Potential Role of Phosphatidylinositol 3 Kinase, rather than DNA-dependent Protein Kinase, in CpG DNA–induced Immune Activation

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Abstract

Unmethylated CpG motifs present in bacterial DNA stimulate a strong innate immune response. There is evidence that DNA-dependent protein kinase (DNA-PK) mediates CpG signaling. Specifically, wortmannin (an inhibitor of phosphatidylinositol 3 kinase [PI3]-kinases including DNA-PK) interferes with CpG-dependent cell activation, and DNA-PK knockout (KO) mice fail to respond to CpG stimulation. Current studies establish that wortmannin actually inhibits the uptake and colocalization of CpG DNA with toll-like receptor (TLR)-9 in endocytic vesicles, thereby preventing CpG-induced activation of the NF-κB signaling cascade. We find that DNA-PK is not involved in this process, since three strains of DNA-PK KO mice responded normally to CpG DNA. These results support a model in which CpG signaling is mediated through TLR-9 but not DNA-PK, and suggest that wortmannin-sensitive member(s) of the PI3-kinase family play a critical role in shuttling CpG DNA to TLR-9.

Key words: toll-like receptors • wortmannin • dendritic cells • innate immunity • cytokines

Introduction

Toll-like receptors (TLRs) mediate host recognition of "pathogen-associated molecular patterns" expressed by infectious microorganisms (1). Engagement of TLR triggers an innate immune response that promotes the elimination of foreign pathogens and supports the development of adaptive immunity (2). For example, "CpG motifs" present at high frequency in bacterial, but not mammalian DNA, interact with TLR-9, stimulating an immune response characterized by the production of cytokines, chemokines, and Ig by B lymphocytes, NK cells, macrophages, and dendritic cells (DCs; references 3–6).

Previous studies established that CpG DNA interacts with TLR-9 in endosomal vesicles, triggering a signaling cascade involving NF-κB–dependent pathways (7–9). Independently, Chu et al. recently reported that the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) has a central role in CpG-mediated immune activation (10). Their findings imply that CpG motifs must interact with two independent receptor molecules (TLR-9 and DNA-PKcs) before eliciting an innate immune response (11).

DNA-PK consists of three subunits (DNA-PKcs, Ku70, and Ku80) that play a critical role in DNA repair and *V(D)J* recombination (19–21). Other members of the phosphatidylinositol 3-kinase (PI3)-kinase family contribute to the phosphorylation of membrane inositol lipids involved in signal transduction and membrane transport (12, 13), facilitate the uptake and processing of foreign molecules, and contribute to phagosome formation and maturation (14–17). Wortmannin inhibits these events by blocking the conserved ATP-binding site present in the catalytic domain of the PI3-kinase family (18).

This work examines the mechanism by which wortmannin inhibits CpG-induced cellular activation and reexamines the involvement of DNA-PK in this process. Results indicate that wortmannin inhibits the uptake and/or entry of CpG DNA into TLR-9-containing endocytic vesicles. In contrast to the report by Chu et al. (10), DNA-PK was not involved in this process, since knockout (KO) mice lacking each of the three functional subunits of DNA-PK responded normally to CpG DNA. These findings support a model in which one or more members of the wortmannin-sensitive PI3-kinase family facilitate the interaction of CpG DNA with TLR-9 in endocytic vesicles, thereby triggering immune activation.

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Materials and Methods

Mice. Female C57BL/6 mice (6–10-wk old) were obtained from The Jackson Laboratory while DNA-PKcs$^{-/-}$ and Ku70$^{-/-}$ were obtained from Frederick Alt (Harvard Medical School, Boston, MA; references 19 and 20). Ku80$^{-/-}$ mice were generated as described previously (21). All studies were approved by the CBER Animal Care and Use Committee.

