.7 cells. (A,B) Expression of mouse TNF-α in CpG-ODN 2006s-treated RAW 264.7 cells. Cells were treated with CpG-ODNs (3 μM) for the indicated time periods (A) or increasing amounts of CpG-ODN 2006(S) for 2 h (B), and expression of TNF-α mRNA was analyzed by RT-PCR. M, DNA standard markers. (C) Effect of LPS and CpG-ODNs on the mouse TNF-α promoter activity. RAW 264.7 cells were transiently transfected with TNF-α promoter-luciferase construct for 24 h and then stimulated with LPS (100 ng/ml) or CpG-ODNs (3 μM) for 8 h. Cultures were harvested and assayed for luciferase activity.

that in the cells stimulated with CpG-ODN 2006(O). However, the CpG-ODN 1826(S) sequence did influence TNF-α promoter activity at a higher level compared with the activity in CpG-ODN 1826(O) stimulation.

Consistent with the ability of the CpG-ODN 2006 sequence to induce TNF-α promoter activation, CpG-ODN 2006(O) led to nuclear accumulation of NF-κB p65 in a partial population of the cells (Table 2). In contrast to the results obtained with CpG-ODN 1826 in Table 2, CpG-ODN 2006(S) induced NF-κB p65 nuclear accumulation in smaller population of the cells than CpG-ODN 2006(O) did. These results showed that PS-ODN of CpG-ODN 2006 active in human cells failed to efficiently stimulate NF-κB and TNF-α promoter activity in mouse macrophages. NF-κB p65 nuclear localization was also monitored in LPS-treated cells to compare its nuclear accumulation extent.

Association of signal-induced degradation of IκBα with nuclear accumulation of NF-κB in the CpG-ODN stimulated RAW 264.7 cells

To observe the CpG-ODN-induced NF-κB activation, indirect immunofluorescence was used to determine the signal-induced IκBα degradation and subcellular localization of NF-κB p65. Confocal images revealed that NF-κB p65 was normally sequestered in the cytoplasmic compartment by physical association with IκBα (Fig. 3, PBS panel). Degradation of IκBα and nuclear accumulation of NF-κB p65 were strongly induced 40 min after stimulation of the RAW 264.7 cells with CpG-ODN 1826(S) (Fig. 3, 1826(S) panel). In contrast, CpG-ODN 1826(O) had almost no such effect. In the case of CpG-ODN 2006(O) treatment, the stimulation led to the degradation of IκBα and nuclear accumulation of NF-κB p65, although to a lesser extent than in the case with CpG-ODN 1826(S). PS-ODN of the CpG-ODN 2006(S) sequence influenced degradation of IκBα and nuclear accumulation of NF-κB p65 at much lower levels compared with the potency of PO-ODN of the CpG-ODN 2006(O).

Effect of CpG sequences and phosphorothioate backbone modification in the CpG-ODNs on MyD88- and TRAF6-mediated TNF-α promoter activation

To determine if MyD88 is involved in CpG-ODN-induced TNF-α promoter activation, expression plasmid encoding a dominant negative version of MyD88 (ΔMyD88) was cotransfected into RAW 264.7 cells with

