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CUTTING EDGE

Cutting Edge: Rapid Induction of Mitogen-Activated Protein Kinases by Immune Stimulatory CpG DNA[1]

Ae-Kyung Yi* and Arthur M. Krieg[2]*†

Unmethylated CpG motifs in bacterial DNA or synthetic oligodeoxynucleotides (CpG DNA) rapidly activate B cells and monocyte-derived cells; however, the intracellular signaling pathways involved in this process are unclear. Here, we demonstrate that CpG DNA induces the activation of c-Jun NH_2-terminal kinase and p38 but does not activate the extracellular receptor kinase in murine B and monocyte-like cell lines. CpG DNA also induces the phosphorylation of activating transcription factor-2, c-Jun, and mitogen-activated protein kinase (MAPK)-activated protein kinase 2 as well as the activation of activator protein-1 (AP-1) DNA binding. Inhibition of p38 led to the suppression of CpG DNA-induced AP-1 DNA-binding activity and cytokine production, indicating that the p38 pathway is required for mediating these immune stimulatory effects of CpG DNA. Chloroquine, an endosomal acidification inhibitor, selectively abolished CpG DNA-mediated MAPK activation. Our results indicate that CpG DNA activates the p38 and c-Jun NH_2-terminal kinase MAPK and leads to the activation of AP-1 via a pathway which is sensitive to chloroquine. ***The Journal of Immunology*****, 1998, 161: 4493–4497.**

The genomic DNAs of bacteria and vertebrates differ in the frequency and methylation of CpG dinucleotides, which are relatively common in bacterial DNA (~1/16 bases) but are underrepresented ("CpG suppression"; 1/50–1/60 bases) and methylated in vertebrate DNA (1). Bacterial DNA or synthetic oligodeoxynucleotides (ODNs)[3] containing unmethylated CpG dinucleotides in particular base contexts (CpG motifs) induce B cell proliferation, IL-6 and Ig secretion, and apoptosis resistance (2–7). Monocyte-derived cells are directly activated by CpG motifs to secrete the Th1-like cytokine IL-12 and type I IFNs, and NK cells respond with increased lytic activity and IFN-γ secretion, enhancing protective immune responses (8–16). Methylated bacterial DNA or ODNs in which the cytosines of CpG have been converted to 5-methyl-cytosine (the form present in vertebrate DNA) fail to induce immune activation (3). Thus, this simple structural difference in the frequency of CpG motifs between vertebrate and prokaryotic genomic DNAs appears to function as a "danger signal" to trigger innate immune defenses against infection and initiate a specific immune response (reviewed in Ref. 17). Indeed, ODNs containing CpG motifs (CpG DNA) can be mixed with Ags to promote strong Th1-like immune responses (18–24).

Some of the stimulatory effects of CpG motifs may be mediated by the activation of NF-κB (7, 11, 25, 26). However, the molecular mechanisms by which CpG DNA mediates leukocyte activation are not clearly understood at the present time. Mitogen-activated protein kinases (MAPKs) are important mediators of many cellular responses to stress and mitogenic signals. Therefore, we investigated whether CpG DNA-induced B cell or monocyte activation is associated with the activation of one or more members of the MAPK superfamily.

Materials and Methods

Oligodeoxynucleotides

Nuclease-resistant phosphorothioate ODNs (S-ODNs) were purchased from Hybridon Specialty Products (Milford, MA) and had no detectable endotoxins by *Limulus* assay. The sequences of the S-ODNs used are 5′TCCATGACGTTCCTGACGTT3′ (CpG DNA: 1826) and 5′TCCAG GACTTTCCTCAGGTT3′ (non-CpG DNA: 1911). For the sake of consistency, only the results using these two ODNs are shown herein. However, essentially the same results have been obtained in experiments with other CpG and control non-CpG DNA.

Culture conditions and reagents

A murine B lymphoma, WEHI-231 (clone 28), and a monocyte-like line, J774 (American Type Culture Collection, Manassas, VA), were cultured at 37°C in a 5% CO_2-humidified incubator and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% (v/v) heat-inactivated FCS (Sigma, St. Louis, MO), 1.5 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Anti-IgM, LPS, PMA, and chloroquine, an endosomal acidification inhibitor, were purchased from Sigma. SB202190, a p38 kinase inhibitor, and PD98059, a MEK inhibitor, were purchased from Calbiochem (La Jolla, CA). Anti-murine CD40 Ab was purchased from PharMingen (San Diego, CA) and used at 2 μg/ml.

