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In Vivo Role of p38 Mitogen-Activated Protein Kinase in Mediating the Anti-inflammatory Effects of CpG Oligodeoxynucleotide in Murine Asthma

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DNA containing unmethylated CpG motifs is intrinsically immunostimulatory, inducing the production of a variety of cytokines and chemokines by immune cells. The strong Th1 response triggered by CpG oligodeoxynucleotide (ODN) inhibits the development of Th2-mediated allergic asthma in mice. This work documents that CpG ODN-induced IL-12 production plays a critical role in this process, because intrapulmonary CpG ODN inhibits allergic inflammation in wild-type but not IL-12−/− mice. CpG ODN rapidly localized to alveolar macrophages (AM), thereby triggering the phosphorylation of p38 mitogen-activated protein kinase (MAP kinase). AM cultured with CpG but not control ODN up-regulated IL-12 p40 expression and release, and these effects were blocked by the highly specific p38 MAP kinase inhibitor SB202190. Intrapulmonary administration of this inhibitor blocked the ability of CpG ODN to produce IL-12 in the lungs and reversed the anti-inflammatory effects of CpG ODN on allergic lung inflammation. These findings indicate that IL-12 production by AM is stimulated by intrapulmonary CpG ODN administration through a p38 MAP kinase-dependent process, and IL-12 is a key cytokine that mediates CpG ODN-induced protection against allergic lung inflammation. The Journal of Immunology, 2002, 169: 5955–5961.

Asthma is an inflammatory disease of the airways that affects 5–10% of the general population. Airway eosinophilia and Th2 cytokine production are characteristic of asthma (1–3). In contrast to the permissive role of Th2 cytokines in asthma, IFN-γ, a product of Th1 cells, potently inhibits eosinophilic lung inflammation (4, 5). IL-12 is a cytokine that potentiates differentiation of naïve T cells into Th1 effector cells while inhibiting their differentiation into Th2-secreting cells (6, 7). We and others have shown that IL-12 inhibits eosinophil recruitment, decreases IgE levels, and suppresses bronchial hyperresponsiveness in murine models of allergic asthma when it is given systemically within 4–72 h of allergen challenge (8–10).

It is well documented that synthetic oligodeoxynucleotides (ODN) containing CpG motifs potently stimulate the mammalian immune system. These CpG ODN rapidly stimulate T cells, B cells, NK cells, and macrophages to proliferate, secrete Abs, and induce the proinflammatory and Th1-associated cytokines, such as IL-12 (11–13). Investigators have reported that administering CpG ODN before sensitization or challenge by a variety of allergens inhibits the development of allergic inflammation (14, 15). We extended these observations by demonstrating that intrapulmonary administration of CpG ODN provided long term protection against allergic inflammation in a murine model of asthma and documented that the inhibitory effects of CpG ODN were mediated through IFN-γ (16).

The mitogen-activated protein kinases (MAP kinases), p38 and c-Jun N-terminal kinase (JNK) are activated by a variety of environmental stresses, and some evidence suggests that MAP kinases play a role in T cell activation and cytokine production. The role of the MAP kinase JNK2 has been investigated in Th1 differentiation, and IL-12 fails to induce differentiation of JNK2-deficient CD4+ T cells into effector Th1 cells (17, 18). These observations suggest that the p38 and JNK MAP kinase-signaling pathways play an important role in the induction of Th1 immune responses. CpG have been shown to activate MAP kinases and regulate cytokine production from B cell lines and monocytic cell lines (19–21). On the basis of these in vitro studies suggesting an important role of MAP kinases in the production of Th1-associated cytokines, we examined their role in mediating the anti-inflammatory effects of CpG ODN in vivo. Our results suggest that p38 MAP kinase is critical for mediating the anti-inflammatory effects of CpG ODN in murine asthma.

