Myeloid differentiation factor 88-dependent post-transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: Tumor necrosis factor-α receptor-associated factor 6, a diverging point in the Toll-like receptor 9-signaling

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SUMMARY

The immune stimulatory unmethylated CpG motifs present in bacterial DNA (CpG DNA) induce expression of cyclooxygenase-2 (COX-2). The present study demonstrates that CpG DNA can upregulate COX-2 expression by post-transcriptional mechanisms in RAW264.7 cells. To determine the CpG DNA-mediated signaling pathway that post-transcriptionally regulates COX-2 expression, a COX-2 translational reporter (COX2-3’UTR-luciferase) was generated by inserting sequences within the 3’ untranslated region (UTR) of COX-2 to the 3’ end of the luciferase gene under control of the SV40 promoter. CpG DNA-induced COX2-3’UTR-luciferase activity was completely inhibited by an endosomal acidification inhibitor chloroquine, a TLR9 antagonist inhibitory CpG DNA, or overexpression of a dominant negative (DN) form of MyD88. However, overexpression of DN-IRAK1 or DN-TRAF6 resulted in substantial, but not complete, inhibition of the CpG DNA-induced COX2-3’UTR-luciferase activity. Activation of all three mitogen-activated protein kinases (MAPKs), ERK, p38, and JNK, was required for optimal COX2-3’UTR-luciferase activity induced by CpG DNA. Overexpression of DN-TRAF6 suppressed CpG DNA-mediated activation of p38 and JNK, but not ERK, explaining the partial inhibitory effects of DN-TRAF6 on CpG DNA-induced COX2-3’UTR-luciferase activity. Co-expression of DN-TRAF6 and N17Ras completely inhibited CpG DNA-induced COX2-3’UTR-luciferase activity, indicating the involvement of Ras in CpG DNA-mediated ERK and COX2-3’UTR regulation. Collectively, our results suggest that MyD88 and MAPKs play a key regulatory role in CpG DNA-mediated COX-2 expression at the post-transcriptional level and that TRAF6 is a diverging point in the TLR9-signaling pathway for CpG DNA-mediated MAPK activation.
INTRODUCTION

A motif of unmethylated CpG dinucleotides in a particular nucleotide context (CpG motif: GACGTT for murine, GTCGTT for human) in bacterial DNA is one of the newly identified pathogen associated molecular patterns (PAMPs), and is capable of activating cells of the immune system, including B lymphocytes, monocytes/macrophages and dendritic cells (DCs), that play a critical role in directing the host immune response to infection (reviewed in ref. 1). The ability of CpG motifs in bacterial DNA to activate innate immunity can be mimicked by synthetic oligodeoxynucleotides containing the CpG motif (CpG DNA). CpG DNA is endocytosed by leukocytes, acidified, and then recognized by a pattern recognition receptor (PRR), Toll-like receptor 9 (TLR9), in an endosomal compartment (2-4). Upon recognition of CpG DNA, TLR9 recruits the adaptor molecule myeloid differentiation factor 88 (MyD88) through an interaction between their C-terminal Toll/IL-1 receptor (TIR) domains. This recruitment of MyD88 to the TIR domain of TLR9 initiates a signaling pathway that sequentially involves IL-1R-associated kinases (IRAKs) and tumor necrosis factor-α receptor-associated factor 6 (TRAF6) (3-8). Studies using gene-deficient mice and RAW264.7 cells transiently transfected with the dominant negative (DN) forms of these molecules indicated that this MyD88-mediated signaling pathway is essential for the CpG DNA-induced activation of NF-κB and c-Jun NH2-terminal kinase (JNK) (3, 5, 8). In addition to MyD88, DNA activated protein kinase (DNA-PK) and intracellular reactive oxygen species (ROS) have also been demonstrated to be required for the CpG DNA-mediated activation of NF-κB and JNK, which in turn lead to the expression of various oncogenes and proinflammatory cytokines and mediators (2, 9).
The effects of CpG DNA on the immune system have been extensively studied. CpG DNA rapidly activates B cells to proliferate, to produce TNF-α, IL-6, IL-10 and immunoglobulin (Ig), and to express increased levels of costimulatory molecules (10-13). CpG DNA synergizes with B cell receptor (BCR)-mediated signals, amplifying Ag-specific responses including TNF-α, IL-6, IL-10 and Ig production and B cell proliferation, thus indicating its function as a co-stimulatory factor in the presence of specific Ag (10, 13, 14). In addition, CpG DNA rescues mature splenic B cells from spontaneous apoptosis and WEHI-231 cells and primary immature B cells from Ag receptor-mediated apoptosis (15-18, Yi and Krieg unpublished data). Addition to its profound effects on B cells, CpG DNA directly activates DCs and macrophages/monocytes to express increased levels of costimulatory molecules, and to increase antibody-dependent cellular cytotoxicity (ADCC) activity, antigen presentation and cross priming (19, 20). CpG DNA also leads to the production of various cytokines and chemokines including TNF-α, IFN-α/β, IL-6, IL-10, IL-12, MIP1α, MIP1β, IP-10 and RANTES (2, 21-25). In addition, recent studies have demonstrated that CpG DNA induces the release of arachidonic acid (AA), which is converted to prostaglandin E2 (PGE2) by cyclooxygenase (COX), and that CpG DNA induces the expression of COX-2 and the production of PGE2 (26-28).

Prostaglandins (PGs) play a critical role in several physiological and pathological processes including inflammatory responses, bone development, wound healing, reproductive function, and blood clotting (reviewed in refs. 29, 30). Dysregulated PG production occurs in chronic inflammation, arteriosclerosis, cardiovascular diseases, and various neoplastic diseases (reviewed in refs. 29, 30). The biosynthesis of PGs is tightly controlled by the activity of the COX enzymes that convert AA to PGs (31, 32). There are two isoforms of COX, COX-1 and COX-2, produced from differently regulated genes. COX-1 is constitutively expressed in most
cell types and is thought to be involved in the maintenance of physiological functions (33, 34).
In contrast, COX-2 is undetectable in normal tissues or resting immune cells, but is rapidly
induced by cytokines, growth factors, tumor promoters, and bacterial products such as LPS and
CpG DNA (26, 27, 35-38). Dysregulation of COX-2 gene expression is correlated with the
pathogenesis of inflammatory diseases, developmental events, and tumorigenesis (39-44).