Reagents. Genomic DNA from *E. coli* and calf thymus, LPS, and wortmannin were obtained from Sigma-Aldrich. Genomic DNA was repurified by treatment with Triton-X114 to remove LPS as described previously (22). Single-stranded DNA was prepared by heat denaturing double-stranded DNA at 95°C for 5 min followed by immediate cooling on ice. Phosphorothioate, Cy3-, and FITC-labeled oligodeoxynucleotides (ODNs) were synthesized at the CBER core facility. The sequence of the ODNs used in this work are: CpG ODN$_{1555}$; GCTAGACGTTAGCGT, CpG ODN$_{1466}$; TCAACGTTGA, human CpG ODN$_{K3}$; ATCGACTCTCGAGCGTTCTC, control ODN$_{1612}$; GCTAGATGTTAGCGT, control ODN$_{1471}$; TCAAGCTTGA, and human control ODN$_{K10}$; ATGCACTCTGCAGGCTTCTC (9, 23). Less than 0.01 U/μg of endotoxin was present in any DNA used in this work, as determined by Limulus amebocyte lysate (LAL) assay (Bio-Whittaker).

Cell Transfection and Luciferase Assay. HEK-293 (5×10^4) cells (American Type Culture Collection) were transfected with 0.8 μg of vector plasmid (pCIneo; Promega), pCIneo-hTLR9, or pCIneo-hTLR4 (9) plus 0.1 μg of p5xNF-κB-luc (Stratagene) and 0.1 μg of pSV-β-galactosidase (Promega) and incubated for 24 h at 37°C. The cells were then stimulated with ODNs or LPS for 24 h. Luciferase assays were performed according to the manufacturer's instructions (Promega). β-galactosidase activity was used to normalize the values.

Flow Cytometric Analysis of Cell Surface Molecule Expression. In brief, ODN-stimulated cells were washed with PBS, fixed with 4% paraformaldehyde for 5 min at 37°C, and stained with FITC- or PE-labeled Abs in the presence of anti-CD16 Ab for 30 min at room temperature (22). Stained cells were washed, resuspended in PBS/0.1% BSA plus azide, and analyzed by FACSort™ (BD Biosciences). Abs specific for the following molecules were obtained from BD PharMingen: CD11c; CD40; CD80; and CD86.

Cytokine-specific ELISA. Immulon 2 microtiter plates (96-well) were coated with anti–IL-12 (BD PharMingen) in PBS for 4 h. After the plates were blocked and washed, supernatants from stimulated cells were added for 2 h at room temperature. The plates were then washed and treated with biotinylated anticytokine Ab followed by phosphatase-streptavidin (BD PharMingen) as described previously (22). A standard curve generated using recombinant IL-12 was used to determine cytokine concentration.

CpG ODN Internalization. Cells were incubated with 1–3 μM FITC-labeled ODN plus 1 μM wortmannin at 37°C. Cells were washed after 1 h, and surface-bound FITC was quenched by trypan blue (1:4 dilution; Sigma-Aldrich). Internalized FITC was quantified by FACSort™ (BD Biosciences) as described previously (24).

Confocal Microscopy. HEK-293 cells that had been stably transfected with human TLR-9 fused with HA (9) were incubated with Cy3-labeled ODN for 10–120 min at 37°C. Cells were washed, fixed, permeabilized, and stained with FITC–anti-HA Ab (clone 3F10; Roche Laboratories). The subcellular localization of Cy3 and FITC signals was determined by confocal microscopy (LSM5 PASCAL; Carl Zeiss).