*Department of Internal Medicine and Interdisciplinary Immunology Program, University of Iowa College of Medicine, Iowa City, IA 52242; and †Department of Veterans Affairs and CpG ImmunoPharmaceuticals Inc., Iowa City, IA 52246

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[2] Address correspondence and reprint requests to Dr. Arthur M. Krieg, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242. E-mail address: arthur-krieg@uiowa.edu

[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; MAPK, mitogen-activated protein kinase; EMSA, electrophoretic mobility shift assay; ERK, extracellular receptor kinase; MEK, MAPK/ERK kinase; JNK, c-Jun NH_2-terminal kinase; ATF, activating transcription factor; AP-1, activator protein-1; MAPKAP, MAPK-activated protein; GST, glutathione *S*-transferase.

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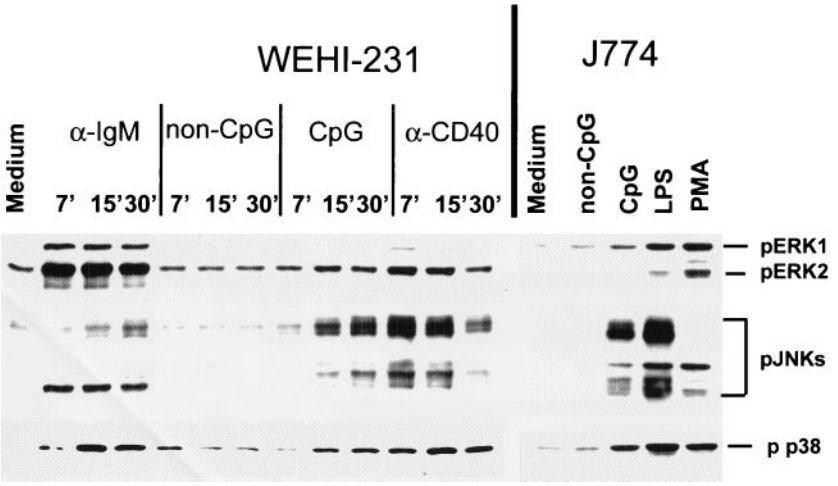

FIGURE 1. CpG DNA induces phosphorylation of JNK and p38 but not ERK in murine B and monocyte-like cell lines. WEHI-231 cells (2×10^6/ml) were stimulated with medium, anti-IgM (10 μg/ml), anti-CD40 (2 μg/ml), CpG DNA (2 μM), or non-CpG DNA (2 μM) for various time periods. J774 cells (2×10^6/ml) were stimulated with medium, CpG DNA (2 μM), or non-CpG DNA (2 μM) for 30 min or with LPS (1 μg/ml) or PMA (100 ng/ml) for 10 min. Whole cell lysates (50 μg/lane) were analyzed by Western blot using specific Abs against the phosphorylated forms of ERK (pERK), JNK (pJNK), or p38 (p p38). The experiment was repeated more than five times with similar results.

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

WEHI-231 cells or J774 cells (2×10^6 cells/ml) were treated with medium, CpG or non-CpG DNA (2 μM), anti-IgM (10 μg/ml), LPS (1 μg/ml), or PMA (100 ng/ml). In some experiments, cells were pretreated with various inhibitors 2 h before the stimulation with DNA. Cells were harvested at the indicated timepoints, and then whole cell lysates or nuclear extracts were prepared as described previously (7, 26). To detect phosphorylated extracellular receptor kinase (ERK), c-Jun NH_2-terminal kinase (JNK), p38, activating transcription factor-2 (ATF-2), or c-Jun, equal amounts of whole cell lysates (50 μg/lane) were subjected to electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE); next, Western blots were performed as described previously (7) using a specific Ab against the phosphorylated form of each protein. Specific Abs against the phosphorylated form of ERK, JNK, p38, ATF-2, or c-Jun were purchased from New England BioLabs (Beverly, MA). To detect the DNA-binding activity of the transcription factor activator protein-1 (AP-1), nuclear extracts (3 μg/lane) were analyzed by EMSA as described previously (26) using ^{32}P-labeled dsODNs containing the AP-1 (GATCTAGTGATGAGTCAGC CGGATC) binding sequence as a probe.