The regulation of COX-2 expression occurs at both transcriptional and post-
transcriptional levels. Post-transcriptional regulation is critical in determining the strength and
duration of COX-2 gene induction by external stimuli, yet is poorly understood. The
3’untranslated region (UTR) of COX-2 is highly conserved across species and contains an AU-
rich element (ARE) with multiple repeats of the regulatory sequence AUUUA (45, 46).
AUUUA sequences are found in 3’UTRs of numerous unstable mRNAs and are regulatory sites
for mRNA stability (47). The 3’UTR AUUUA sequences present in COX-2 transcripts have
also been demonstrated to be involved in COX-2 mRNA stability and translational efficiency
(45, 46, 48). ARE/poly (U) binding factor 1 (AUF1) and CArG box-binding factor-A (CBF-A)
have been shown to interact specifically with the AU-rich RNA stability determinants in COX-2
transcripts (48, 49). Receptor-mediated signaling pathways that lead to the regulation of COX-2
mRNA stability have not been extensively studied. Three mitogen-activated protein kinase
(MAPK) family members, extracellular signal-regulated kinase (ERK), JNK, and p38, have been
reported to play an important regulatory role in mRNA stability of various cytokines and
oncogenes (48, 50-56). In particular, it has been demonstrated that activation of p38 leads to the
activation of MAPKAPK-2, which in turn activates heat shock protein 27 (HSP27). HSP27 has
been shown to be involved in COX-2 mRNA stability via AUF1 (48). Through the
TLR9/MyD88-signaling pathway, CpG DNA induces activation of all three MAPKs in
macrophages (3, 5). Of the three MAPKs activated by CpG DNA, p38, by leading to activation of the transcription factor cAMP-responsive element binding protein (CREB), plays a critical regulatory role in the CpG DNA-mediated transcriptional regulation of COX-2 expression (28). However, previous studies have not addressed whether CpG DNA induces COX-2 mRNA stabilization nor have they determined what are the relative contributions of different TLR9/MyD88-signaling mediators to CpG DNA-induced COX-2 post-transcriptional regulation in macrophages. In the present study, we have demonstrated that CpG DNA upregulates COX-2 expression, at least in part, through post-transcriptional regulation mechanisms that include mRNA stabilization. CpG DNA-mediated post-transcriptional regulation of COX-2 expression is dependent on the endosomal processing of CpG DNA and the TLR9/MyD88-dependent signaling pathway. In addition, our results demonstrated that all three MAPKs (ERK, p38, and JNK) are required for the CpG DNA-mediated post-transcriptional regulation of COX-2 expression and that TRAF6 only partially contributes to this post-transcriptional regulation process, due to its differential regulation of the different MAPKs. Furthermore, our results demonstrated that CpG DNA induces activation of the small GTP-binding protein Ras, and this CpG DNA-activated Ras, presumably through activation of ERK, positively regulates CpG DNA-mediated COX-2 expression at the post-transcriptional level.
METHODS AND MATERIALS

Oligodeoxynucleotides. Nuclease resistant phosphorothioate oligodeoxynucleotides (S-ODN) were purchased from Operon (Alameda, CA) and further purified by ethanol precipitation. S-ODN had no detectable endotoxins by Limulus assay. The sequences of S-ODN used are 5’TCCATGACGTTCCCTGACGTT3’ (CpG DNA: 1826), 5’TCCAGGACTTCTCTCAGGTT3’ (non-CpG DNA: 1982), and 5’TCCTGGCGGGGAAGT3’ (inhibitory CpG DNA: iCpG DNA: 2088).

Cell lines, culture conditions, and reagents. RAW264.7 cells (ATCC, Rockville, MD) were maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin and cultured at 37°C in a 5% CO2 humidified incubator. All culture reagents were purchased from Life Technologies (Gaithersburg, MD). LPS (Salmonella minnesota Re 595) was purchased from Sigma Chemical Co. (St. Louis, MO). LPS purity was confirmed by its inability to induce IL-6 production in macrophages isolated from the LPS-unresponsive C3H/HeJ mouse strain. SB202190, U0126, and SP600125 were purchased from Calbiochem (La Jolla, CA). Recombinant murine IFN-γ was purchased from R&D System (Minneapolis, MN).

Preparation of RNA and Real-time polymerase chain reaction (PCR). RAW 264.7 cells (2x10^6) were plated in 15 mm diameter dishes and then incubated for 24 h. Cells were stimulated with medium, CpG DNA (3 µg/ml) or non-CpG DNA (3 µg/ml) for designated time periods. In some experiments, RAW264.7 cells pretreated with CpG DNA (3 µg/ml) for 12 h were stimulated with medium, CpG DNA (3 µg/ml), or non-CpG DNA (3 µg/ml) for designated time periods in the presence of Actinomycin D (ActD; 10 µg/ml) to inhibit new mRNA transcription. Cells were harvested and total RNA was isolated by using RNeasy mini kit
(Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. Ten µg of total RNA served as the template for single strand cDNA synthesis in a reaction using oligo(dT) primers and M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. To measure the relative amount of COX-2 gene transcripts, amplification of cDNA was monitored with the fluorescent DNA binding dye SYBR Green (PE Applied Biosystems, Foster City, CA) in combination with the ABI 7900 sequence detection system (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Forward and reverse primers were designed using primer Express software (PE Applied Biosystems, Foster City, CA). GAPDH was used for an internal control. The sequences of real-time PCR primers used were: COX-2 sense, 5’-CAGAACCCGCATTGCCTCTG-3’ and COX-2 antisense, 5’-TTGAAGGTTGTCGGGCAGC-3’; GAPDH sense, 5’-TTCACCACCATGGAGAAGGC-3’ and GAPDH antisense, 5’-GGCATGGACTGTGGTCATGA. ActD was purchased from Sigma Chemical Co. (St. Louis, MO). All real time PCR primers used were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

**Plasmids.** The 3’UTR of COX-2 containing 763 base pairs was amplified by polymerase chain reaction (PCR) using 5’-TCTAGAGTTCAACTGAGCTGAAAGTCTG-3’ as a forward primer and 5’-TCTAGACCCAGCATTTTGGACTACAG-3’ as a reverse primer. The resulting 3’ UTR of COX-2 was ligated into pGEM-T easy vector (Promega, Madison, WI) and then subcloned into the 3’end of the luciferase gene in pGL3-control vector (Promega, Madison, WI) to generate the COX-2 translational reporter (COX2-3’UTR-luciferase). All PCR primers used for cloning were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Generation of expression constructs encoding a dominant negative form (DN) of IRAK-1 (amino
acids 1-215), DN-TRAF6 (amino acids 305-531), DN-p38, DN-JNK1, or DN-MEK1 were previously described (28). DN-MyD88 expression construct pIRES2-EGFP-DN-MyD88 was provided by Dr. S.-C. Hong (Indiana Univ., Indianapolis, IN). An AP-1-β-galactosidase construct, pCDNA3-JNK1 expression construct, and pCDNA3-N17Ras expression construct were provided by Dr. G. Koretzky (University of Pennsylvania, Philadelphia, PA).

