

# CD83 expression induced by CpG-DNA stimulation in a macrophage cell line RAW 264.7

Min Chul Park<sup>1</sup>, Dongbum Kim<sup>2</sup>, Younghee Lee<sup>3</sup> & Hyung-Joo Kwon<sup>1,2,\*</sup>

<sup>1</sup>Department of Microbiology, College of Medicine, Hallym University, <sup>2</sup>Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 200-702, <sup>3</sup>Department of Biochemistry, College of Natural Sciences, Chungbuk National University, Cheongju 361-763, Korea

CpG-DNA has various immunomodulatory effects in dendritic cells, B cells, and macrophages. While induction of cytokines by CpG-DNA has been well documented in macrophages, the expression of costimulatory molecules in CpG-DNA treated macrophages has not yet been defined. Therefore, we investigated the effects of CpG-DNA on the expression of costimulatory molecules in RAW 264.7 cells. The surface expression of CD80 was slightly increased and CD83 expression was significantly increased in response to CpG-DNA. However, the expression of CD86 and MHC class II was not changed. As expression of CD83 mRNA was also increased by CpG-DNA, CD83 expression is regulated at a transcriptional level. To understand the contribution of signaling pathways to CD83 induction, we used pathway specific inhibitors. The NF- $\kappa$ B inhibitor significantly reduced surface expression of CD83 as well as phagocytic activity of RAW 264.7 cells. Therefore, CD83 expression may contribute to the immunostimulatory effects of CpG-DNA in macrophage cells. [BMB Reports 2013; 46(9): 448-453]

## INTRODUCTION

The innate immune system is the early line of host defense for protection against and elimination of infectious pathogens. The innate immune responses in the infection sites are mediated by dendritic cells and primary phagocytic cells, such as macrophages and neutrophils. Pathogen recognition through pattern recognition receptors (PRRs) enables the innate immune cells to discriminate themselves from components of foreign pathogens referred to as pathogen-associated molecular

patterns (PAMPs) (1). PRRs such as Toll-like receptors (TLRs) recognize a wide variety of PAMPs including lipopolysaccharides (LPS), lipids, lipoproteins, glycoproteins, proteins, and nucleic acids derived from a wide range of microbes such as bacteria, viruses, parasites, and fungi (2-4).

TLR9 has emerged as a key sensor of innate immune responses to synthetic oligodeoxynucleotides (ODNs) and bacterial DNA that contain unmethylated CpG dinucleotides in the context of particular base sequences (CpG-DNA) (5, 6). The myeloid differentiation protein (MyD88)/IL-1R-associated kinase (IRAK) and MAP kinase pathways are activated by exposure of TLR9 to CpG-DNA. Stimulation of these pathways upregulates the expression of various genes through activation of various transcription factors such as nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) (3, 7, 8).

TLR9 is expressed in a variety of cells such as B cells, dendritic cells (DCs), and macrophages. The effects of TLR9 activation varies according to cell type. CpG-DNA facilitates B cell proliferation and induces expression of IL-6, costimulatory molecules (CD40, CD54, CD80, CD86), and MHC class I and II in B cells (9, 10). DCs are major antigen-presenting cells and plasmacytoid DCs (pDCs) strongly express TLR9. CpG-DNA activates pDCs, resulting in upregulated IFN- $\gamma$  secretion, expression of proinflammatory cytokines (TNF- $\alpha$ , IL-6, IL-12), chemokines (IL-8 and IP-10), major histocompatibility (MHC) class II molecules, and costimulatory molecules (CD40, CD54, CD80, and CD86). Activation of TLR9 induces DCs maturation, and thereby upregulates the expression of CD83 as a marker for DC maturation together with the co-stimulatory molecules CD80 and CD86 (11-13).

Macrophages are also crucial effector cells for the innate immune responses. CpG-DNA affects a variety of murine macrophage functions. The treatment of macrophages with CpG-DNA induces a range of cytokines and chemokines including TNF- $\alpha$ , IL-1, IL-6, IL-12, and MIP-2. The induction of IL-12 and IFN- $\gamma$  by CpG-DNA enhances Th1 adjuvant activity, characterized by Th1-associated Ag-specific IgG2a production (14, 15). In addition, the enhanced Th1 responses by CpG-DNA result in the protection of the host from infectious pathogens (16, 17). CpG-DNA-stimulated macrophages enhance antimicrobial activity, as CpG-DNA induces the expression of inducible NO

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

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synthase (iNOS) and production of NO after pretreatment of macrophages with IFN- $\gamma$  (18). However, the effects of CpG-DNA on the expression of costimulatory molecules (CD80, CD83, and CD86 etc) in macrophages have not been clearly examined.