Materials and Methods

Animals and immunization

Experiments were performed on 6- to 8 wk-old female BALB/c mice that were purchased from Harlan Sprague Dawley (Indianapolis, IN). Some experiments were conducted on wild-type (WT) and IL-12−/− BALB/c mice purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a pathogen-free environment throughout the experiment at University of Texas Medical Branch (Galveston, TX). The Institutional Animal Care and Use Committee of the Academic Medical Center approved all animal experiments.
Cpg and control GpC ODN

Two immunostimulatory ODN containing Cpg motifs (GCTAGACGT TACGCT and TCAAGCTT) were synthesized using a phosphorothioate backbone as previously described (16). Control ODN were of the same sequence except the Cpg motif was inverted to GpC (GCTAGACGT TACGCT and TCAAGCTT). All ODN were produced on the same synthesizer and were column purified. These ODN contained undetectable levels of endotoxin (<0.02 U/kg, as determined using a Limulus amoebocyte lysate analysis kit (QCL-1000; BioWhittaker, Walkersville, MD). For in vivo experiments, all ODN were administered intranasally (i.n.) at a dose of 35 μg/animal in 100 μl PBS.

In vivo experiments

The first set of in vivo experiments (Figs. 1A and 2A) were designed to determine the role of Cpg ODN in mediating IL-12 production in naive BALB/c mice. Either control GpC or Cpg ODN were given i.n. After 48 h, bronchoalveolar lavage (BAL) was performed. In the second set of experiments (Fig. 1B), the role of IL-12 in mediating the effects of Cpg ODN was determined. We used a protocol similar to the one described by us previously (16). Briefly, WT and IL-12−/− BALB/c mice were sensitized by two injections of 150 μg endotoxin-free ragweed (RWC; Greer Laboratories, Lenoir, NC) adsorbed with 150 μg of aluminum hydroxide (alum) in 100 μl PBS on days 0 and 4. Cpg ODN (35 μg/100 μl/mouse) was administered i.n. on day 9, 48 h before allergen challenge. Allergen challenge was performed i.n. on day 11 with 100 μg RW. Three days later, on day 14, the mice were sacrificed, and BAL fluids were collected. The third set of experiments (Fig. 1C) were designed to determine the role of p38 MAP kinase in mediating the effects of Cpg ODN in a mouse model of asthma. BALB/c mice were sensitized to RW as outlined above. On day 9, water-soluble p38 MAP kinase inhibitor SB202190 was administered via the intrapulmonary route in a dose of 5 mg/kg body weight, divided into two equal doses, and administered 90 and 30 min before Cpg ODN administration. RW challenge was performed i.n. 48 h later on day 11 with 100 μg of RW. Three days later, on day 14, the mice were sacrificed.

Uptake of Cpg ODN after intrapulmonary administration

To track uptake of Cpg and GpC ODN, naive BALB/c mice were challenged i.n. with FITC-labeled Cpg and Rhodamine-labeled GpC. The sequence of labeled Cpg and GpC ODN were identical with those listed above. At various time points after intrapulmonary administration, a BAL was performed. A cytospin preparation was analyzed by krypton-argon laser two-color confocal microscopy (Olympus, Salt Lake, UT).

IL-12 expression in alveolar macrophages (AM)

AM 99% pure were isolated from BAL fluids of five naive BALB/c mice and pooled. These cells were cultured with PBS, 2 μM Cpg ODN, or 2 μM control ODN (GpC) for 2 h and then lysed to extract total RNA. In some experiments, AM were pretreated for 1 h with various concentrations of SB202190 and then cultured with 2 μM Cpg ODN for 2 h. The mRNA expression level of IL-12 p40 was determined by either relative RT-PCR or real time quantitative RT-PCR performed in TaqMan ABI Prism. In both types of PCR, the IL-12 p40 primer sequences were as follows: sense 5‘-ACA TCT ACC GTA CAA TGC A-3‘; antisense, 5‘-GGA ATT GTA ACG ATC CTG ACG-3‘. For TaqMan, the internal probe sequence was: 5‘-6FAM-TGC AAG CAC ACA TCG CCT TAMRA-3‘. In separate experiments AM were pretreated with or without various concentrations of SB202190 and then cultured either with PBS, 2 μM Cpg ODN, or 2 μM control GpC for 12 h, and cell supernatants were collected to measure IL-12 release.