**Transient Transfection and reporter gene assays.** RAW 264.7 cells (1.5x10^6 cells/plate) were plated into 60-mm cell culture plates and then incubated for 48 hrs to reach approximately 80% confluence. Cells were transfected with pRL-TK-luciferase (0.5 µg) and COX2-3’UTR-luciferase (1 µg) or pGL3-luciferase (1 µg), using LipofectAMINE Plus (Life Technologies, Gaithersburg, MD). In some experiments, cells were co-transfected with an equal amount (2 µg) of control empty vector (pIRES-EGFP, pCDNA3, or pUSE), DN-MyD88, DN-IRAK1, DN-TRAF6, DN-MEK1, DN-p38, or DN-JNK1 and COX2-3’UTR-luciferase (1 µg) plus pRL-TK-luciferase (0.5 µg) or AP-1-β-galactosidase (2 µg) and then incubated for 6 hrs. Transfected cells were pooled and washed 3 times with culture media. Cells (1x10^6 cells/15-mm cell culture plate) were stimulated with medium, CpG DNA (6 µg/ml), non-CpG DNA (6 µg/ml), IFN-γ (166 ng/ml), or LPS (50 ng/ml) for 12 h. In some experiments, transfected cells were pretreated with medium, iCpG DNA (6 µg/ml), Chloroquine (2.5 µg/ml), DMSO, U0126 (1.25 µM), SB202190 (2.5 µM), or SP600125 (5 µM) for 15 min before the stimulation. β-galactosidase and luciferase activities in cell extracts were analyzed according to manufacturer's protocol using Galacto-Light Plus Reporter gene assay (Tropix, Bedford, MA) and Dual-Luciferase Reporter Assay System (Promega, Madison, WI), respectively. Luciferase activity was normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. For AP1-β-galactosidase assay, equal concentrations of cell lysates were used.
Generation of DN-TRAF6 expressing RAW264.7 stable transfectants (TRAF6DN-RAW264.7). To generate RAW264.7 stable transfectants constitutively expressing DN-TRAF6, RAW264.7 cells (approximately 80% confluent in a 100 mm tissue culture dish) were transfected with pOPTRAF6DN constructs (30 µg) using LipofectAMINE PLUS (Life Technologies, Gaithersburg, MD). To generate vector control cell line, RAW264.7 cells (approximately 80% confluent in a 100 mm tissue culture dish) were transfected with pOPRSVI.mcs1 cloning vector (30 µg) using LipofectAMINE PLUS (Life Technologies, Gaithersburg, MD). Transfected cells (TRAF6DN-RAW264.7 cells and vector control cells) were selected and maintained in complete DMEM medium plus 500 µg/ml of geneticin (G418; Calbiochem, La Jolla, CA). pOPTRAF6DN construct and pOPRSVI.mcs1 cloning vector were provided by Dr. G. Bishop at University of Iowa (Iowa City, IA) (57).

Preparation of whole cell lysates and Western blot analysis. RAW264.7 cells (2 × 10^6 cells/ml) were pretreated with DMSO or SP600125 (5 µM) for 15 min and then stimulated with medium or CpG DNA (6 µg/ml) for 45 min. In some experiments, TRAF6DN-RAW264.7 (2 × 10^6 cells/ml) were stimulated with medium, CpG DNA (6 µg/ml), or PMA (50 ng/ml) for 45 min. Whole cell lysates were prepared as previously described (58). To detect the presence of a specific protein or the phosphorylation status of a specific protein, equal amounts (15 µg/lane) of whole cell lysates were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS and then western blots were performed as previously described (58). Actin was used as a loading control. Antibodies specific for actin and the FLAG epitope tag were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Eastman Kodak (New Haven, CT), respectively. Antibodies specific for the phosphorylated forms of ERK, JNK, or p38 were purchased from Cell Signaling (Beverly, MA).
**Ras activation assay.** Ras Activation Assay Kit was purchased from Upstate Inc. (Charlottesville, VA) and Ras activation assay was performed according to the manufacturer’s protocol. Briefly, RAW264.7 cells (1 x 10^7 cells) were stimulated with medium or CpG DNA (6 µg/ml) for the indicated time periods (0-30 min). Cells were harvested and lysed in Mg^{2+} lysis buffer (MLB buffer). GTP bound Ras (active Ras) in cell lysates was precipitated using bead-bound Raf1-RBD-GST fusion proteins that specifically bind to GTP-bound Ras. Precipitates were subjected to electrophoresis on a 14% polyacrylamide gel containing 0.1% SDS and then analyzed by western blot using an antibody specific for Ras.
RESULTS

CpG DNA regulates COX-2 expression at the post-transcriptional level. As recently demonstrated (26, 27), CpG DNA induced increases in the levels of COX-2 mRNA and protein in a murine macrophage-like cell line RAW264.7 (Fig. 1A and data not shown). Expression of COX-2 can be regulated at both transcriptional and post-transcriptional levels. We have investigated whether CpG DNA has the ability to regulate COX-2 expression post-transcriptionally by mechanisms including mRNA stabilization. RAW264.7 cells were pre-stimulated with CpG DNA for 12 h to induce COX-2 mRNA expression. Actinomycin D (ActD) was added to stop transcription and then cells were further incubated for the designated time periods in the presence of medium, CpG DNA, or control non-CpG DNA. Total RNA was isolated and COX-2 mRNA levels were analyzed using Real-time PCR. As demonstrated in Figure 1B, levels of COX-2 mRNA were substantially reduced within 6 h after ActD addition in cells incubated in either medium or control non-CpG DNA. The estimated relative half-lives ($t_{1/2}$) of COX-2 mRNA (extrapolated from Figure 1B) were approximately 4.4 h and 5.2 h in the cells treated with medium alone and with control non-CpG DNA, respectively. In contrast, levels of COX-2 mRNA were maintained up to 6 h and then slowly declined in cells incubated in the presence of CpG DNA ($t_{1/2} = 8.6$ h) (Fig. 1B). Compared to COX-2 mRNA levels in cells incubated with medium alone or control non-CpG DNA, COX-2 mRNA levels in the cells incubated with CpG DNA were substantially higher even at 9 h after ActD addition (Fig. 1B). These results indicate that CpG DNA may have the ability to transduce a signal that regulates COX-2 mRNA stability.

It has been reported that the 3’UTR of COX-2 contains multiple repeats of the regulatory sequence AUUUA, which is critical for post-transcriptional control of the gene, and that the first
600 nt of the COX-2 3’UTR is necessary and sufficient for the regulation of mRNA stability and translational efficiency (45, 46, 48). To further investigate how CpG DNA post-transcriptionally regulates COX-2 expression, a COX-2 translational reporter gene construct (COX2-3’UTR-luc) was generated by inserting the 3’-UTR (nucleotides 1-792) of the COX-2 sequence to the 3’ end of the luciferase gene under control of the SV40 promoter and enhancer elements (Fig. 2A). Since both the pGL3-luciferase reporter construct and the COX2-3’UTR-luciferase reporter construct have identical promoter elements, differences in luciferase activity reflect differences in regulation as a result of post-transcriptional events. The negative regulatory function of the COX-2 3’UTR was analyzed. As shown in Figure 2B, insertion of the COX-2 3’UTR to the 3’ end of the luciferase gene in the pGL3-control vector resulted in an approximately 95% reduction in the luciferase activity, indicating the presence of a functional negative regulatory element within the 3’UTR of the COX-2.