In vitro kinase assays

JNK and p38 in vitro kinase assays were performed as described previously (27), and the MAPK-activated protein (MAPKAP) kinase 2 in vitro kinase assay was completed using the MAPKAP kinase 2 assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol. Polyhistidine-tagged ATF-2 and Ab against p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The c-Jun-glutathione *S*-transferase (GST) fusion protein (1–79 aa) was a generous gift of Dr. Gary Koretzky (University of Iowa).

Cytokine ELISA

WEHI-231 cells (10^6 cells/ml for IL-6 and 10^7 cells/ml for TNF-α) were treated with medium, CpG or non-CpG DNA (1 μM), anti-IgM (10 μg/ml), or PMA (100 ng/ml) plus ionomycin (1 μM) for 4 h (for TNF-α) or 12 h (for IL-6) in the presence or absence of SB202190 (5 or 10 μM). Culture supernatants were analyzed by ELISA for TNF-α or IL-6 as described previously (6).

Results

CpG DNA induces phosphorylation of JNK and p38 but not ERK

CpG DNA, but not control non-CpG DNA, induced the phosphorylation of several isoforms of JNK in both the murine B cell line WEHI-231 and the monocyte-like line J774 (Fig. 1). The JNK isoforms that were phosphorylated after CpG DNA stimulation were similar to those activated by anti-CD40 or LPS stimulation but somewhat different from those induced by anti-IgM or PMA (Fig. 1). The kinetics of induction of JNK phosphorylation by CpG DNA (within 15 min) were slightly slower compared with stimulation by anti-CD40 or LPS (within 3 min). CpG DNA also induced the phosphorylation of p38 in both WEHI-231 cells and J774 cells. However, CpG DNA failed to induce a substantial phosphorylation of ERK within 1 h in these cell lines. ERK phosphorylation in these cells was inducible, because it was markedly increased by treatment with anti-IgM (WEHI-231) or PMA (J774; Fig. 1).

CpG DNA activates JNK and p38 kinase activity

To determine whether the phosphorylation of JNK and p38 was associated with an increase in their enzyme activity, in vitro kinase assays for JNK or p38 kinase were performed using c-Jun-GST (1–79 aa) or polyhistidine-tagged recombinant ATF-2, respectively, as substrates. These substrates showed a selective induction of JNK and p38 activities in the CpG DNA-treated cells within 15 min (Fig. 2, *A* and *B*).

CpG DNA induces phosphorylation of c-Jun and ATF-2 and induces MAPKAP kinase 2 enzyme activity

The phosphorylation status of the downstream effectors of JNK and p38 kinase, such as c-Jun, ATF-2, and MAPKAP kinase 2, was assessed directly in CpG DNA-treated cells by Western blot assay using an Ab specific for the phosphorylated form of c-Jun or ATF-2; MAPKAP kinase 2 was assessed using an in vitro kinase assay. As shown in Figure 3*A*, CpG DNA, but not non-CpG DNA, induced the phosphorylation of both c-Jun and ATF-2 within 15 min in the murine B cell lines WEHI-231 and CH12.LX and in the monocyte-like cell line J774. The enzyme activity of MAPKAP kinase 2 was increased in cells stimulated with CpG DNA and could be blocked by SB202190, indicating that CpG DNA-mediated MAPKAP kinase 2 activation was due to the activation of p38 (Fig. 3*B*).

DNA-binding activity of transcription factor AP-1 is induced by CpG DNA and is sensitive to chloroquine and p38 inhibitor

Since c-Jun is a component of the transcription factor AP-1, we investigated whether CpG DNA can induce the DNA-binding activity of this transcription factor by EMSA using a double-stranded probe containing an AP-1 binding site. As demonstrated in Figure 4, CpG DNA induced the DNA-binding activity of AP-1 within 30 min in WEHI-231 and J774 cells (Fig. 4*A*). The CpG DNA-induced DNA-binding activity of AP-1 was suppressed by the p38 inhibitor SB202190, suggesting that the activation of p38 by CpG DNA may contribute to the activation of transcription factor AP-1 by CpG DNA (Fig. 4*C*). CpG DNA-mediated activation of AP-1