Determination of IL-12 levels

IL-12 levels in BAL fluids and culture supernatants were determined using two-site immunoenzymometric ELISA (Endogen, Woburn, MA) according to the manufacturer’s instructions. Each value represents the mean of duplicate values.

Activation of MAP kinases in AM and peritoneal macrophages (PM)

AM and PM were isolated from naive BALB/c mice by alveolar and peritoneal lavage, respectively. For AM, BAL lavage cells were pooled from 25 naive BALB/c mice. Cells were plated in complete medium (RPMI supplemented with 10% FBS, 2 mM l-glutamine; Life Technologies, Gaithersburg, MD). Four hours after plating, nonadherent cells were removed by extensive washing. For each stimulation, 4 × 106 AM or 2 × 106 PM were cultured for 12 h. The next morning, cells were supplemented with PBS, 2 μM Cpg ODN, or 2 μM control ODN for 5–120 min. Immediately after incubation, cells were washed, pelleted, and lysed in 100 μl of radioimmunoprecipitation buffer (50 mM Tris (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40) and supplemented with 1 mM DTT, 2 μg/ml pepstatin, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Cell lysates were used for Western blot analyses. Briefly, the whole cell lysate was removed, combined with an equal volume of 2× Laemmli sample loading buffer, and boiled for 2 min; 50 μl of this preparation was added to each lane of a 10% SDS-polyacrylamide gel. The proteins were transferred to a polyvinylidine difluoride membrane. Western blot analyses were performed with an affinity purified rabbit polyclonal Ab against phosphorylated p38 MAP kinase (New England Biolabs, Beverly, MA). After a washing step with TBS-T, the membrane was incubated with a goat anti-rabbit IgG secondary Ab conjugated to HRP for 30 min. Chemiluminescent detection of phosphorylated p38 was performed using ECL Western blotting detection reagents (Amersham Life Sciences, Piscataway, NJ) followed by film exposure. Sequential Western blot analyses were performed after repeatedly strip-washing the same blot, using a mAb to pERKs, a mouse mAb (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal Ab to pJNKs, (BioSource International, Camarillo, CA), and a rabbit affinity-purified polyclonal Ab to whole p38 MAP kinase (Santa Cruz Biotechnology).

Data analysis

BAL cell counts and IL-12 levels in BAL fluids are presented as means ± SEM. The difference in outcome variables between treatment groups was analyzed by ANOVA. Significant ANOVAs were further analyzed by the Bonferroni/Dunn post hoc test, and p < 0.05 was considered statistically significant.

Results

IL-12 is critical to Cpg ODN-mediated inhibition of allergic lung inflammation

We and others have previously shown that intrapulmonary administration of Cpg ODN but not control ODN inhibits allergic inflammation (14, 16). On the basis of reports that Cpg ODN stimulates macrophages to produce IL-12 (11, 12) and that IL-12 potently inhibits Th2-mediated allergic lung inflammation (8), we...
examined the ability of intrapulmonary administration of CpG ODN to induce IL-12 production in the lungs. Forty-eight hours after intrapulmonary administration CpG ODN in naive BALB/c mice (Fig. 1A) induced a 10-fold higher IL-12 level in BAL fluids than administration of GpC ODN (p < 0.00001; Fig. 2A). We sought to determine the role of this increase in IL-12 levels in mediating the anti-inflammatory effects of CpG. CpG ODN was administered 48 h before RW challenge to optimize its effects in the mouse model of asthma (16) and potently induce IL-12 production in BAL fluids of naive mice (Fig. 2A). Consistent with our previous findings (16), intrapulmonary CpG ODN treatment of WT mice significantly inhibited recruitment of eosinophils after RW challenge (p < 0.01; Fig. 2B). However, CpG ODN treatment in IL-12−/− mice failed to inhibit eosinophil infiltration (p = NS). RW challenge induced eosinophil recruitment in IL-12−/− mice to the same extent as WT mice (p = NS), indicating that the genotypic difference does not alter eosinophil recruitment. These data indicate that IL-12 plays an important role in mediating the anti-inflammatory effects of CpG ODN in a mouse model of asthma.