Results

CpG DNA–induced Immune Activation Is Independent of DNA-PK. It was previously reported that DNA-PK plays a critical role in mediating CpG DNA–dependent immune activation (10). To confirm that finding, we examined the response of three different strains of KO mice, each lacking a different functional subunit of DNA-PK (DNA-PKcs, Ku70, and Ku80) to various CpG motifs. These animals had no mature T or B cells, as expected for the KO phenotype (19–21 and unpublished data). PCR analyses confirmed that the relevant DNA-PK genes were disrupted in these animals (unpublished data). When spleen cells from the KO mice were stimulated with bacterial DNA or CpG ODNs (both phosphodiester and phosphorothioate), their IL-12, IL-6, and IFN-γ responses did not differ significantly from wild-type mice (Fig. 1 and unpublished data). Similarly, bone marrow–derived DCs (BMDCs) from these mice underwent normal cellular maturation (characterized

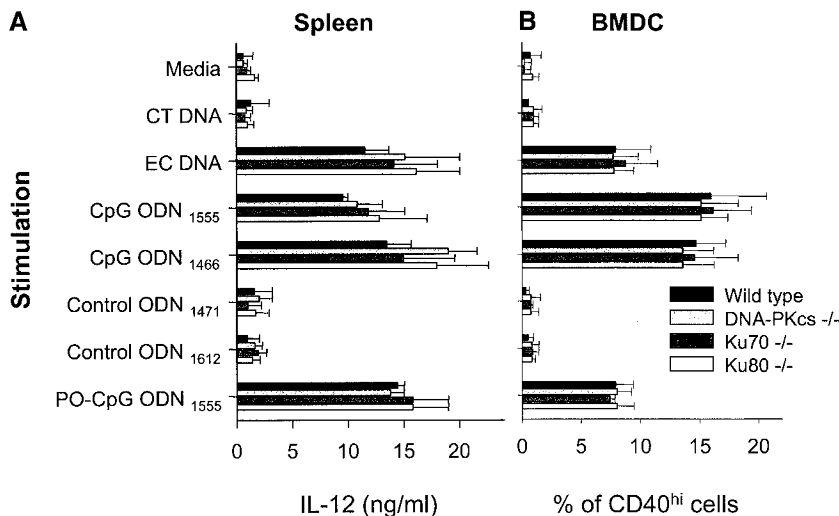

Figure 1. CpG DNA–induced cytokine production and BMDC maturation are independent of DNA-PK. Spleen cells (2.5×10^6 cells/ml) or BMDCs (5×10^5 cells/ml) from DNA-PKcs$^{-/-}$, Ku70$^{-/-}$, Ku80$^{-/-}$, and wild-type mice were incubated with 50 μg/ml of calf thymus (CT) or *E. coli* (EC) DNA, 1 μM for phosphorothioate ODNs, or 10 μM of phosphodiester ODNs (PO) for 48 h. IL-12 levels in culture supernatants were measured by ELISA, while the percentage of mature DCs (expressing high levels of both CD11c and CD40) was evaluated by FACS®. Data represent the average ± SD of the samples from three mice per group.

by the upregulation of CD40, CD80, and CD86) when stimulated with CpG DNA (Fig. 1 and unpublished data). Thus, in contrast to the results reported by Chu et al. we found no deficit in the immune response of DNA-PK KO mice to CpG motifs.

CpG DNA–induced Immune Activation Is Wortmannin Dependent. Chu's finding that wortmannin could inhibit CpG DNA–induced immune activation was then examined. BMDCs were isolated from wild-type and DNA-PKcs KO mice and stimulated with CpG ODN, bacterial DNA, LPS, or double-stranded RNA. Wortmannin significantly inhibited CpG-induced cytokine production and BMDC maturation by cells isolated from both wild-type and KO mice (Fig. 2 and unpublished data). In contrast, wortmannin had no effect on the immune activation induced by LPS or double-stranded RNA (Fig. 2 and unpublished data). These findings confirm that DNA-PK was not involved in CpG-induced immune activation, and suggest some other wortmannin-sensitive molecule (presumably a different member of the wortmannin-sensitive PI3-kinase family) plays a crucial role in mediating CpG-dependent immune activation.