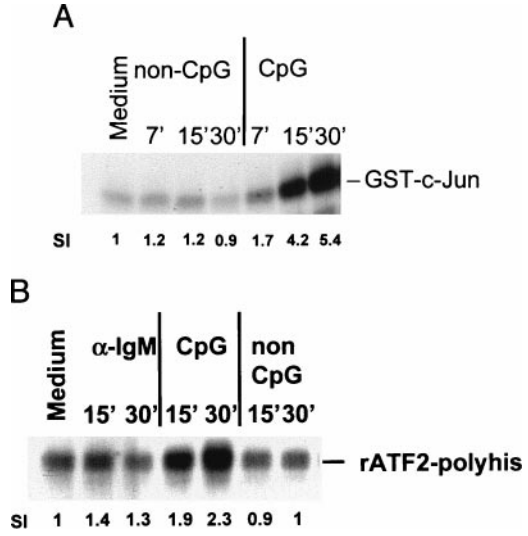

FIGURE 2. CpG DNA activates enzyme activities of JNK and p38. WEHI-231 cells (10^7/ml) were stimulated with medium, anti-IgM (10 μg/ml), CpG DNA (2 μM), or non-CpG DNA (2 μM) for various time periods. Whole cell lysates were immunoprecipitated with agarose bead-bound c-Jun-GST fusion proteins (10 μg/sample) (*A*) or anti-p38 Abs (*B*). In vitro kinase assays were performed at 30°C for 30 min. Polyhistidine-tagged ATF-2 (rATF-2-polyhis) was used as a substrate for the p38 in vitro kinase assay (*B*). SI = stimulation index (numbers indicate the relative intensity of the band compared with that of medium control as 1). The experiment was repeated five times with similar results.

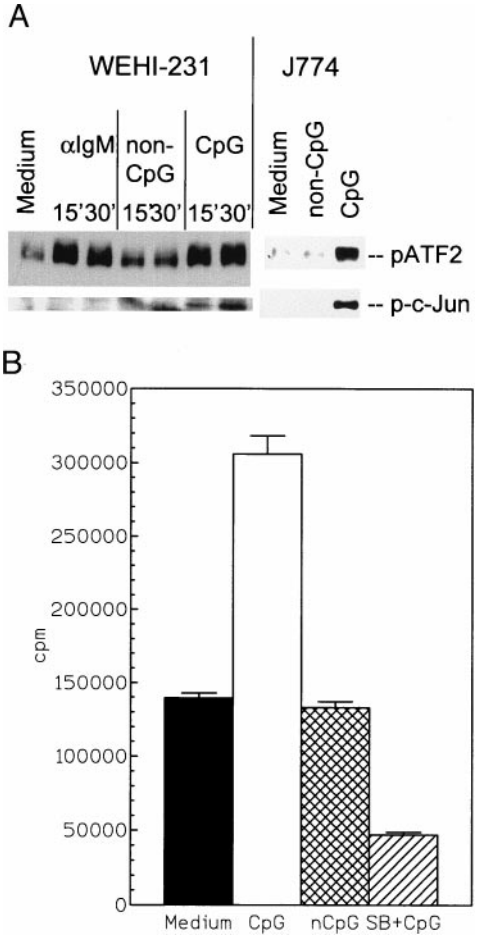

FIGURE 3. CpG DNA induces phosphorylation of ATF-2 and c-Jun and activates MAPKAP kinase 2 enzyme activity. *A*, CpG DNA induces phosphorylation of ATF-2 and c-Jun. WEHI-231 cells or J774 cells (2×10^6/ml) were stimulated with medium, anti-IgM (10 μg/ml), CpG DNA (2 μM), or non-CpG DNA (2 μM) for 15 min (for WEHI-231) or 30 min (for WEHI-231 and J774). Whole cell lysates (50 μg/lane) were analyzed by Western blot using a specific Ab against the phosphorylated forms of ATF-2 (pATF-2) and c-Jun (p-c-Jun). *B*, CpG DNA induces MAPKAP kinase 2 enzyme activity. J774 cells (5×10^6/5 ml) were pretreated with medium or SB202190 (10 μM) for 1 h and then stimulated with medium, CpG DNA (1 μM), or non-CpG DNA (nCpG: 1 μM) for 30 min. Whole cell lysates were immunoprecipitated with anti-MAPKAP kinase 2 Ab and an in vitro kinase assay was performed. The experiment was repeated more than three times with J774 or WEHI-231 cells.

was not affected by PD98059, a specific MEK inhibitor (Fig. 4*C*). Of note, SB202190 showed no effects on CpG DNA-mediated NF-κB activation in either J774 or WEHI-231 cells (data not shown).