CpG ODN are internalized by AM

To determine which cells mediate the anti-inflammatory effects of CpG ODN in the mouse model of asthma, the uptake and internalization of CpG ODN after intrapulmonary administration were monitored. Intrapulmonary challenge was performed with FITC-labeled CpG ODN and Rhodamine-labeled GpC ODN administration in naive BALB/c mice, and BAL cells were collected at various time points after administration and analyzed by confocal microscopy. Results indicate that AM internalized both CpG (green fluorescence; Fig. 3A) and control GpC ODN (red fluorescence; Fig. 3B) within 30 min. Furthermore, CpG and GpC ODN colocalized in the same vesicle within AM as indicated by yellow color (Fig. 3C). When these cells were counterstained with Wright-Giemsa stain, they were confirmed to be AM. Thus, AM internalize CpG ODN within 30 min into the cytosolic compartment.

CpG ODN up-regulates IL-12 p40 mRNA expression and release from AM

Building on the observations that AM rapidly internalize CpG ODN into the cytosolic compartment, and that macrophages are frequently an important source of IL-12, the effect of CpG ODN on IL-12 p40 mRNA expression was examined. As seen in Fig. 4A, culture of AM with CpG ODN up-regulated expression of IL-12 p40, unlike cells culture with PBS or GpC ODN. Real time quantitative RT-PCR confirmed these findings and demonstrated that CpG ODN increased IL-12 p40/β-actin expression levels 80-fold over PBS and GpC treatment (data not shown). CpG also induced
IL-12 p40 up-regulation to a similar extent in peritoneal macrophages (data not shown). In additional studies, culture of AM with CpG ODN augmented IL-12 production, unlike cells cultured with PBS or GpC ODN (Fig. 4B). These data indicate that CpG ODN specifically up-regulates IL-12 p40 expression and release from AM.

CpG ODN triggers rapid phosphorylation of p38 MAP kinases in macrophages

Previous studies have shown that CpG ODN induces the phosphorylation of MAP kinases, a major cytosolic signaling pathway. Because it is difficult to obtain large numbers of AM, initial cell experiments were conducted on PM. CpG ODN, but not PBS or GpC ODN, triggered rapidly phosphorylation of p38 MAP kinase in PM in 15 min (Fig. 5A). This rapid phosphorylation of p38 MAP kinase was not associated with any change in total p38 MAP kinase (Fig. 5A). None of these agents induced phosphorylation of extracellular regulated kinase (ERK) 1/2 and JNK 1/2 MAP kinases. Next we examined the effects of CpG ODN on phosphorylation of p38 MAP kinase in AM. As shown in Fig. 5B, CpG and LPS, but not PBS or GpC ODN, induced phosphorylation of p38 MAP kinase within 5 min in AM.

The p38 MAPK inhibitor decreases CpG ODN-induced IL-12 production from AM

The specific p38 MAP kinase inhibitor SB202190 was used to examine the role of CpG ODN-induced phosphorylation on IL-12 p40 expression. As shown in Fig. 6A, CpG rapidly up-regulated IL-12 p40 mRNA expression in cultured AM. This effect was suppressed in a dose-dependent manner by pretreatment of AM with SB202190. Furthermore, CpG ODN rapidly induced IL-12 production from cultured AM, and this induction was also inhibited in a dose-dependent manner, in which half-maximal inhibition was observed at the concentration of 100 nM (Fig. 6B). These data...
flammation in anti-inflammatory effects of CpG ODN on allergic airway

The p38 MAPK inhibitor reverses IL-12-mediated anti-inflammatory effects of CpG ODN in the mouse model of asthma

By blocking CpG ODN-induced IL-12 production from AM, SB202190 may decrease IL-12 production in the lungs and consequently reverse the beneficial effects of CpG ODN on allergic lung inflammation. To test this hypothesis, 5 mg/kg SB202190 were administered into the lungs shortly before CpG administration in the mouse model of asthma. Similar to our results in naïve mice (Fig. 2A), administration of CpG ODN increased IL-12 levels in BAL fluids in the mouse model of asthma (p < 0.0001), and this increase was blocked by prior intrapulmonary administration of SB202190 (p < 0.001; Fig. 7). However, CpG ODN treatment failed to increase serum levels of IL-12 (data not shown).