To investigate the ability of CpG DNA to transduce signals post-transcriptionally regulating COX-2 expression, RAW264.7 cells were transfected with a COX-2 translational reporter (COX2-3’UTR-luc) and then stimulated with medium, CpG DNA, control non-CpG DNA, or LPS. As shown in Figure 2C, both CpG DNA and LPS, but not control non-CpG DNA, induced increases of COX2-3’UTR-luciferase activity. This indicates that, like LPS, CpG DNA also has ability to induce COX-2 expression by post-transcriptional regulation mechanisms. To further confirm that COX2-3’UTR-luciferase activity induced by CpG DNA was mediated through a TLR9-dependent pathway, and not due to LPS contamination, RAW264.7 cells were transfected with COX2-3’UTR-luc reporter and then stimulated with medium, CpG DNA, or LPS in the presence or absence of a TLR9 antagonist inhibitory CpG DNA (iCpG DNA). Of note, the recently identified iCpG DNA specifically blocks the effects of
CpG DNA that include activation of all proximal signaling mediators, such as NF-κB and MAPKs, in B cells and macrophages (59-61 and Yi, unpublished data). As demonstrated in figure 2D, CpG DNA-mediated induction of COX2-3’UTR-luciferase activity was completely abolished in the presence of iCpG DNA. In contrast, the LPS-mediated induction of COX2-3’UTR-luciferase activity was not suppressed by iCpG DNA. Taken together, these results demonstrate that CpG DNA has the ability to transmit signals that lead to the induction of COX-2 expression through post-transcriptional regulation mechanisms including mRNA stabilization in a sequence specific manner.

**Endosomal acidification is required for CpG DNA-mediated post-transcriptional regulation of COX-2 expression.** It has been previously demonstrated that CpG DNA is endocytosed by leukocytes and endosomal acidification/processing/maturation is required for its action (2, 4). To investigate whether this endosomal acidification of DNA is also required for the CpG DNA-mediated post-transcriptional regulation of COX-2 expression, RAW264.7 cells were transfected with COX2-3’UTR-luc. Cells were stimulated with medium, CpG DNA or LPS in the presence or absence of chloroquine, an inhibitor of endosomal acidification. As demonstrated in figure 3, induction of COX2-3’UTR-luciferase activity by CpG DNA was completely inhibited by chloroquine. In contrast, chloroquine failed to suppress the COX2-3’UTR-luciferase activity induced by LPS. These results indicate that CpG DNA upregulates COX-2 expression at the post-transcriptional level through an endosomal acidification-dependent pathway.

**CpG DNA post-transcriptionally regulates COX-2 expression through an MyD88-dependent signaling pathway.** It has recently been demonstrated that after it is endocytosed, CpG DNA is co-localized in an endosomal compartment with TLR9, a putative CpG DNA
receptor (3, 4). After recognizing CpG DNA, TLR9 binds to MyD88 and initiates a signal transduction cascade that is mediated through MyD88 downstream effectors including IRAK-1 and TRAF6 (3-5, 8). MyD88, IRAK-1, and TRAF6 have been demonstrated to be required for the CpG DNA-mediated activation of NF-κB and JNK, which in turn regulates gene expression (5, 8). However, to date it is not known whether these signaling modulators play a role in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression. To investigate whether MyD88, IRAK-1, and TRAF6 play an essential role in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression, RAW264.7 cells were co-transfected with expression vectors encoding DN-MyD88, DN-IRAK-1, or DN-TRAF6 and reporter constructs COX2-3’ UTR-luc or AP-1-β galactosidase. Overexpression of DN-MyD88 abolished CpG DNA-induced COX2-3’UTR-luciferase activity as well as CpG DNA-mediated AP-1 activation (Figs. 4A). In contrast, overexpression of DN-MyD88 showed little or no suppressive effects on IFN-γ-induced COX2-3’UTR-luciferase activity, verifying the pathway specificity of DN-MyD88 (Fig. 4A). This result demonstrates that MyD88 is required for the CpG DNA-mediated post-transcriptional regulation of COX-2 expression.

As shown in Figure 4B, overexpression of DN-IRAK1 substantially, but not completely, inhibited CpG DNA-induced COX2-3’UTR-luciferase activity while it showed no inhibitory effect on COX2-3’UTR-luciferase activity induced by IFN-γ. There was no further suppression of CpG DNA-induced COX2-3’-UTR-luciferase activity in cells transfected with higher doses of DN-IRAK-1 expression vector (data not shown). However, overexpression of DN-IRAK-1 completely abolished activation of AP-1 induced by CpG DNA, verifying the inhibitory potency of DN-IRAK-1 and suggesting that IRAK-1 is required for CpG DNA-mediated AP-1 activation.
(Fig. 4B). These results indicate that IRAK-1 is necessary for optimal post-transcriptional induction of COX-2 expression in response to CpG DNA.

In the TLR9/MyD88-signaling pathway, activated IRAK-1 interacts with TRAF6, which in turn leads to the activation of NF-κB and MAPK. As demonstrated in figure 4C, CpG DNA-induced COX2-3’UTR-luciferase activity was only partially inhibited in RAW264.7 cells overexpressing DN-TRAF6. There was no further suppression of CpG DNA-induced COX2-3’-UTR-luciferase activity in the cells transfected with higher doses of the DN-TRAF6 expression vector (data not shown). However, overexpression of DN-TRAF6 resulted in complete inhibition of CpG DNA-mediated AP-1 activation (Fig. 4C). This verifies the inhibitory efficacy of DN-TRAF6 and indicates that TRAF6 is required for AP-1 activation induced by CpG DNA. COX2-3’UTR-luciferase activity induced by IFN-γ was not affected by overexpression of DN-TRAF6, demonstrating the pathway specificity of DN-TRAF6 (Fig. 4C). These results demonstrate that TRAF6 is necessary for the optimal induction of COX-2 expression regulated through post-transcriptional mechanisms in response to CpG DNA. Taken together, our results demonstrate that for the post-transcriptional regulation of COX-2 expression by CpG DNA, MyD88 is indispensable but TRAF6 and IRAK-1 are only partially required, suggesting that MyD88 signaling effectors other than IRAK-1 and TRAF6 may also be involved in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression.

**MAPKs play a critical role in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression.** Three members of the MAPK family, ERK, JNK, and p38, are known to play a critical role in CpG DNA-mediated gene expression and are proposed downstream effectors in the MyD88/IRAK/TRAF6-signaling pathway (3-5, 58, 62). In addition, these MAPKs have been shown to stabilize various cytokine and chemokine mRNAs (52-56). To
investigate the role of MAPKs in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression, three specific MAPK inhibitors U0126 (a MEK inhibitor), SB202190 (a p38 inhibitor), and SP600125 (a JNK inhibitor) were employed. RAW264.7 cells transiently transfected with the translational reporter COX2-3’UTR-Luc were stimulated with CpG DNA in the presence of DMSO, U0126, SB202190, or SP600125. As shown in Figure 5A, CpG DNA-induced COX2-3’UTR-luciferase activity was partially suppressed in the presence of U0126, SB202190, or SP600125. In addition, the combination of any two MAPK inhibitors further suppressed CpG DNA-induced COX2-3’UTR-luciferase activity. Furthermore, CpG DNA-induced COX2-3’UTR-luciferase activity was completely abolished in the presence of all three MAPK inhibitors. These results indicate that each different MAPK contributes partially to the CpG DNA-mediated induction of COX2-3’ UTR-luciferase activity and that all three MAPKs, ERK, p38, and JNK, are necessary to mediate the CpG DNA signal for optimal post-transcriptional regulation of COX-2 expression. Of note, SP600125 specifically inhibited phosphorylation of JNK and c-Jun without effecting phosphorylation of ERK or p38 in RAW264.7 cells, confirming the specific inhibitory effects of SP600125 on the JNK pathway (Fig. 5B). The specificity of U0126 and SB202190 in our experimental system has previously been reported (58).