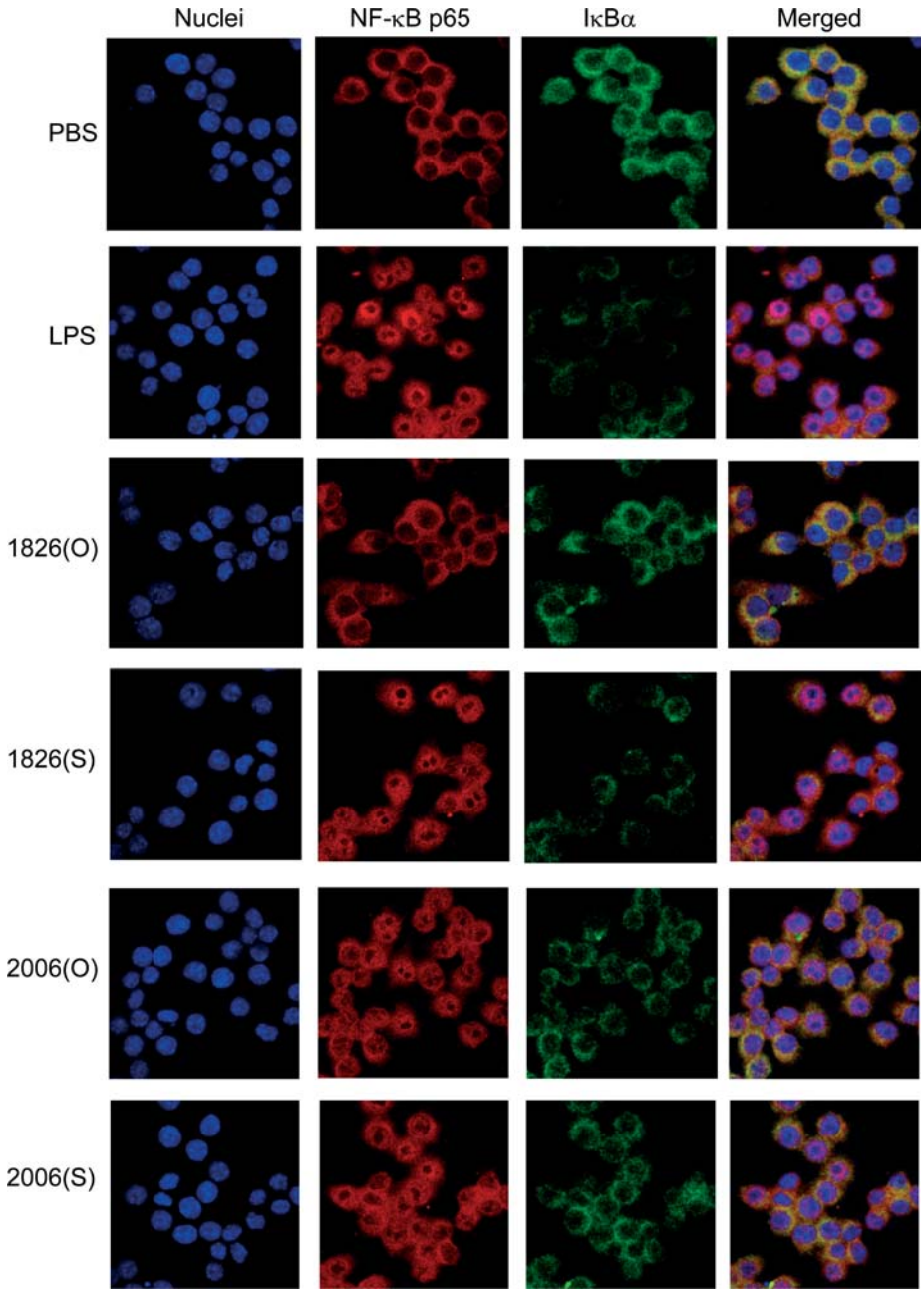

Fig. 3. Degradation of IκBα and nuclear localization of NF-κB p65 in CpG-ODNs stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/ml) or CpG-ODNs (3 μM) for 40 min. Cultures were fixed with 4% paraformaldehyde and processed for indirect immunofluorescence using NF-κB p65 (Red) and IκBα (Green) antisera, and images were visualized with a confocal laser scanning microscope. Cells were stained with Hoechst No. 33258 to visualize the nuclei (column labeled Nuclei).

a TNF-α promoter–reporter construct. The mutant, acting as a dominant negative molecule in TLR/IL-1R-dependent signaling, inhibited LPS- and CpG-ODN-mediated TNF-α promoter activation (Fig. 4).

It was attempted to examine the requirement for the TRAF6 in CpG-ODN-induced TNF-α promoter activation. As shown in Figs. 4A and B, ΔTRAF6 but not ΔTRAF2 significantly reduced LPS or CpG-ODN-induced activation of the TNF-α promoter. These data indicate that the signal transduction molecules MyD88 and TRAF6 in the TLR/IL-1R signaling pathway are required for induction of TNF-α gene expression in the mouse macrophage stimulated by LPS or CpG-ODNs. Similar inhibition of TNF-α promoter activity was observed in ΔMyD88 and ΔTRAF6 when the cells were treated with other CpG-ODNs (Figs. 4B–F).

Then, we examined whether TNF-α promoter activation is modulated by CpG-ODNs signaling-dependent IκBα degradation. When RAW 264.7 cells were transfected with a mutant IκBα protein (IκBα Super

