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Enhancement of immunomodulatory activity by liposome-encapsulated natural phosphodiester bond CpG-DNA in a human B cell line

Dongbum Kim[1], Jae Won Rhee[2], Sanghoon Kwon[1], Young-Eun Kim[3], Soo-Young Choi[4], Jinseu Park[4], Younghee Lee[3] & Hyung-Joo Kwon[1,2,]*

[1]Department of Microbiology, College of Medicine, Hallym University, [2]Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 200-702, [3]Department of Biochemistry, College of Natural Sciences, Chungbuk National University, Chungju 361-763, [4]Department of Biomedical Science and Research Institute for Bioscience and Biotechnology, Hallym University, Chuncheon 200-702, Korea

Natural phosphodiester bond CpG-DNA that contains immunomodulatory CpG motifs (PO-DNA) upregulates the expression of proinflammatory cytokines and induces an Ag-driven Th1 response in a CG sequence-dependent manner in mice. In humans, only phosphorothioate backbone-modified CpG-DNA (PS-DNA) and not PO-DNA has immunomodulatory activity. In this study, we found that liposome-encapsulated PO-DNA upregulated the expression of human β-defensin-2 (hBD-2) and major histocompatibility class II molecules (HLA-DRA) in a CG sequence-dependent and liposome- dependent manner in human B cells. Of the three different liposomes, DOTAP has the unique ability to enhance the immunomodulatory activity of PO-DNA. In contrast, HLA-DRA and hBD-2 promoter activation can be induced by liposome-encapsulated PS-DNA in a CG sequence-independent manner, depending on the CpG-DNA species. Our observations demonstrate that, when encapsulated with a proper liposome in the immune system, natural PO-DNA has the potential to be a useful therapy for the regulation of the innate immune response. [BMB reports 2010; 43(4): 250-256]

INTRODUCTION

Various microbial products such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid and bacterial DNA have pathogen-associated molecular pattern (PAMP) signatures; they are known as polyclonal activators of rapid immune responses. The innate immune system expresses conserved pattern recognition receptors, such as Toll-like receptors (TLRs), to discriminate itself from the PAMP signatures of infectious agents (1, 2).

*Corresponding author. Tel: 82-33-248-2635; Fax: 82-33-241-3640; E-mail: hjookwon@hallym.ac.kr

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A growing body of evidence demonstrates that the innate immune system can use TLR9 to recognize bacterial DNA as non-self. TLR9 recognizes bacterial DNA and synthetic oligodeoxynucleotides that contain unmethylated CpG dinucleotides in the context of particular base sequences (CpG-DNA) (3, 4). Experimental evidence suggests that CpG-DNA plays a significant immunomodulatory role in B lymphocytes, macrophages, dendritic cells and natural killer cells (5-8). The application of CpG-DNA is known to preferentially induce a Th1 response with increased levels of proinflammatory cytokines (for example IL-6, IL-12, IFN-α and IFN-γ) (9-11) as well as the regulation of Th1/Th2 immune responses (4). Moreover, CpG-DNA upregulates the expression of major histocompatibility (MHC) class II molecules and costimulatory molecules (CD40, CD80 and CD86) in the immune system (10, 12). Recently, we reported that the expression of human β-defensin-2 (hBD-2) is upregulated in response to CpG-DNA in human B cells (13). Therefore, CpG-DNA is considered a potentially useful therapeutic substance for immune adjuvants, allergic diseases and infectious diseases (4, 10).

Several investigators have used phosphorothioate backbone-modified CpG-DNA (PS-DNA), in which sulfurs replace the nonbridging oxygen atoms in the backbone, to provide resistance to nuclease activity and to improve the efficiency of CpG-DNA uptake into cells (14-16). PS-DNA has been used for clinical applications due to its ability to dramatically increase the activation of CpG-DNA-induced innate immunity (17-19). However, several investigators have suggested that PS-DNA induces severe side effects, such as arthritis, transient splenomegaly, lymphoid follicle destruction, immunosuppression and PS-DNA-specific IgM production in PS-DNA-treated mice in a CG sequence-dependent and backbone modification-dependent manner (20-23). Thus, the identification of potent immunomodulatory CpG-DNA that does not severe side effects is required for inducing a well-controlled immune response.