Effect of Wortmannin on CpG ODN Uptake. The mechanism by which wortmannin inhibited CpG-dependent immune activation was then examined. The interaction of CpG DNA with TLR-9 triggers a signaling cascade involving the rapid upregulation of NF-κB and the subsequent production of immunostimulatory cytokines and chemokines (9, 23, 25). To monitor this activity, HEK-293 cells were transiently transfected in vitro with a TLR-9–encoding plasmid (pCIneo-TLR-9) plus a NF-κB–dependent luciferase reporter construct (p5xNF-κB-luc). Consistent with previous findings, transfected cells stimulated with CpG (but not control) ODNs increased luciferase activity by more than ninefold ($P < 0.01$, Fig. 3 and reference 9). Wortmannin reduced this NF-κB–mediated increase in luciferase expression in a dose-dependent manner ($P < 0.01$, Fig. 3). This effect of wortmannin was CpG specific, since HEK-293 cells cotransfected with the luciferase reporter plus a TLR-4–encoding plasmid (which confers responsiveness to LPS) responded normally to LPS stimulation in the presence of wortmannin (Fig. 3). These findings are consistent with a wortmannin-sensitive PI3-kinase contributing to the signaling process by which CpG DNA activates NF-κB.

To further clarify the nature of this contribution, the uptake and localization of CpG DNA was monitored by confocal microscopy. HEK-293 cells were stably transfected with an HA-tagged TLR-9 construct, and then incubated with Cy-3–labeled CpG ODN (red) and then stained with anti-HA Ab (green). As seen in Fig. 4, CpG ODNs readily entered transfected cells and colocalized in endocytic vesicles with HA-tagged TLR-9. This interaction triggered the activation of NF-κB and the production of immunostimulatory cytokines such as IL-8 (Fig. 3 and unpublished data). However, when transfected cells were treated with wortmannin, both the size and number of endocytic vesicles containing CpG ODNs plus TLR-9 decreases significantly, as did NF-κB activation (Figs. 3 and 4). These findings suggest that wortmannin interferes with the endosomal uptake and/or colocalization of CpG ODNs with TLR-9 in target cells.

To confirm this finding in cells that respond physiologically to CpG ODNs, the effect of wortmannin on the internalization of CpG ODNs and subsequent production of IL-12 by BMDCs was examined. As seen in Fig. 5, wortmannin reduced the internalization of CpG ODNs by 90% and the IL-12 response elicited by CpG ODNs by 85% ($P <$ 0.01) when compared with baseline. Similar results were obtained using BMDCs from DNA-PK KO mice, establishing that wortmannin inhibits ODN internalization in a

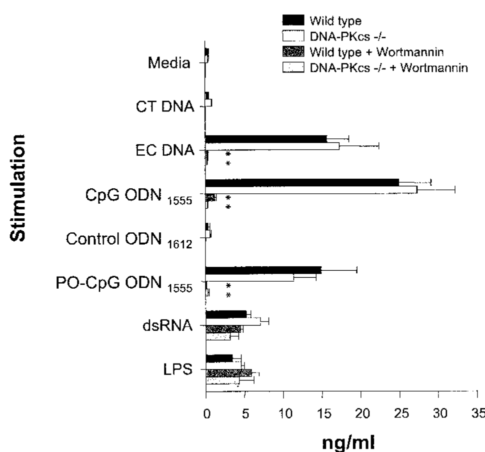

Figure 2. Wortmannin inhibits IL-12 production by CpG DNA in both wild-type and DNA-PKcs KO mice. The effect of wortmannin on the production of IL-12 by BMDCs from both wild-type and DNA-PKcs$^{-/-}$ mice was evaluated as described in the legend to Fig. 1. Additional controls include double-stranded RNA (Poly IC, 50 μg/ml) and LPS (1 μg/ml). Data represent the average ± SD of three mice per group. *$P < 0.01$.