To further confirm the role of MAPKs in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression, RAW264.7 cells were co-transfected with COX2-3’UTR-Luc or AP-1-β galactosidase reporters and expression vectors encoding DN-p38, DN-JNK1, or DN-MEK1. As demonstrated in figure 5C, CpG DNA-induced COX2-3’UTR-luciferase activity was substantially inhibited in cells overexpressing DN-MEK1, DN-p38, or DN-JNK1. As with pharmacological inhibitors (Fig 5A), inhibition of ERK activation by overexpressing DN-MEK1
showed the least suppressive effect on CpG DNA-induced COX2-3’UTR-luciferase activity (Fig. 5C). Co-expression of DN-p38 and DN-JNK1 further reduced CpG DNA-induced COX2-3’UTR-luciferase activity compared to cells overexpressing DN-p38 or DN-JNK1 alone (Fig. 5C). However, co-expression of DN-MEK1 and DN-p38 or DN-MEK1 and DN-JNK1 did not further inhibit the CpG DNA-induced COX2-3’UTR-luciferase activity beyond either DN-p38 or DN-JNK alone. Similar to the result seen in the study with pharmacological inhibitors (Fig 5A), co-expression of all three DN-MAPKs, DN-p38, DN-JNK1, and DN-MEK1, completely suppressed CpG DNA-induced COX2-3’UTR-luciferase activity (Fig. 5C). Overexpression of DN-MEK1, DN-p38, or DN-JNK1 completely abolished CpG DNA-induced AP-1 activation (Fig. 5D). This indicates that each MAPK plays an indispensable role in CpG DNA-mediated AP-1 activation and that overexpressed DN-MEK1, DN-p38, and DN-JNK1 are sufficiently potent dominant negatives under our experimental conditions. Our results demonstrate that activation of all three MAPKs, ERK, p38, and JNK, is required for the optimal post-transcriptional regulation of COX-2 expression by CpG DNA.

**Differential roles of TRAF6 for activating the different MAPK by CpG DNA in RAW264.7 cells.** Our results demonstrated that each MAPK activated by CpG DNA only partially contributes to the post-transcriptional regulation of COX-2 expression and that all three active MAPKs are required for the optimal COX-2 post-transcriptional regulation by CpG DNA (Figs. 5A and 5C). Although there has been no formal demonstration in the literature showing that TRAF6 is actually required for the activation of all three MAPKs induced by CpG DNA, TRAF6 has been posited to be an upstream modulator of MAPK activation (5, 8). However, our results showed that overexpression of DN-TRAF6 only partially inhibits CpG DNA-induced COX2-3’UTR luciferase activity (Fig. 4C). Therefore, we hypothesized that this partial
contribution of TRAF6 to CpG DNA-induced COX2-3’UTR-luciferase activity may be due to a differential contribution of TRAF6 to CpG DNA-mediated MAPK activation. To investigate this hypothesis, we generated a RAW264.7 stable transfectant that constitutively expresses Flag-tagged DN-TRAF6 (TRAF6DN-RAW264.7). The expression of Flag-tagged DN-TRAF6 protein in this stable transfectant was confirmed by western blot using an antibody against Flag (Fig. 6A). As expected, CpG DNA induced activation of AP-1 in RAW264.7 cells transfected with a control vector (vector control cell), but failed to induce AP-1 activation in TRAF6DN-RAW264.7 transfectants (Fig. 6B). This result confirms that constitutively expressed DN-TRAF6 in this stable transfectant acts as a sufficiently potent dominant negative. Figure 6C showed that CpG DNA failed to induce phosphorylation of JNK and p38 in TRAF6DN-RAW264.7 cells. However, phosphorylation of ERK induced by CpG DNA was not inhibited in the TRAF6DN-RAW264.7 cells. In addition, CpG DNA-induced COX2-3’UTR-luciferase activity was partially inhibited in TRAF6DN-RAW264.7 cells. These results demonstrate that TRAF6 is an upstream signaling modulator for CpG DNA-mediated JNK and p38 activation but not for ERK activation, and indicate that the partial contribution of TRAF6 to the CpG DNA-mediated post-transcriptional regulation of COX-2 expression may due to differential regulation of the MAPKs by TRAF6.

**CpG DNA induces activation of the small GTP-binding protein Ras, which contributes to the post-transcriptional regulation of COX-2 expression in RAW264.7 cells.** Our results demonstrated that CpG DNA activates ERK through a TRAF6-independent manner (Fig. 6C). Previous studies have demonstrated that CpG DNA induces activation of Raf1 and MEK1/2, and that active Raf1 and MEK1/2 are required for CpG DNA-mediated ERK activation (58, 63). In the classical ERK activation pathway, the small GTP-binding protein Ras is the upstream
signaling modulator of Raf1. Therefore, we have investigated whether CpG DNA induces Ras activation and whether active Ras plays a functional regulatory role in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression. To investigate whether CpG DNA induces activation of Ras, RAW264.7 cells were stimulated with CpG DNA. The presence of the active form of Ras (GTP-bound Ras) in cell lysates prepared from the CpG DNA-stimulated cells was detected using bead-bound Raf1-RBD-GST fusion proteins followed by western blot detection using an antibody specific for Ras. As demonstrated in Figure 7A, CpG DNA induced Ras activation within 3 min in RAW264.7 cells. To investigate whether Ras contributes to the post-transcriptional regulation of COX-2 expression by CpG DNA, TRAF6DN-RAW264.7 transfectants and vector control cells were co-transfected with COX2-3’UTR-Luc reporter and expression vectors encoding DN-Ras (N17Ras). As shown in Figure 7B, overexpression of N17Ras partially inhibited CpG DNA-induced COX-2-3’UTR-luciferase activity in vector control cells, indicating the functional regulatory role of Ras in the post-transcriptional regulation of COX-2 expression by CpG DNA. Furthermore, overexpression of N17Ras completely inhibited CpG DNA-induced COX-2-3’UTR-luciferase activity in TRAF6DN-RAW264.7 transfectants (Fig. 7B). These results indicate that CpG DNA-mediated post-transcriptional regulation of COX-2 expression requires activation of both TRAF6 and Ras, and that CpG DNA-activated Ras may contribute to the post-transcriptional regulation of COX-2 expression by leading to the activation of ERK.
DISCUSSION

In recent years, much progress has been made in uncovering the proximal signaling pathways responsible for CpG DNA-mediated innate immune cell activation. However, it is yet to be understood how individual signaling modulators in the newly identified CpG DNA-signaling pathway contribute to the expression of each specific pro-inflammatory cytokine and mediator. Moreover, it has not been studied whether CpG DNA can regulate gene expression by post-transcriptional mechanisms and if so, how the TLR9/MyD88-signaling pathway transmits the CpG DNA signal post-transcriptionally regulating gene expression. In the present study, we investigated whether CpG DNA utilizes a TLR9/MyD88-dependent pathway to regulate COX-2 expression post-transcriptionally by mechanisms including mRNA stabilization.