To develop potent CpG-DNA without severe side effects,

we identified natural phosphodiester bond CpG-DNA (PO-DNA) through a computer-assisted analysis and by screening the chromosomal DNA sequences of *M. bovis* having immunomodulatory activity (24). Potent PO-DNA, specifically MB-ODN 4531(O) containing immunomodulatory CpG motifs, is an inducer of the innate immune response and a powerful adjuvant for the induction of Ag-driven Th1 responses without severe side effects, such as PS-DNA-specific IgM production and transient splenomegaly (23, 24). However, the activity of PO-DNA with immunomodulatory activity in mice is relatively low in human cells (3). Previously, it was reported that activation of innate immune responses in human cells could be induced by PO-DNA encapsulated in liposomes (25, 26). In this study, we report that expression of hBD-2 and MHC class II molecule (HLA-DRA) is upregulated by liposome-encapsulated PO-DNA in a CG sequence-dependent and liposome-dependent manner in human B cells.

RESULTS AND DISCUSSION

Effect of liposome-encapsulated PO-DNA on HLA-DRA and hBD-2 promoter activation

Previously, we reported that PO-DNA upregulates the expression of proinflammatory cytokines (TNF-α and IL-12) and induces Ag-driven Th1 responses in a CG sequence-dependent manner in mice (23, 24). In contrast to PS-DNA, the effects of PO-DNA are not evident in human cells (3). For this study, we examined the effects of liposome-encapsulated PO-ODN on HLA-DRA and hBD-2 expression in the human B cell line RPMI 8226. First, we determined the activation of the HLA-DRA gene promoter in RPMI 8226 cells by stimulating PO-DNA. As we have shown previously and in Fig. 1A, PS-DNA upregulates HLA-DRA expression in RPMI 8226 cells (12). In contrast, the HLA-DRA promoter was not activated by treatment with PO-DNA [MB-ODN 4531(O)] (Fig. 1A). However, the HLA-DRA promoter was activated when the cells were treated with liposome-encapsulated MB-ODN 4531(O) (Fig. 1A). The liposomes used for this study are DOTAP, Lipofectamine and Lipofectin. As shown in Fig. 1A, the luciferase activity was significantly greater in cells treated with DOTAP-encapsulated MB-ODN 4531(O) than in Lipofectamine- or Lipofectin-encapsulated-MB-ODN 4531(O)-treated cells.

Previously we showed that both expression of the hBD-2 gene and the release of hBD-2 protein are upregulated in PS-DNA-treated RPMI 8226 cells (13). However, hBD-2 expression was not induced by MB-ODN 4531(O) alone. Here, we found that activation of the hBD-2 promoter is significantly increased when cells are treated with DOTAP-encapsulated MB-ODN 4531(O) (Fig. 1B).

Expression of HLA-DRA and hBD-2 in RPMI 8226 cells activated by liposome-encapsulated PO-DNA

To further investigate whether liposome-encapsulated PO-DNA induces the synthesis of endogenous HLA-DRA and hBD-2, we prepared and applied DOTAP-encapsulated MB-ODN 4531 to RPMI 8226 cells. RT-PCR was used to examine the mRNA expression of HLA-DRA and hBD-2. As shown in Fig. 2A, HLA-DRA and hBD-2 were barely detected in RPMI 8226 cells. Furthermore, DOTAP-encapsulated MB-ODN 4531(O) induced expression of HLA-DRA and hBD-2 as potently as DOTAP-encapsulated MB-ODN 4531(S). The results of MB-ODN 4531(S) did not differ from that of DOTAP-encapsulated MB-ODN 4531(S) (data not shown). Next, we used an ELISA kit to determine whether hBD-2 protein is secreted to the culture medium in response to DOTAP-encapsulated MB-ODN