The p38 MAPK inhibitor reverses IL-12-mediated anti-inflammatory effects of CpG ODN in allergic airway inflammation

Consistent with our earlier results (16), CpG ODN potently inhibited influx of total inflammatory cells (p < 0.05; Fig. 8A) and total eosinophils (p < 0.0001; Fig. 8B) into BAL fluids after RW challenge. Administration of SB202190 before administration of CpG ODN reversed these beneficial effects of CpG ODN. However administration of SB202190 by itself had no direct effect on RW challenge-induced allergic inflammation. These observations indicate that p38 MAP kinase mediate the anti-inflammatory effects of CpG ODN in a mouse model of asthma.

Discussion

We and others have previously reported that CpG but not control ODN inhibits allergic lung inflammation (14–16). We previously reported that intrapulmonary CpG ODN administration is most effective when it is administered 48 h before RW challenge (16). The current work documents that CpG ODN treatment fails to inhibit RW challenge induced eosinophilic inflammation in IL-12−/− mice. These data indicate that IL-12 plays a crucial role in mediating the anti-inflammatory effects of CpG ODN in this mouse model of asthma, a conclusion consistent with evidence that IL-12 potently inhibits Th2-mediated allergic lung inflammation (8, 9). However, our findings differ from those of Kline et al. (22), who reported that the anti-inflammatory effects of CpG ODN in the mouse model of asthma are independent of IL-12. In that report, CpG conferred protection against both airway eosinophilia and bronchial hyperreactivity in the absence of IFN-γ or IL-12 or both IFN-γ and IL-12 (22). It is possible that differences in the allergen model used (schistosome egg-Ag vs RW) to induce asthma or timing of CpG doses (during allergic sensitization in the Kline study and 48 h before RW challenge in sensitized mice in our study) may account for these differences.

It is well established that CpG ODN stimulates monocytes and macrophages to secrete IL-12 (11, 12, 21, 23, 24). However, other reports indicate that in vivo CpG ODN treatment fails to increase systemic levels of IL-12 (25). These reports are consistent with our finding that CpG ODN treatment in murine asthma increased local IL-12 levels in the lungs by stimulating AM but failed to increase the serum IL-12 levels. We previously reported that very low levels of mucosally administered IL-12 profoundly inhibit allergic lung inflammation and airway reactivity (26, 27). It is therefore likely that the increase in airway IL-12 levels after intrapulmonary administration of CpG is sufficient to potentiate inhibiting eosinophilic lung inflammation, possibly via inhibition of eosinophilopoiesis (28).

Previous studies document that CpG ODN activates MAP kinases in dendritic cells and B- and monocytic cell lines (19–21). For example, Yi and Krieg (20) reported that CpG but not control ODN phosphorylated p38 MAP kinase and several isoforms of JNK in murine B cell line WEHI-231 and in monocyte-like cell line J774. They also reported that the p38 MAP kinase inhibitor SB202190 inhibited CpG ODN-induced IL-6 and TNF-α production from the murine WEHI-231 B cell line (20). Hacker et al. (21) initially reported that CpG ODN activates p38 MAP kinase in ANA-1 and RAW264.7 macrophage cell lines and that p38 MAP kinase regulated TNF-α and IL-12 production in these cells and in bone marrow-derived dendritic cells. Hacker et al. (29) subsequently reported that CpG activates the mitogen-activated protein kinase kinase-ERK pathway in RAW264.7 macrophage cell line and a mitogen-activated protein kinase kinase inhibitor blocks IL-12 production from these cells. In contrast to RAW264.7, they