Like many cytokine genes, the COX-2 gene has conserved ARE sequences, which regulate message stability, within the 3’UTR. The COX-2 gene produces two major transcript isoforms, COX-24.6 (4.6 kb) and COX-22.8 (2.8 kb), which are derived by alternative polyadenylation in the 3’UTR (64, 65). The most abundant COX-2 transcript (COX-24.6) contains a 2515-nt 3’UTR, while the second most abundant COX-2 transcript (COX-22.8) contains a 603-nt 3’UTR (64, 65). Previous studies have demonstrated that insertion of the 3’UTR of COX-2 to the 3’ end of a reporter gene alters its expression, and the ARE sequences are crucial for this response (45, 46, 48). These studies demonstrated that a region 123 nt immediately 3’ to the translation termination codon contains a highly conserved ARE (CR1), composed of 6 AUUUA elements, and that CR1 is necessary and sufficient to regulate both COX-2 message stability and translational efficiency (45, 46, 48). The ability of CpG DNA to induce COX-2 expression through post-transcriptional mechanisms in RAW264.7 cells was demonstrated by showing that CpG DNA stimulation prevented COX-2 mRNA degradation in
the presence of the transcriptional inhibitor ActD (Fig. 1B) and reversed the inhibition of luciferase activity caused by insertion of COX-2 3’UTR (Fig. 2C). These results suggest that CpG DNA stabilizes COX-2 mRNA and that increases in COX2-3’UTR-luciferase activity induced after CpG DNA stimulation may be due, at least in part, to increased mRNA stability.

The involvement of TLR9 in transducing the CpG DNA signal to post-transcriptionally regulate COX-2 expression was indirectly shown by using a TLR9 antagonist iCpG DNA (Fig. 2D). Although there has been no formal demonstration showing that either CpG DNA or iCpG DNA directly binds to TLR9, and the precise molecular mechanism by which iCpG DNA specifically blocks the activity of CpG DNA has not been revealed, previous studies suggest that iCpG DNA may compete with CpG DNA for binding to TLR9 (59-61). Utilization of TLR9 for CpG DNA-mediated post-transcriptional regulation of COX-2 expression was also supported by demonstrating that chloroquine, an endosomal acidification inhibitor, completely blocked CpG DNA-induced COX2-3’UTR-luciferase activity (Fig. 3). It has previously been demonstrated that an endosomal acidification inhibitor such as chloroquine abolishes the biologic effects of CpG DNA by inhibiting binding of CpG DNA to TLR9 and the subsequent recruitment of MyD88 to TLR9 (2, 4, 62, 63). Recent studies have demonstrated the absolute requirement of MyD88 for the CpG DNA-mediated activation of NF-κB, JNK, and p38, and their subsequent biologic effects including proinflammatory cytokine production and B cell proliferation (3-5). In addition, we have recently demonstrated that MyD88 is indispensable for CpG DNA-mediated COX-2 transcriptional regulation (28). In agreement with these previous observations, our results also showed that transient overexpression of DN-MyD88 completely inhibits the ability of CpG DNA to induce COX2-3’UTR-luciferase activity and AP-1 transcriptional activity (Fig. 4A) (28).
MyD88 bound to TLRs recruits IRAK-1 via a death domain-death domain interaction. IRAK-1 is activated at the receptor complex, becomes rapidly phosphorylated, and leaves the receptor complex to interact with the adapter molecule TRAF6 (66-68). It has also been demonstrated that the CpG DNA signal is transduced through IRAK-1 and TRAF6 via the TLR9/MyD88-dependent mechanism (5, 8). Overexpression of DN-IRAK-1 inhibits CpG DNA-induced NF-κB activation in HEK293 cells when transiently co-transfected with mouse TLR9 (8). In addition, CpG DNA-induced AP-1 transcriptional activity was completely inhibited in RAW264.7 cells overexpressing DN-IRAK-1 (Fig. 4B) (28). In contrast, CpG DNA-induced NF-κB transcriptional activity and COX-2 promoter activity are substantially, but not completely, inhibited in RAW264.7 cells overexpressing DN-IRAK-1 (28). This incomplete inhibition of COX-2 transcriptional regulation by DN-IRAK-1 may be due to the inability of DN-IRAK-1 to completely inhibit the CpG DNA-mediated activation of NF-κB, a transcription factor established to indispensable for CpG DNA-induced COX-2 promoter activity (28). Similarly, transient overexpression of DN-IRAK-1 dramatically diminished, but not completely abolished, the CpG DNA-induced COX2-3’UTR-luciferase activity in RAW264.7 cells (Fig. 4B). Our results indicate that additional signaling modulators other than IRAK-1 may function downstream of MyD88 for the post-transcriptional regulation of COX-2 expression in RAW264.7 cells in response to CpG DNA stimulation. In addition to IRAK-1, three more IRAK family members, IRAK-2, IRAK-4 and IRAK-M, have recently been reported (6, 7, 69, 70). The role of IRAK-2 in TLR9 signal transduction is currently unknown, and IRAK-M has been shown to play a negative regulatory role in TLR9 signal transduction (6). IRAK-4-deficient mice are severely impaired in their response to CpG DNA, indicating the indispensable role of IRAK-4 in TLR9 signal transduction (7). Therefore, it is possible that in addition to IRAK-1, other IRAK
family members, such as IRAK-2 and IRAK-4, may be required for CpG DNA-mediated post-transcriptional regulation of COX-2 expression in RAW264.7 cells. It is yet to be determined what relative contribution each IRAK family member makes to the CpG DNA-mediated activation of NF-κB and MAPKs, and to the transcriptional and post-transcriptional regulation of COX-2 expression.