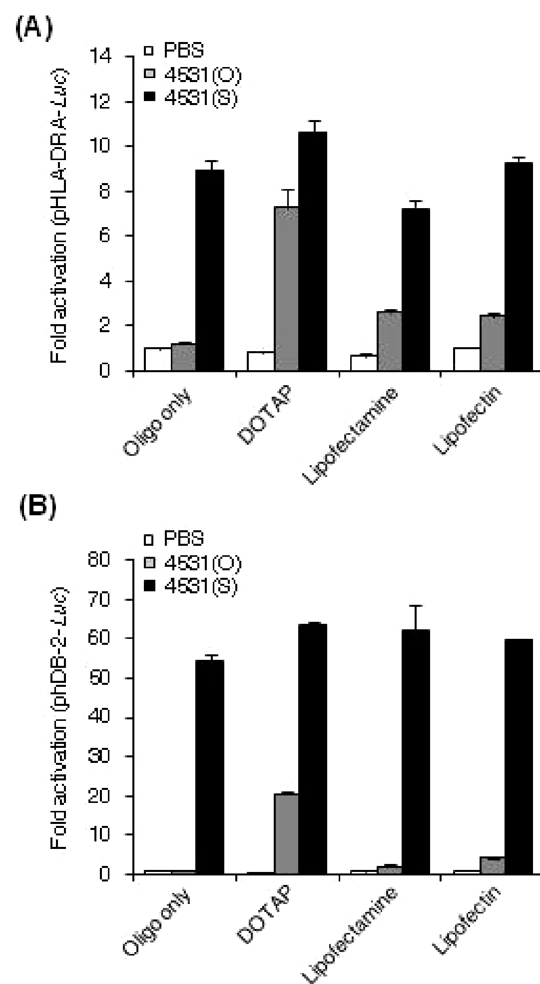

Fig. 1. Activation of the HLA-DRA and hBD-2 promoter in RPMI 8226 cells treated with liposome-encapsulated PO-DNA. RPMI 8226 cells were transiently transfected with HLA-DRA promoter-luciferase (A) or hBD-2 promoter-luciferase (B) construct for 24 h. The cells were then stimulated with the indicated liposome-encapsulated CpG-DNA (5 μM) for 12 h. The cells were harvested and assayed for luciferase activity as described under "Materials and methods". The results are presented as fold activation compared to control.

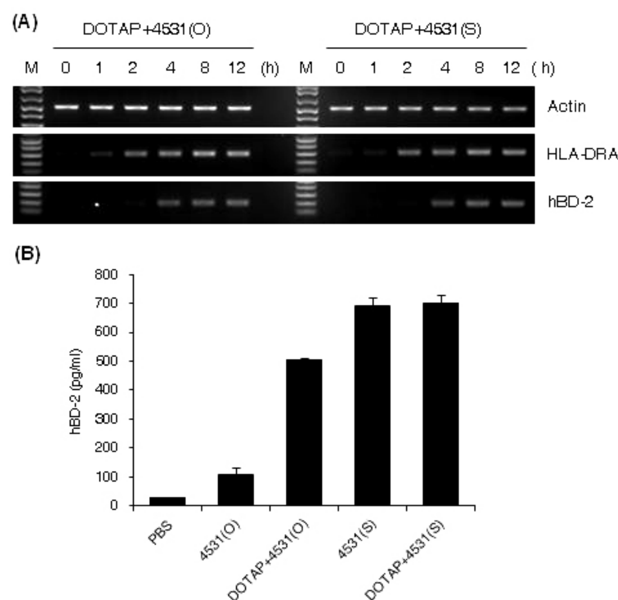

Fig. 2. HLA-DRA and hBD-2 expression induced by liposome-encapsulated CpG-DNA in RPMI 8226 cells. (A) Time course expression of HLA-DRA and hBD-2 mRNA induced by liposome-encapsulated CpG-DNA. RPMI 8226 cells were treated with the indicated liposome-encapsulated CpG-DNA (5 μM) for the indicated time periods. The expression of HLA-DRA and hBD-2 mRNA was analyzed by RT-PCR. The expression of β-actin was used as the control. The letter M denotes a standard DNA marker. (B) Production of hBD-2 induced by liposome-encapsulated CpG-DNA. Cells were treated with the indicated liposome-encapsulated CpG-DNA (5 μM) for 24 h. Culture supernatants were harvested and the levels of hBD-2 were measured by ELISA.