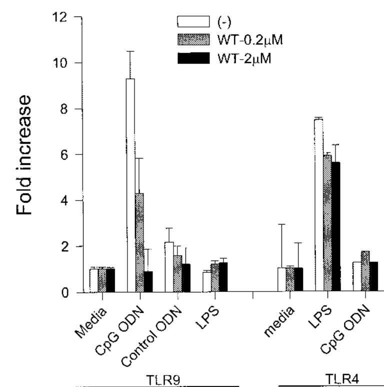

Figure 3. Wortmannin selectively inhibits CpG-induced NF-κB activation. HEK 293 (5×10^4) cells were transfected with plasmids encoding hTLR-9 or hTLR-4 plus p5xNF-κB-luciferase. Cells were stimulated the next day with 1 μM of CpG (K3), control (K10) ODN, or 1 μg/ml of LPS in the presence or absence of wortmannin (WT). The increase in luciferase activity at 24 h is shown. Results represent the average ± SD of triplicate cultures in experiments that were repeated three times. *$P <$ 0.05, **$P < 0.01$.

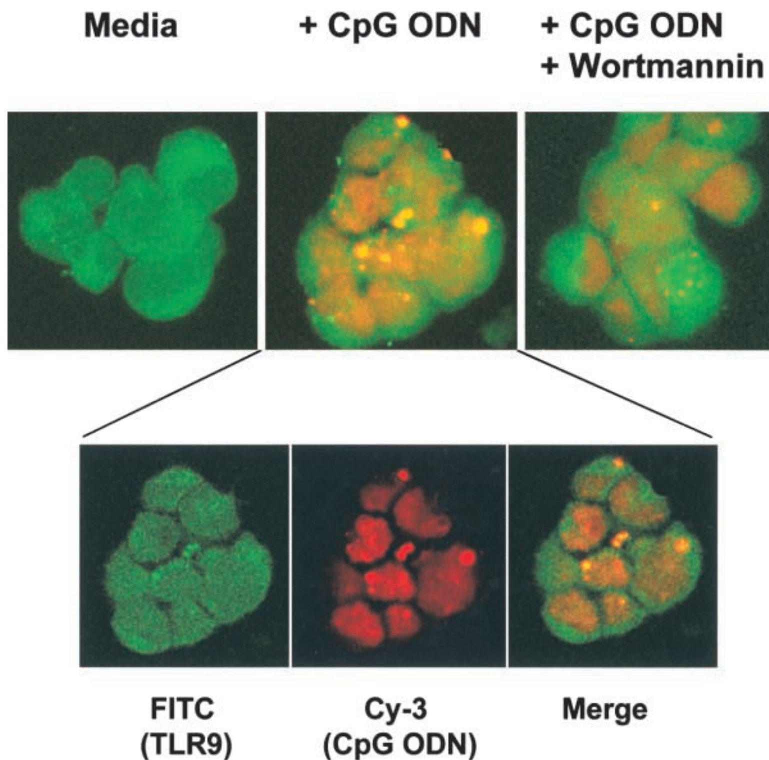

Figure 4. Wortmannin reduces the size and number of vesicles containing CpG ODN plus TLR-9. HEK-293 cells were transfected HA-tagged TLR-9, and cultured with 1 μM of wortmannin plus Cy3 (red) labeled CpG ODN (K3) for 2 h. Cells were then fixed, stained with FITC-labeled anti-HA Ab (green), and analyzed by confocal microscopy. The top panel shows the 3-dimensional stacked pictures of 15 sections with 0.8-μm thickness and the bottom panel shows the representative section of the cells treated with CpG ODNs alone. Each panel is representative of multiple samples from repeat experiments.

DNA-PK–independent manner (unpublished data). Taken together, these data strongly suggest that wortmannin-sensitive PI3-kinase(s) plays a critical role in the uptake and/or localization of CpG ODNs with TLR-9.

Discussion

CpG motifs in bacterial DNA act as pathogen-associated molecular patterns, interacting with TLR-9 to induce a robust innate immune response (7–9). Previous studies established that CpG DNA and TLR-9 colocalize in endocytic vesicles and that cellular activation is associated with an increase in the size and number of these vesicles (9). The process involved in the uptake, transport, and colocalization of CpG DNA with TLR-9 have not been established. Current results indicate that the internalization of CpG ODNs is wortmannin sensitive, suggesting that one or more members of the PI3-kinase family contribute to this process.