The contribution of TRAF6 to CpG DNA-mediated NF-κB activation is controversial and is different in different types of cells. Overexpression of DN-TRAF6 has been shown to inhibit CpG DNA-induced NF-κB activation in HEK293 cells (8) and substantially inhibits CpG DNA-induced IκB phosphorylation in RAW264.7 cells (5). In contrast, we have demonstrated that overexpression of DN-TRAF6 only partially inhibits the CpG DNA-induced transcriptional activity of NF-κB in RAW264.7 cells (28). This partial NF-κB inhibition might be a reason that DN-TRAF6 only partially inhibits CpG DNA-mediated transcriptional regulation of COX-2 (28). In contrast to the effects on NF-κB activation, DN-TRAF6 overexpression in RAW264.7 cells abrogated CpG DNA-induced transcriptional activity of AP-1 and phosphorylation of JNK and p38 (Figs. 4C, 6B, and 6C) (28). However, overexpression of DN-TRAF6 did not inhibit ERK activation induced by CpG DNA (Fig. 6C), indicating that CpG DNA induces activation of ERK through a signaling pathway that is independent of TRAF6, and that TRAF6 may be a diverging point for the activation of MAPKs in the TLR9/MyD88-signaling pathway. While p38 is the only MAPK required for the transcriptional regulation of COX-2 expression by CpG DNA (28), the optimal induction of COX2-3’UTR-luciferase activity by CpG DNA requires all three active MAPKs, ERK, JNK, and p38 (Fig. 5). Therefore, this TRAF6-independent activation of ERK by CpG DNA may explain the reason that DN-TRAF6 only partially blocks the CpG DNA-mediated post-transcriptional regulation of COX-2 expression (Figs. 4C and 6D).
In the classical ERK activation pathway, the small GTP-binding protein Ras is the upstream activator of Raf1. Previous studies have demonstrated that CpG DNA induces activation of Raf1 and MEK1/2, and that active Raf1 and MEK1/2 are required for CpG DNA-mediated ERK activation (58, 63). In the present study, we have found that CpG DNA rapidly induces activation of Ras in RAW264.7 cells (Fig. 7A) and that Ras plays a functional regulatory role in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression (Fig. 7B). Moreover, co-expression of both DN-TRAF6 and N17Ras results in complete inhibition of COX-2-3’UTR-luciferase activity in response to CpG DNA (Fig. 7B). Taken together, our results indicate that CpG DNA may activate ERK through a Ras-dependent pathway and that this Ras-dependent activation of ERK and TRAF6-dependent activation of p38 and JNK play indispensable roles in the post-transcriptional regulation of COX-2 expression by CpG DNA. Of note, while revising our current manuscript, Dr. Cao and his colleagues reported that CpG DNA induces activation of Ras in RAW264.7 cells through a TLR9-dependent pathway, and that CpG DNA-activated Ras contributes to the activation of ERK, JNK, and NF-κB and the production of NO and TNF-α in RAW264.7 cells (71).

Previous studies have demonstrated that MAPKs activated by various stimuli play critical roles in the post-transcriptional regulation of COX-2 gene expression by stabilizing COX-2 message, which is dependent on regulatory elements contained within its 3’UTR. MAPK p38 has been shown to be critical for COX-2 mRNA stabilization by cytokines, serum withdrawal, or LPS (48, 50, 51, 72). The ERK1/2 pathway has been shown to be essential for stabilizing COX-2 mRNA in response to IL-1β in human endometrial stromal cells (73). Ceramide-induced COX-2 mRNA stabilization in rat intestinal epithelial cells requires both p38 and ERK but not JNK (74). Our results demonstrated that in addition to ERK1/2 and p38, JNK also contributes to
the CpG DNA-mediated post-transcriptional regulation of COX-2 expression in RAW264.7 cells (Fig. 5). Post-transcriptional regulation is not limited to changes in message stability, and sequences within the 3’UTR of mRNAs have also been shown to be important for enhancing message translation as well as for translational silencing (45, 75, 76). Since the 3’UTR of murine COX-2 also contains multiple regulatory elements that alter message stability and translational efficiency (45), results from the assay system we employed for the present study collectively reflect post-transcriptional regulatory mechanisms but do not differentiate translational efficiency or translational silencing from message stabilization. Therefore, it is yet to be determined whether each MAPK plays a different regulatory role in CpG DNA-regulated COX-2 message stability, translational efficiency and translational silencing.

In summary, the present study demonstrates for the first time that CpG DNA has ability to post-transcriptionally regulate gene expression through a TLR9/MyD88-dependent signaling pathway and that CpG DNA induces ERK activation through a TRAF6-independent manner. Our results indicate that TRAF6 may be a diverging point for the activation of MAPKs in the TLR9/MyD88-signaling pathway.
REFERENCES


FOOTNOTE

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FIGURE LEGENDS

Figure 1. Induction of COX-2 mRNA stability by CpG DNA. Panel A. RAW264.7 cells were stimulated with medium, CpG DNA (3 μg/ml), or non-CpG DNA (3 μg/ml) for the designated time periods. Total RNA was isolated and the presence of mRNA for COX-2 and GAPDH in each sample was detected by real-time PCR using SYBR green. GAPDH was used as an endogenous control. Data present the mean (fold induction from unstimulated control) ± SD of triplicates. The experiment was done three times with similar results. Panel B. RAW264.7 cells pretreated with CpG DNA (3 μg/ml) for 12h were stimulated with medium, CpG DNA (3 μg/ml), or non-CpG DNA (3 μg/ml) for the designated time periods in the presence of Actinomycin D (10 μg/ml). Total RNA was isolated and the presence of mRNA for COX-2 and GAPDH in each sample was detected by real-time PCR using SYBR green. GAPDH was used as an endogenous control. Data present the mean (% of unstimulated control) ± SD of triplicates. The experiment was done three times with similar results.

Figure 2. CpG DNA regulates COX-2 expression at the post-transcriptional level.

Panel A. COX-2 translational reporter (COX2-3’UTR-Luciferase) was constructed by inserting a portion (nucleotides 1-792) of the 3’UTR of COX-2 message into the 3’ end of the luciferase gene in pGL3-control vector. Panel B. RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and pGL3-control vector or COX2-3’-UTR-Luciferase reporter gene vector and then incubated for 12h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. Data present the mean (% of luciferase activity measured in cells transfected with pGL3-control) ± SD of triplicates. The experiment was done three times with similar results. Panel C. RAW264.7 cells were transiently co-transfected with pRL-TK-
luciferase and COX2-3’UTR-luciferase reporter gene vectors and then stimulated with medium, CpG DNA (6 µg/ml), non-CpG DNA (6 µg/ml), or LPS (50 ng/ml) for 12h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity (Renilla luciferase activity) in each sample. Data present the mean (fold increase in luciferase activity compared with cells stimulated with medium) ± SD of triplicates. **Panel D.** RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and COX2-3’-UTR-Luciferase. Cells were stimulated with medium, CpG DNA (6 µg/ml), or LPS (50 ng/ml) in the presence or absence of iCpG DNA (6 or 12 µg/ml) for 12h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (fold induction from unstimulated controls) ± SD of triplicates. The experiment was done three times with similar results.

**Figure 3. Endosomal acidification is required for the CpG DNA-mediated post-transcriptional regulation of COX-2 expression.** RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and COX2-3’-UTR-Luciferase. Transfected cells were pooled and washed 3 times with culture media. Cells were stimulated with medium, CpG DNA (6 µg/ml), or LPS (50 ng/ml) in the presence or absence of an inhibitor of endosomal acidification, chloroquine (2.5 µg/ml) for 12h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (fold induction from unstimulated controls) ± SD of triplicates. The experiment was done three times with similar results.