4531(O) treatment. As shown in Fig. 2B, DOTAP-encapsulated MB-ODN 4531(O) significantly induces the release of hBD-2 protein into the culture medium of RPMI 8226 cells, which is consistent with the RT-PCR results. When the cells were stimulated with MB-ODN 4531(S) or DOTAP-encapsulated MB-ODN 4531(S), there was no difference in terms of hBD-2 production and secretion, probably because MB-ODN 4531(S) is potent enough to trigger maximum activation of hBD-2 gene expression.

Effect of CG sequence and phosphorothioate backbone modification on liposome-encapsulated PO-DNA-induced HLA-DRA and hBD-2 promoter activation

To confirm that the CpG dinucleotide sequence promotes activation of the HLA-DRA and hBD-2 promoters, we used PO-DNA and PS-DNA derivatives of CpG-DNA 2006 and MB-ODN 4531, which differ only in the reversal of one CpG dinucleotide to GpC dinucleotide. When RPMI 8226 cells were treated with DOTAP-encapsulated CpG-DNA 2006(O)GC or MB-ODN 4531(O)GC, luciferase activity was decreased more dramatically than that of HLA-DRA and hBD-2 promoter activation in PO-DNAs-treated cells (Fig. 3A, C). These results suggest that liposome-encapsulated PO-DNA is capable of activating the human B cell line RPMI 8226 in a CG sequence-dependent manner. Basal luciferase activity was also detected in cells treated with MB-ODN 4531(S)GC (Fig. 3B, D). However, treatment with liposome-encapsulated CpG-DNA 2006 (S)GC led to activation of the HLA-DRA and hBD-2 promoter. This is comparable to activation by CpG-DNA 2006(S) (Fig. 3B, D). Furthermore, luciferase activity was also detected in cells stimulated with liposome-encapsulated non-CpG-DNA 2041(S) and non-CpG-DNA 2041(S). These results show that, depending on the CpG-DNA species, HLA-DRA and hBD-2 promoter activation is induced by liposome-encapsulated PS-DNA in a CG sequence-independent manner.

Effect of liposome-encapsulated PO-DNA on MyD88-mediated and NF-κB activation-mediated HLA-DRA and hBD-2 promoter activation

We examined the requirement for MyD88 in liposome-encapsulated PO-DNA-induced HLA-DRA and hBD-2 promoter activation. As shown in Fig. 4A, B, a dominant negative version of MyD88 (ΔMyD88) significantly reduces liposome-encapsulated PO-DNA-induced HLA-DRA and hBD-2 promoter activation. These results show that the signal transduction molecule MyD88 of the TLR/IL-1R signaling pathway is required for activation of the HLA-DRA and hBD-2 promoter in RPMI 8226 cells stimulated by liposome-encapsulated PO-DNA; the same requirement also exists for PS-DNA. To determine whether NF-κB activation is affected in liposome-encapsulated PO-DNA-induced HLA-DRA and hBD-2 promoter activation, we cotransfected an expression plasmid encoding mutant IκBα protein (IκBα Super Repressor, IκBα SR) into RPMI 8226 cells with the promoter-reporter construct. The induction of HLA-DRA and hBD-2 promoter activity was inhibited by the expression of IκBα SR in cells treated with liposome-encapsulated PO-DNA (Fig. 4). These results confirm that both IκBα degradation and NF-κB activation are involved in liposome-encapsulated PO-DNA-induced HLA-DRA and hBD-2 promoter activation, similar to that of PS-DNA.

Although investigators mainly use PS-DNA for the clinical application of therapeutic CpG-DNA, the phosphorothioate backbone linkage reportedly induces CpG dinucleotide sequence-independent and backbone modification-dependent side effects (20-23). We therefore identified natural PO-DNA with immunomodulatory activity from bacterial chromosomal DNA. Immunomodulatory PO-DNA, named MB-ODN 4531(O), plays a functional role as a Th1-responsive adjuvant in mice (24, 27). However, PO-DNA was much less potent in inducing HLA-DRA and hBD-2 promoter activation in RPMI 8226 cells than in mice (Fig. 1).