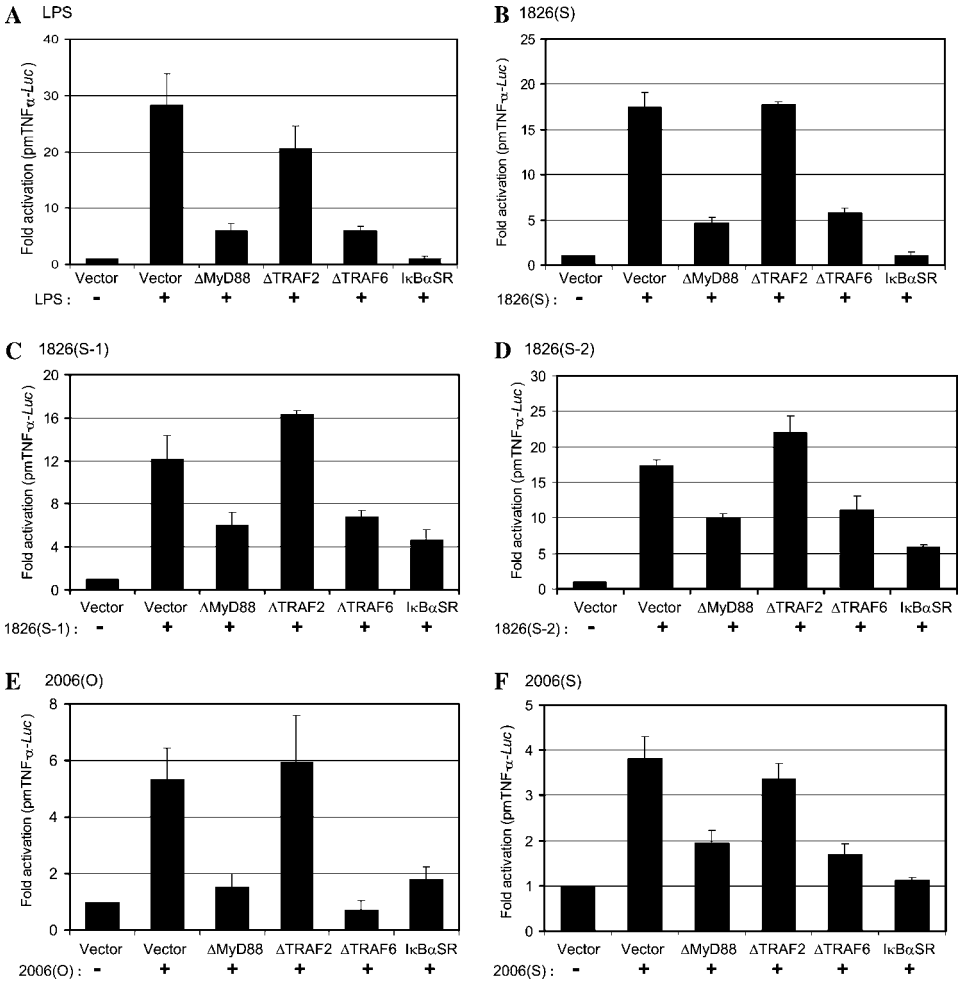

Fig. 4. CpG-ODN-induced TNF-α promoter activity in RAW 264.7 cells is inhibited by dominant negative mutants of MyD88 and TRAF6 but not by TRAF2. RAW 264.7 cells were transiently cotransfected with mouse TNF-α promoter–luciferase construct and control vector, or IκBαSR, dominant negative mutants of MyD88, TRAF2, and TRAF6 for 24 h. The cells were treated with 100 ng/ml LPS (A), or 3 μM of CpG-ODN 1826(S) (B), CpG-ODN 1826(S-1) (C), CpG-ODN 1826(S-2) (D), CpG-ODN 2006(O) (E), or CpG-ODN 2006(S) (F) for 8 h. Cells were harvested, and cell lysates were obtained by freeze–thaw. Luciferase activity was measured as relative light units (RLU), which was normalized to *Renilla* activity. The results are represented as fold activation compared with control vector alone.

Repressor, IκBαSR) which cannot be phosphorylated on serines 32 and 36, induction of TNF-α promoter activity was inhibited in the cells stimulated with CpG-ODNs, further confirming that degradation of IκBα is necessary for the CpG-ODN-induced TNF-α promoter activation (Figs. 4A–F).

Discussion

Synthetic oligodeoxynucleotides containing CpG motifs have been found to have potent immunostimulatory qualities of native bacterial DNA [1–6]. CpG-ODNs being used in several application studies were synthesized with a phosphorothioate backbone modification to resist nuclease digestion [13–16]. The phosphorothioate modification of ODN was necessary for the effective immunostimulatory actions to be seen in previous investigations [3,18,27–30]. However, phosphorothioate modification of the non-bridging oxygen atom on the phosphate group with a sulfur atom is associated with a number of side effects including massive lymphadenopathy [31], autoimmune disease [32,33], and granuloma formation [34].