![FIGURE 7.](image-url)  
**FIGURE 7.** p38 MAP kinase mediates CpG ODN-induced IL-12 release in the airways in a mouse model of asthma. BALB/c mice were sensitized to RW and treated with either CpG ODN or CpG ODN plus SB202190 administered via the intrapulmonary route at a dose of 5 mg/kg body weight on day 9 (Fig. 1C). On day 11, a challenge was performed with RW. On day 14, the mice were euthanized, and the BAL fluids were analyzed for IL-12 levels. PBS/RW, PBS i.n. on day 9 and RW i.n. on day 11; CpG/RW, CpG i.n. on day 9 and RW i.n. on day 11; SB+CpG/RW, SB202190 plus CpG i.n. on day 9 and RW i.n. on day 11. n = 7–12 per group.

![FIGURE 8.](image-url)  
**FIGURE 8.** p38 MAP kinase mediates the anti-inflammatory effects of CpG ODN on allergic lung inflammation. The BAL fluids from the animals in Fig. 7 were analyzed for total inflammatory cells (A) and eosinophils (B) on day 14. CpG/RW = CpG i.n. on day 9 and RW i.n. on day 11. SB+CpG/RW, SB202190 plus CpG i.n. on day 9 and RW i.n. on day 11; SB/RW, SB202190 i.n. on day 9 and RW i.n. on day 11. n = 7–12 per group.
reported that CpG ODN activates JNK and p38 MAP kinases in bone marrow-derived dendritic cells (29). In another study, Yi et al. (30) reported that p38 activity was essential for both IL-10 and IL-12 production from RAW 264.7 cell line. Our work extends their findings to physiologically relevant PM and demonstrates that CpG phosphorylates p38 MAP kinase, but not ERK or JNK MAP kinases, in these cells. The differences in signaling pathways used by macrophage cell lines and normal PM may reflect physiological differences between these cell types. Ours is the first report showing that CpG ODN induces phosphorylation of p38 MAP kinase in AM and that this event is critical for mediating CpG-induced production of IL-12 from AM.

A notable finding in our study is that p38 MAP kinase mediates the anti-inflammatory effects of CpG ODN in a mouse model of asthma. We show that administration of p38 MAP kinase inhibitor just before CpG ODN administration in the present study blocked the anti-inflammatory effects of CpG ODN in murine asthma. These observations are somewhat surprising because p38 MAP kinase inhibitors can block allergic lung inflammation when they are administered at the time of allergen challenge in animal models of asthma (31, 32). Furthermore, p38 MAP kinase inhibitors can inhibit other inflammatory diseases such as LPS-induced neutrophilic inflammation and LPS-induced IL-6 and MMP-9 production (33–35). Interestingly, p38 MAP kinase inhibitors have also been reported to prevent arthritis, osteoporosis, and brain injury (36–38). In light of these potent anti-inflammatory effects, it seems puzzling that these inhibitors block the anti-inflammatory effects of CpG ODN in the present study. A likely explanation for our results is that the timing of administration of these inhibitors with respect to the causative agent being tested is a critical determinant of the final outcome. Consistent with this hypothesis, administration of this inhibitor had no direct effect on allergic inflammation in our study because it was administered 48 h before RW challenge. These findings extend our knowledge from prior reports that administration of p38 MAP kinase inhibitors just before allergen challenge blocked allergen-induced eosinophilic inflammation (31).

In summary, this work clarifies the mechanism by which intrapulmonary CpG ODN prevent allergen-induced lung inflammation. We show that intrapulmonary CpG ODN are internalized by AM, triggering them to up-regulate production of IL-12. IL-12 is critical for mediating the beneficial effects of CpG ODN on allergic inflammation. We further show that IL-12 expression and release are critically mediated through CpG ODN-induced phosphorylation of p38 MAP kinase in AM and in vivo in the lungs and that p38 MAP kinase mediates the anti-inflammatory effects of CpG ODN on allergic inflammation. These findings may facilitate development of CpG ODN as a therapeutic agent in allergic asthma.

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