The use of liposomes as vehicles for CpG-DNA delivery has been extensively evaluated for the purpose of developing therapeutic CpG-DNA (28, 29). Encapsulated liposomes can increase the half-life of CpG-DNA and protect it from nuclease

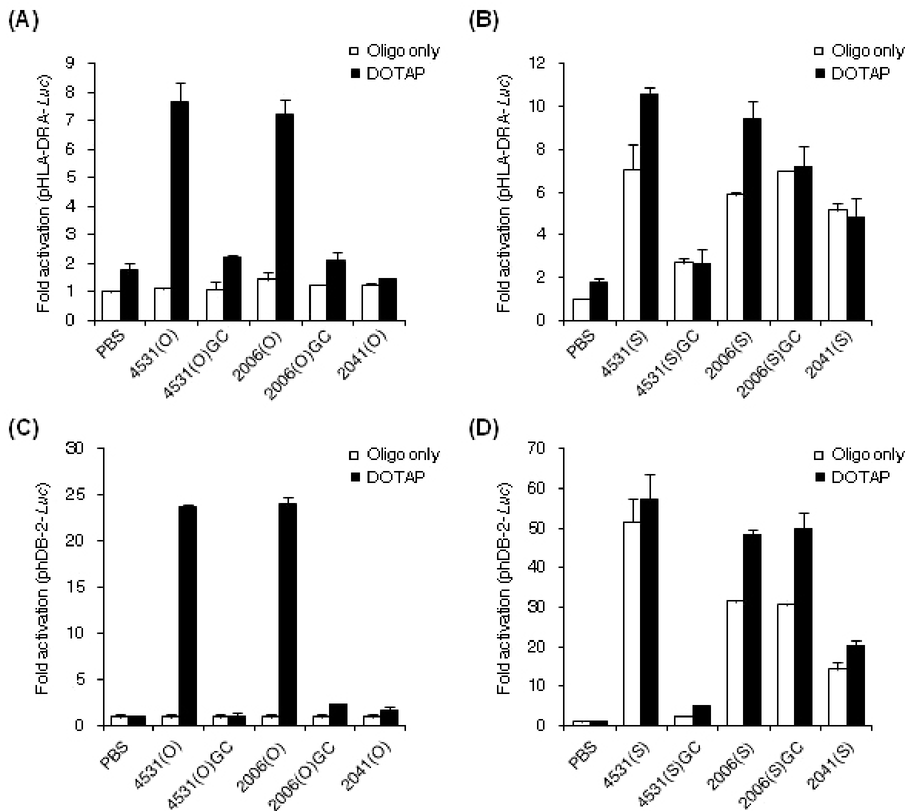

Fig. 3. Effect of CpG sequences and phosphorothioate backbone modification on HLA-DRA and hBD-2 promoter activation induced by liposome-encapsulated CpG-DNA. RPMI 8226 cells were transiently transfected with a HLA-DRA promoter-luciferase (A and B) or hBD-2 promoter-luciferase (C and D) construct for 24 h. The cells were then stimulated with the indicated liposome-encapsulated CpG-DNA (5 μM) for 12 h. The cells were harvested and assayed for luciferase activity as described under "Materials and methods". The results are presented as fold activation compared to control.

activity. Several investigators have shown that CpG-DNA upregulates antigen-presenting cell activity, Th1 immune responses, and immunoglobulin (Ig) isotype switching (30, 31). The role of immunomodulatory species as potent adjuvants is enhanced by liposome-encapsulated CpG-DNA (PS-DNA) in mice. Suzuki et al. showed that PS-DNA encapsulated in cationic liposomes induces the expression of IL-12 and IFN-γ and that PS-DNA-liposome coencapsulated with ovalbumin (OVA) activates OVA-specific cytotoxic T lympocytes, which exhibit potent cytotoxicity against OVA-expressing tumors (32). On the other hand, PO-DNA encapsulated in liposomes, especially DOTAP and Lipofectin, reportedly increases the expression of proinflammatory cytokines in human cells (25, 26). However, there have been no precise analytical investigations into the relationship between liposome-encapsulated PO-DNA and immunomodulatory function in human immune cells. Our work demonstrates that PO-DNA encapsulated in DOTAP can elicit HLA-DRA and hBD-2 promoter activation and gene expression in RPMI 8226 cells in a CG sequence-dependent manner (Fig. 3A, C). In contrast to PO-DNA, the promoter activities of HLA-DRA and hBD-2 are induced by liposome-encapsulated PS-DNA in a CG sequence-independent manner, though the extent of induction depends on the CpG-DNA species (Fig. 3B, D).