Contrary to the conclusions in a previous report (10), we find that CpG-mediated immune activation is a DNA-PK–independent process. Supporting this conclusion is evidence that cells from DNA-PK KO mice (lacking functional DNA-PKcs, Ku70, or Ku80) internalize and respond normally to CpG DNA. PCR analyses confirmed that the relevant DNA-PK genes were disrupted in these KO mice. In addition, phenotypic studies established that the expected abnormalities in T and B cell maturation/function were present in these animals. Results shown in Figs. 1 and 2 are inconsistent with those of Chu et al. (10), who reported that a different strain of DNA-PKcs KO mouse failed to produce cytokine when stimulated by CpG DNA. Unfortunately, that strain of DNA-PKcs KO mouse was unavailable, preventing our performing a comparative study of potential epigenetic differences. However, results

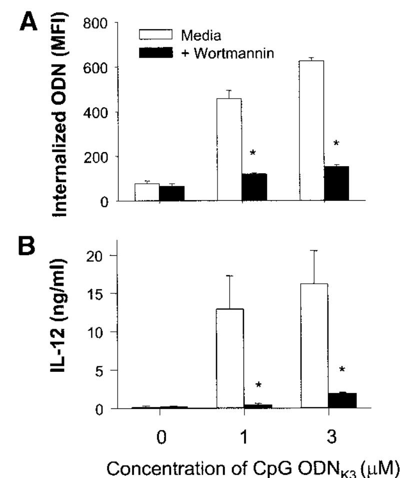

Figure 5. CpG ODN internalization and subsequent activation of BMDCs is inhibited by wortmannin. (A) 10^6 BMDC/ml were incubated with FITC-labeled CpG ODN_{1555} plus 1 μM of wortmannin. 60 min later cells were washed on ice and the signal from surface-bound FITC quenched using trypan blue. Internalized FITC was detected by FACS®. Data represent the mean fluorescein intensity (± SD) from three independently studied mice/group. $*P < 0.01$. (B) BMDCs were treated as described in A for 48 h. IL-12 levels in culture supernatants were measured by ELISA. Data represent the averages ± SD of IL-12 concentrations in four mice per group. The experiment was repeated five times with similar results. $*P < 0.01$.

from all three strains of DNA-PK KO mice used in this work strongly suggest that DNA-PK plays no role in mediating the immune response to CpG DNA.

Chu et al. also observed that wortmannin significantly reduced the immune activation triggered by CpG DNA and attributed this effect to the inhibition of DNA-PK function (10). Our results exclude DNA-PK as the wortmannin-sensitive mediator of CpG DNA–induced cell stimulation (Fig. 2). Wortmannin blocks a conserved ATP-binding site located in the catalytic domain of many members of the PI3-kinase family. Class I and III PI3-kinases facilitate phagocytosis, endocytosis, and endosomal maturation (15–17). Our results suggest that wortmannin-sensitive PI3-kinases contribute to the internalization and/or transport of CpG ODNs to TLR-9–containing endosomes. Consistent with such a model, it was recently shown that PI3-kinases are activated after CpG-mediated stimulation of DCs (26).

Due to the large number and overlapping activities of members of the PI3-kinase family, additional studies are needed to identify the specific kinase(s) that contribute to CpG-mediated signaling. Indeed, wortmannin sensitivity does not unequivocally establish that PI3-kinases are involved in this process. However, considerable data (including results shown in Figs. 2 and 3) establish that the inhibitory effects of wortmannin are selective for PI3-kinases, having no impact on responses mediated by other members of the TLR family.

Taken together, the present findings strongly suggest that CpG DNA–induced innate immune activation is mediated through TLR-9 but not DNA-PK and that wortmannin-sensitive member(s) of the PI3-kinase family play a critical role in shuttling CpG DNA to TLR-9.

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