SB202190, a specific inhibitor of p38, blocks CpG DNA-mediated cytokine production

To investigate whether CpG DNA-mediated p38 activation led to the production of cytokines, WEHI-231 cells were stimulated with CpG DNA or anti-IgM in the presence or absence of p38 inhibitor SB202190 (Table I). The addition of SB202190 markedly suppressed CpG DNA-mediated TNF-α and IL-6 production in WEHI-231 cells. SB202190 had less of an effect on anti-IgM- or PMA and ionomycin-induced cytokine production (Table I). These results indicate that CpG DNA-mediated p38 activation contributes to the activation of AP-1 and leads to the production of cytokines such as IL-6 and TNF-α.

CpG DNA-mediated JNK and p38 activation is dependent upon a chloroquine-sensitive step

CpG DNA triggers the activation of the transcription factor NF-κB within 15 min in B and monocytic cells through a pathway that is sensitive to the inhibitor of endosomal acidification, chloroquine (7). With this in mind, we investigated whether CpG DNA-mediated MAPK activation is dependent upon a chloroquine-sensitive step. The CpG DNA-mediated phosphorylation of JNK, p38, c-Jun, and ATF-2; the induction of JNK and p38 kinase activities; and the induction of the DNA-binding activity of AP-1 were inhibited by chloroquine (Figs. 4*B* and 5 and data not shown). The activation of these pathways by anti-CD40 or LPS was unaffected by chloroquine, demonstrating the specificity of this inhibition (Figs. 4*B* and 5 and data not shown).

Discussion

The discovery that the immune system "recognizes" bacterial DNA by its content of unmethylated CpG motifs (3) led to the question of how the recognition of CpG DNA activates B cell and macrophage responses. CpG DNA does not appear to activate cells through a cell surface receptor, but does require cell uptake, through which DNA is internalized into an acidified intracellular compartment (3, 28). It has become evident that one mechanism that transduces the CpG DNA signal into the nucleus of cells exposed to bacterial DNA is the degradation of IκB and the activation of NF-κB, as discussed previously (7, 26). The activation of NF-κB in CpG DNA-treated cells is preceded by and may be dependent upon the generation of reactive oxygen species (7) and also appears to be dependent upon the acidification of the DNA, since inhibitors of endosomal acidification specifically block the

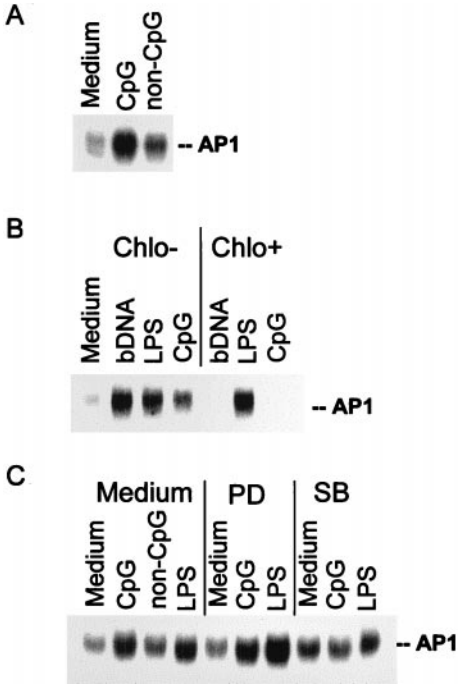

FIGURE 4. CpG DNA induces DNA-binding activity of transcription factor AP-1. *A*, CpG DNA activates AP-1 in WEHI-231 cells. *B*, CpG DNA induces the DNA-binding activity of AP-1 through a chloroquine-sensitive pathway. *C*, p38 inhibitor blocks CpG DNA-mediated AP-1 activation. WEHI-231 cells (*A*) (2×10^6/ml) were stimulated with medium, CpG DNA (2 μM), or non-CpG DNA (2 μM) for 30 min. J774 cells (*B* and *C*) (2×10^6/ml) were pretreated with medium, chloroquine (5 μg/ml) (*B*), PD98059 (100 μM) (*C*), or SB202190 (10 μM) (*C*) for 1 h. Cells were stimulated with medium, *Escherichia coli* DNA (bDNA: 5 μg/ml), LPS (100 ng/ml for *B* and 1 μg/ml for *C*), CpG DNA (1 μM for *B* and 2 μM for *C*), or non-CpG DNA (2 μM) for 1 h (*C*) or 2 h (*B*). Equal amounts (3 μg/lane) of nuclear extracts were analyzed by EMSA for the DNA-binding activities of transcription factor AP-1 using ^{32}P-labeled oligonucleotides containing the AP-1 binding sequence as a probe. The band specificity was confirmed by cross-competition with unlabeled specific and nonspecific oligonucleotides (data not shown). The experiments were repeated three times with similar results using extracts from two B cell lines (WEHI-231 and CH12.LX) and two monocyte-like cell lines (J774 and Raw264.7).