Recognition of CpG-DNA by TLR is known to induce the activation of the MyD88/IL-1R-associated kinase (IRAK) pathway (10). Activation of the MyD88/IRAK pathway stimulates several transcription factors such as NF-κB for cytokine production. We directly demonstrated by promoter-reporter assay that NF-κB activation induces HLA-DRA and hBD-2 promoter activation in response to PO-DNA-encapsulated DOTAP stimulation. We also provide evidence that MyD88 is required for HLA-DRA and hBD-2 promoter activation in response to PO-DNA encapsulated in DOTAP (Fig. 4).

In summary, our observations demonstrate that natural PO-DNA has potential as a useful therapy and as a regulator of the innate immune response when encapsulated in liposome in the human immune system. Of the three different liposomes, DOTAP is the most useful and unique in terms of enhancing immunomodulatory activity. Accordingly, further investigation and screening of other liposomes can be a strategy for developing more potent therapeutics.

MATERIALS AND METHODS

ODNs and reagents

ODNs were purchased from GenoTech (Daejeon, Korea). The CpG-DNA sequences used in this study were either phospho-

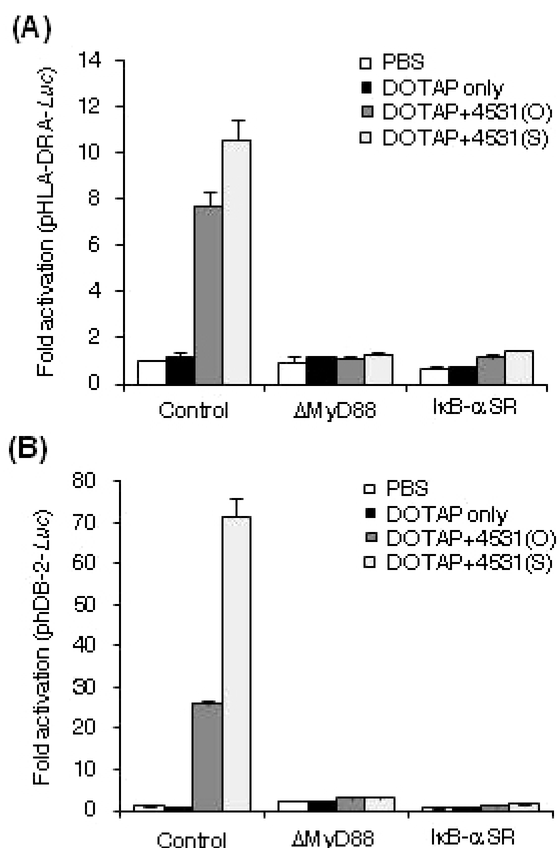

Fig. 4. MyD88 and NF-kB activation is required for HLA-DRA and hBD-2 promoter activation induced by liposome-encapsulated CpG-DNA. RPMI 8226 cells were transiently transfected with a HLA-DRA promoter-luciferase construct (A) or hBD-2 promoter-luciferase construct (B) in combination with IκBα SR or a dominant negative MyD88 mutant (ΔMyD88) for 24 h. Cells were then stimulated with the indicated liposome-encapsulated CpG-DNA (5 μM) for 12 h. The cells were harvested and assayed for luciferase activity as described under "Materials and methods". The results are presented as fold activation compared to control.