This study contributes insights into the immunostimulatory effects of PS-ODN or PO-ODN containing CpG motifs on mouse macrophage cells. In previous studies, PS-ODN containing the CpG motif GACGTT (CpG-ODN 1826, Table 1) showed high activity on mouse B cells [20] and macrophages [35,36]. However, there have not been comparative studies about PO-ODN and PS-ODN of the CpG-ODN 1826 sequence in mouse macrophages. We now report the comparison of effects and mechanism of actions of PS-ODN and

PO-ODN of the CpG-ODN 1826 sequence on the TLR/IL-1R pathway-dependent TNF-α gene expression in mouse macrophage. PS-ODN of CpG-ODN 1826 strongly stimulates TNF-α mRNA expression and TNF-α promoter activation (Figs. 1A and C) in mouse macrophage. We observed that the stimulation of RAW 264.7 cells with CpG-ODN 1826(S) induced significant IκBα degradation and nuclear localization of NF-κB p65 (Fig. 3 and Table 2). However, the CpG-ODN 1826(O) was much less potent than CpG-ODN 1826(S) in inducing TNF-α gene expression, TNF-α promoter activation, IκBα degradation, and nuclear localization of NF-κB p65 (Figs. 1, 3, and Table 2). Therefore, it is evident that the phosphorothioate backbone modification plays a crucial role in the immunostimulatory functions of CpG-ODN 1826 sequence.

It was reported that CpG-ODNs are capable of activating B cells in a CpG sequence-independent manner [8,37]. We further analyzed in the mouse macrophage cell line whether CpG-ODN 1826(S) containing two CpG motifs activates NF-κB and TNF-α promoter in a CpG sequence-dependent manner. Here, we showed that CpG-ODN 1826(S) containing two CpG motifs induced maximal TNF-α promoter activity (Fig. 1D) and nuclear localization of NF-κB (Table 2) compared to CpG-ODNs in which the CpG dinucleotide sequences were replaced by GpC dinucleotides [CpG-ODN 1826(S-1) and CpG-ODN 1826(S-2)]. Non-CpG PS-ODNs (2041(S) and 1826(S-3)) did not enhance the activity of the TNF-α promoter (Fig. 1D) as well as the NF-κB nuclear localization (Table 2). These results clearly demonstrate that CpG motifs are key structural elements in exerting the immunostimulatory function of CpG-ODNs.

Innate immune recognition of CpG motifs varies between species. To date, mouse cells poorly respond to many PS-ODNs containing CpG motifs that are strongly active in the human system [7,8]. Consistent with a previous report by others [7], CpG-ODN 2006 that has strong immunostimulatory activity in human cells was observed to have moderate stimulatory effects on the activity of the TNF-α promoter and NF-κB nuclear localization in the mouse macrophage cell line. However, there have not been comparative studies of PO-ODN and PS-ODN of the CpG-ODN 2006 sequence in mouse macrophages. In contrast to the results of CpG-ODN 1826, CpG-ODN 2006(S) induced lower levels of TNF-α gene expression and TNF-α promoter activation than CpG-ODN 2006(O) (Fig. 2). Sester et al. [38] have suggested that the increased stability and uptake are likely to be responsible for the higher potency of PS-ODN in some assays. Thus, higher potency of PS-ODN of CpG-ODN 1826 in mouse macrophages may reflect this effect. However, they also suggested that PS-ODN failed to efficiently stimulate some responses, and delayed or poor activation of signaling components such as the ERK MAP kinases may contribute to these effects. Therefore, it is likely that the context of the CpG-ODN sequence affects differently immune cell activation in human and mouse as previously shown, and that phosphorothioate modification may modulate the activity of CpG-ODN depending on the sequence context. Mechanisms involved in these features are yet to be determined.

Exposure of macrophages and B cells to CpG-ODN results in activation of the MyD88 (myeloid differentiation protein)/IRAK (IL-1 receptor-associated kinase) pathway which is suggested to be a downstream effector of TLR9 (Toll-like receptor 9). The activation of TLR9 induces recruitment of the adaptor protein MyD88 and sequential activation of signaling molecules such as IRAK, TRAF6 (TNF receptor-associated factor 6), and IκB kinase (IKK) [39,40]. In this study, we demonstrated in RAW 264.7 cells that CpG-ODN-induced TNF-α promoter activation is mediated by TLR/IL-1R signaling molecules, namely MyD88 and TRAF6, but not the TNF signaling molecule, TRAF2 (Fig. 4). We also demonstrated that PS-ODNs and PO-ODNs of the two sets of CpG-ODN sequences (1826 and 2006) activate the TLR/IL-1R pathway-dependent TNF-α gene expression through similar intracellular mechanism in mouse macrophages. Therefore, CpG-ODNs appear to have common activation mechanisms which are independent of the potency. Taken together, our results suggest the possibility that finely tuned combinations of CpG sequence and phosphorothioate modification may trigger optimal innate immune response perhaps without severe side effects.

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