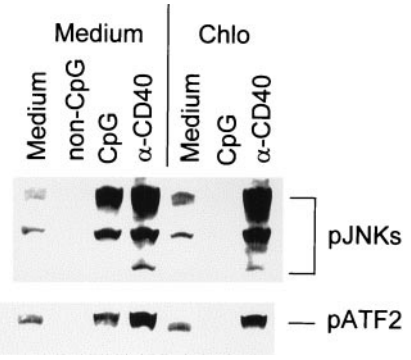

FIGURE 5. Endosomal acidification is required for CpG DNA-mediated MAPK activation. WEHI-231 cells (2×10^6/ml) were pretreated with medium or chloroquine (2.5 μg/ml) for 2 h and then stimulated with medium, CpG DNA (1 μM), non-CpG DNA (1 μM), or anti-CD40 (2 μg/ml) for 30 min. Whole cell lysates (50 μg/lane) were analyzed by Western blot using a specific Ab against the phosphorylated forms of JNK (pJNK) or ATF-2 (pATF-2). Similar results were observed for CpG DNA-mediated p38 activation and c-Jun phosphorylation in both WEHI-231 and J774 cells (data not shown). The experiment was repeated more than three times with similar results.

activation of NF-κB by CpG DNA, but not by LPS, anti-CD40, or anti-IgM (7).

Some of the genes whose expression is induced by CpG DNA do not have known NF-κB binding sites in their promoters, which suggested to us the possibility that additional intracellular signaling pathways may also be induced in CpG DNA-triggered cells. We now report that two of the MAPK pathways, p38 and JNK, are rapidly activated in both B cells and macrophage-like cells following treatment with CpG DNA. Our results suggest that the activation of these kinases may lead to the phosphorylation and activation of the transcription factor AP-1, which may participate in the induction of new gene transcription in CpG DNA-treated cells. We hypothesize that the signals from these different pathways are integrated in the nucleus, where the pathways converge. Inhibition of NF-κB leads to the suppression of cytokine production as well as B cell growth and apoptosis protection mediated by CpG DNA (7, 26). Interestingly, the inhibition of p38 by its specific inhibitor SB202190 led to the inhibition of CpG DNA-mediated cytokine production (Table I). Thus, activation of both NF-κB and AP-1 is required for CpG DNA-mediated cytokine production, but other immune effects of CpG DNA may require only one of these pathways. The present results demonstrate that CpG DNA-mediated MAPK activation is also dependent upon a chloroquine-sensitive step, which might be endosomal acidification. Since chloroquine specifically blocks all of the CpG DNA-induced signaling events identified to date (7, 29), we hypothesize that it blocks a very early step in the signaling pathway triggered by CpG DNA, after which the signal may diverge into the NF-κB and MAPK pathways. Further studies are underway to evaluate these possibilities.

Note added in proof. Since the submission of this manuscript, we have learned that similar results have been obtained by another group (30).

Table I. *P38 inhibitor blocks CpG DNA-mediated cytokine production*[a]

	Medium		Anti-IgM		CpG DNA		NonCpG DNA		PMA + Ionomycin	
	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6	TNF-α	IL-6
Medium	ND	90	281	186	478	3596	ND	114	1256	4773
SB202190 (5 μM)	ND	40	252	236	41	704	ND	22	822	1464
SB202190 (10 μM)	ND	ND	77	177	ND	382	ND	ND	610	1890

[a] WEHI-231 cells (10^6 cells/ml for IL-6 and 10^7 cells/ml for TNF-α) were pretreated with SB202190 (5 or 10 μM) for 1 h and then stimulated with medium, CpG or non-CpG DNA (1 μM), anti-IgM (10 μg/ml), or PMA (100 ng/ml) plus ionomycin (1 μM) for 4 h (for TNF-α) or 12 h (for IL-6). Culture supernatants were analyzed by ELISA for TNF-α or IL-6 (pg/ml). ND stands for not detected. Data (pg/ml) represent the mean of triplicates. SE was <10%. The experiment was repeated three times with similar results.

Acknowledgments

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