diester (O) or phosphorothioate-modified (S). The phosphorothioate versions of CpG-DNA 2006(O) and MB-ODN 4531(O) are CpG-DNA 2006(S) and MB-ODN 4531(S), respectively. MB-ODN 4531 consists of 20 bases containing three (MB-ODN 4531) CpG-motifs (underlined). CpG-DNA 2006 consists of 24 bases containing three CpG-motifs (underlined): CpG-DNA 2006, TCGTCGTTTTGTCGTTTTGTCGTT; MB-ODN 4531, AGCAGCGTTCGTGTCGGCCT. CpG-DNA 2006GC and MB-ODN 4531GC are derivatives of CpG-DNA 2006 and MBODN 4531, respectively, with one of the CG sequences reversed to GC. Non-CpG-DNA 2041 (CTGGTCTTTCTGGTTTTTTTCTGG) served as the negative control. The endotoxin content of the ODNs was less than 1 ng/mg of ODN, as measured by *Limulus amebocyte* assay (Whittaker Bioproducts, Walkersville, MD, USA). The expression vectors encoding IκBαSR and ΔMyD88 were kindly provided by Dr. Harikrishna Nakshatri (Indiana University, Indianapolis, IN, USA) and Dr. Jurg Tschopp (University of Lausanne, Switzerland), respectively. The following liposomes were used in this study: DOTAP (Roche, Penzberg, Germany), Lipofectamine (Invitrogen, Carlsbad, CA USA) and Lipofectin (Invitrogen). Complexes of CpG-DNA with DOTAP, Lipofectamine or Lipofectin were prepared in accordance with the manufacturer's specifications.

Cell culture

The human B cell line RPMI 8226 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/mL of penicillin and 100 μg/mL of streptomycin. Viability was assayed by trypan blue dye exclusion.

Construction of luciferase reporter plasmids

The HLA-DRA promoter fragment −323 to +43 was amplified by PCR using human genomic DNA (Clontech, Palo Alto, CA, USA) as a template with the following primer sets (12): DRA (-323), 5′-GGTACCATCTGTATTTAGCTCT-3′ and DRA(+43), 5′-AAGCTTGGGAGTGAGGCAGAACAGAC-3′. To construct a hBD-2 promoter-reporter plasmid containing −2,531 to +111 (13), we amplified an hBD-2 promoter fragment by PCR using human genomic DNA (Clontech) as a template with the following primer set: HDB (-2531), 5′-GGTACCAATTTCTTTGTC TCCAT-3′ and HDB(+111), 5′-CTCGAGGCATCAGGAATATG A-3′. These fragments were then ligated into the luciferase reporter plasmid pGL3-Basic vector (Promega, Madison, WI, USA), which yielded the reporter constructs pHLA-DRA-*Luc* and phBD-2-*Luc*.

Transfection and luciferase assay

Transfection and the luciferase assay were performed as previously reported (33). RPMI 8226 cells were placed into six-well plates one day before transfection. After transfection, the cells were placed in complete medium for 24 h and then treated with CpG-DNA or liposome-encapsulated CpG-DNA for 12 h. Cells were harvested, washed and lysed by freeze-thawing three times. Luciferase activities were determined by Dual-Luciferase Reporter Assay (Promega) using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). To normalize individual assays, we adjusted transfection efficiency by cotransfecting the promoterless Renilla luciferase vector pRL-null (Promega).

RT-PCR analysis

After cells were treated with CpG-DNA or liposome- encapsulated CpG-DNA for the indicated time periods, total RNA was extracted using an RNeasy RNA isolation kit (Qiagen). Five micrograms of total RNA were reverse-transcribed in the first-strand synthesis buffer containing 6 μg/ml of oligo (dT) primer,

50 U of reverse transcriptase, 4 mM of dNTP, and 40 U of RNase inhibitor. We performed a standard PCR reaction for 25 cycles using a cDNA mixture as a template with the following primer sets: HLA-DRA, 5'-CGAGTTCTATCTGAATCCTG-3′ (sense), 5'-GTTCTGCTGCATTGCTTTTGC-3′ (anti-sense); Human BD-2, 5′-GGG TCT TGT ATC TCC TCT TC-3′ (sense) and 5′-ATT TGG TTT ACA TGT CGC ACG-3′ (anti-sense); human actin, 5'-GGGTCAGAAGGATTCCTATG-3' (sense), 5'-CCTTA ATGTCACGCACGATTT-3' (anti-sense). The PCR products were detected by UV light after being resolved on a 1% agarose gel.

Measurement of hBD-2

To measure the amount of hBD-2, we added CpG-DNA or liposome-encapsulated CpG-DNA to RPMI 8226 cells, followed by incubation at 37°C with 5% CO_2 for 24 h. The culture supernatants were collected and hBD-2 levels were measured using a commercially available ELISA kit for hBD-2 (Peprotech, Rocky Hill, NJ, USA) in accordance with the manufacturer's specifications.

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