**Figure 4.** CpG DNA induces COX-2 mRNA stability through a MyD88-dependent signaling pathway. RAW264.7 cells were transiently co-transfected with plasmids encoding
DN-MyD88 (2 µg) (for Panel A), DN-IRAK-1 (2 µg) (for Panel B), or DN-TRAF6 (2 µg) (for Panel C) and COX2-3′-UTR-luciferase (1 µg) plus pRL-TK-luciferase (0.5 µg) or AP-1-β galactosidase (2 µg). Transfected cells were washed and incubated in complete media for 6h. Cells were stimulated with medium, CpG DNA (6 µg/ml) or IFN-γ (166 ng/ml) for 12 h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. AP1-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 (fold induction from unstimulated controls) ± SD of triplicates. The experiment was done three times with similar results.

**Figure 5. MAPKs play a critical role in the CpG DNA-mediated post-transcriptional regulation of COX-2 expression.** **Panel A.** RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and COX2-3′-UTR-Luciferase. Transfected cells were pooled and washed 3 times with culture media. Transfected cells were pretreated with DMSO, SB202190 (2.5 µM), U0126 (1.25 µM), or SP600125 (5 µM) for 15min and then stimulated with medium or CpG DNA (3 µg/ml) for 12 h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (fold induction from unstimulated controls) ± SD of triplicates. The experiment was done three times with similar results. **Panel B.** RAW264.7 cells (2x10⁶ cells/ml) were treated with DMSO or SP600125 (5 µM) for 15 min and then stimulated with medium or CpG DNA (6 µg/ml) for 45 min. Equal amounts of whole cell lysates (15 µg/lane) were subjected to SDS-PAGE and then western blots were performed using a specific antibody against the phosphorylated form of c-Jun (pc-Jun), JNK (pJNKp52 and pJNKp46), ERK (pERK1
and pERK2) or p38 (pp38). Actin was used as a loading control. The experiment was done three times with similar results. **Panel C and D.** RAW264.7 cells were transiently co-transfected with plasmids encoding DN-p38 (2 µg), DN-JNK1 (2 µg), and/or DN-MEK1 (2 µg) and COX2-3’UTR-luciferase (1 µg) + pRL-TK-luciferase (0.5 µg) (for Panel C) or AP-1-β galactosidase (2 µg) (for Panel D). Transfected cells were washed and incubated in complete media for 6 h. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (% values from CpG DNA-stimulated samples which were transfected with control empty vectors) ± SD. AP1-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 (fold induction from unstimulated control) ± SD of triplicates. The experiment was done three times with similar results.

**Figure 6.** CpG DNA fails to induce phosphorylation of JNK and p38, but not ERK, in RAW264.7 cells constitutively expressing DN-TRAF6. **Panel A.** Whole cell lysates were prepared from vector control cells or TRAF6DN-RAW264.7 cells. Equal amounts of whole cell lysates (15 µg/lane) were subjected to SDS-PAGE and then western blots were performed using a specific antibody against Flag, TRAF6, or Actin. Actin and TRAF6 were used as loading controls. The experiment was done three times with similar results. **Panel B.** Vector control cells or TRAF6DN-RAW264.7 cells were transiently transfected with AP-1-β galactosidase (2 µg). Transfected cells were pooled and washed 3 times with culture media. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 h. AP1-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 (fold induction from unstimulated controls) ± SD of triplicates. The experiment was done three times with similar results. **Panel C.** Vector control cells or TRAF6DN-RAW264.7 cells were stimulated with medium or CpG DNA (6 µg/ml) for 45 min. Equal amounts of whole cell lysates (15 µg/lane) were subjected to SDS-PAGE and then western blots were performed using a specific antibody against the phosphorylated form of JNK (pJNKp52 and pJNKp46), ERK (pERK1 and pERK2) or p38 (pp38). Actin was used as a loading control. The experiment was done three times with similar results. **Panel D.** Vector control cells or TRAF6DN-RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase and COX2-3’-UTR-Luciferase. Transfected cells were pooled and washed 3 times with culture media. Cells were stimulated with medium or CpG DNA (6 µg/ml) for 12 h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (fold induction from unstimulated control) ± SD of triplicates. The experiment was done three times with similar results.

**Figure 7.** CpG DNA induces activation of the small GTP-binding protein Ras which contributes to the post-transcriptional regulation of COX-2 expression in RAW264.7 cells.

**Panel A.** RAW264.7 cells (1x10⁷ cells) were stimulated with medium or CpG DNA (6 µg/ml) for the indicated time periods. GTP-bound Ras in cell lysates was precipitated using bead-bound Raf1-RBD-GST fusion protein. Precipitates were analyzed by western blot using an antibody specific for Ras. The experiment was done twice with similar results. **Panel B.** Vector control cells or TRAF6DN-RAW264.7 cells were transiently co-transfected with pRL-TK-luciferase, COX2-3’-UTR-Luciferase, and N17Ras or control empty vector. Transfected cells were pooled, washed 3 times with culture media, and then incubated in complete media for 6 h. Cells were
stimulated with medium or CpG DNA (6 µg/ml) for 12 h. Luciferase activities in cell extracts were analyzed by Dual-Luciferase Reporter Assay System and normalized using pRL-TK-luciferase activity in each sample. Data present the mean (% values from CpG DNA-stimulated vector control cell samples which were transfected with control empty vectors) ± SD of triplicates. The experiment was done three times with similar results.
Fig. 1

A. Remaining COX-2 mRNA (%)

B. Remaining COX-2 mRNA (%)

Figure 2.

A. COX-2: COX-2

pGL3-Control: SV40 luc

C. COX2-3'UTR-Luc: SV40 luc COX2-3'UTR

D. COX2-3'UTR-Luc (Fold)
Fig. 3

![Graph showing COX2-3'UTR-Luc expression in different conditions.]

Fig. 4

A.

![Graph showing RLU for COX2-3'UTR-Luc and AP-1-beta-galactosidase in different conditions.]

B.

![Graph showing RLU for COX2-3'UTR-Luc and AP-1-beta-galactosidase in different conditions.]

C.

![Graph showing RLU for COX2-3'UTR-Luc and AP-1-beta-galactosidase in different conditions.]

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Figure 5.
Fig. 6

A. Control DNTRAF6
    - Flag-DNTRAF6
    - TRAF6
    - Actin

B. Vector Control TRAF6DN-RAW264.7

C. Con. DNTRAF6
    Medium CpG
    - pJNK (p52)
    - pJNK (p46)
    - pp38
    - pERK1
    - pERK2
    - Actin

D. Vector Control TRAF6DN-RAW264.7

Fig. 7

A. 0 3 7 15 30 (min) -Ras

B. Empty Vector N17Ras
Myeloid differentiation factor 88-dependent post-transcriptional regulation of cyclooxygenase-2 expression by CpG DNA: Tumor necrosis factor-a receptor-associated factor 6, a diverging point in the toll-like receptor 9-signaling
Seon-Ju Yeo, Jae-Geun Yoon and Ae-Kyung Yi

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