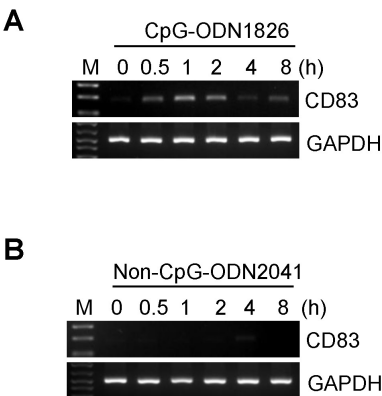
In the present study, we have demonstrated that the stimulation of the macrophage cell line RAW 264.7 with CpG-DNA significantly induces the expression of CD83 in a NF- $\kappa$ B-dependent manner.

RESULTS AND DISCUSSION

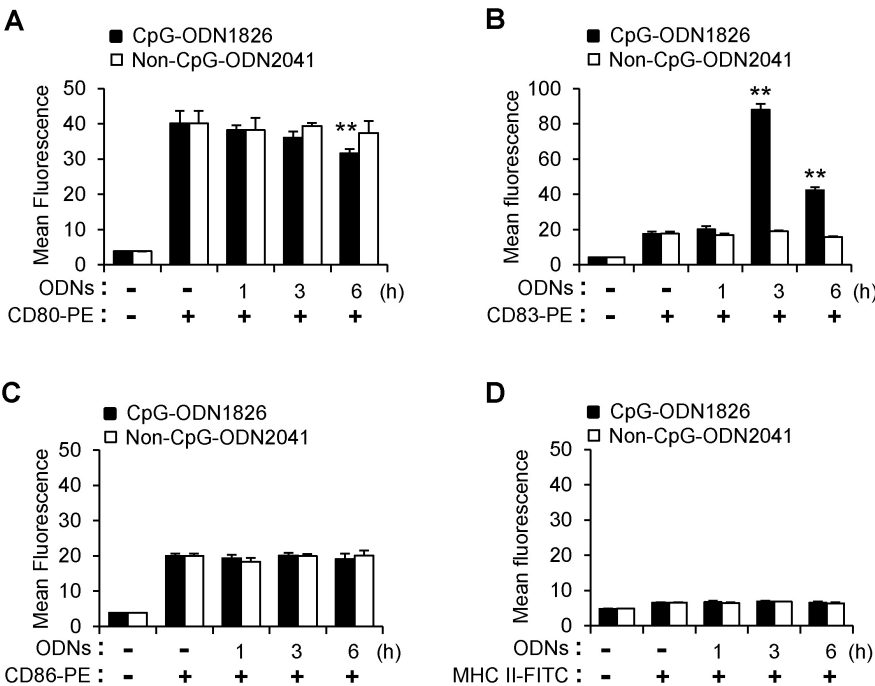
Expression of cell surface markers in macrophage cell line by CpG-DNA treatment

To investigate the role of CpG-DNA on the expression of costimulatory molecules in macrophages, we stimulated RAW 264.7 cells with CpG-DNA (CpG-ODN 1826) and non-CpG DNA control (non-CpG-ODN 2041), and investigated the expression of CD80, CD83, CD86, and MHC class II by a FACS analysis in a time-dependent manner. Unstimulated RAW 264.7 cells expressed CD80, CD83, and CD86, but not MHC class II on the cell surface (Fig. 1 and Supplementary Fig. 1). While treatment with CpG-DNA downregulated macrophage CD80 expression in a time-dependent manner (Fig. 1A), CpG-DNA significantly enhanced CD83 surface expression 3 h after stimulation and the expression level declined in 6 h (Fig. 1B). The non-CpG-DNA control did not show any influence on the expression of CD80 or CD83 (Fig. 1A and 1B). In contrast to previously reported results from DCs (11),

CpG-DNA did not increase the expression of CD86 and MHC class II in RAW 264.7 cells (Fig. 1C and D). This suggests that macrophages may have a limited function compared with DCs, which are professional antigen presenting cells, because of the lack of concerted induction of costimulatory molecules in response to CpG-DNA. In accordance with a FACS analysis, the expression level of CD83 mRNA in RAW 264.7 cells was increased by CpG-DNA with maximum induction at 1 h after



**Fig. 2.** CpG-DNA-induced CD83 mRNA expression in RAW 264.7 cells. RAW 264.7 cells were treated with CpG-ODN1826 (A) and non-CpG-ODN2041 (B) for the indicated periods, and the expression of CD83 mRNA was analyzed by RT-PCR. One representative set of data of three independent experiments is shown.



**Fig. 1.** CpG-DNA-induced CD83 expression in RAW 264.7 cells. RAW 264.7 cells were treated with CpG-ODN1826 and non-CpG-ODN2041 for the indicated periods, and the expression of costimulatory molecules was analyzed by immunostaining and FACS. (A) CD80. (B) CD83. (C) CD86. (D) MHC class II. The Y axis presents the mean fluorescence intensity of the FACS data. These results are representative of three experiments that yielded similar results. \*\*P < 0.05 (vs PBS control).

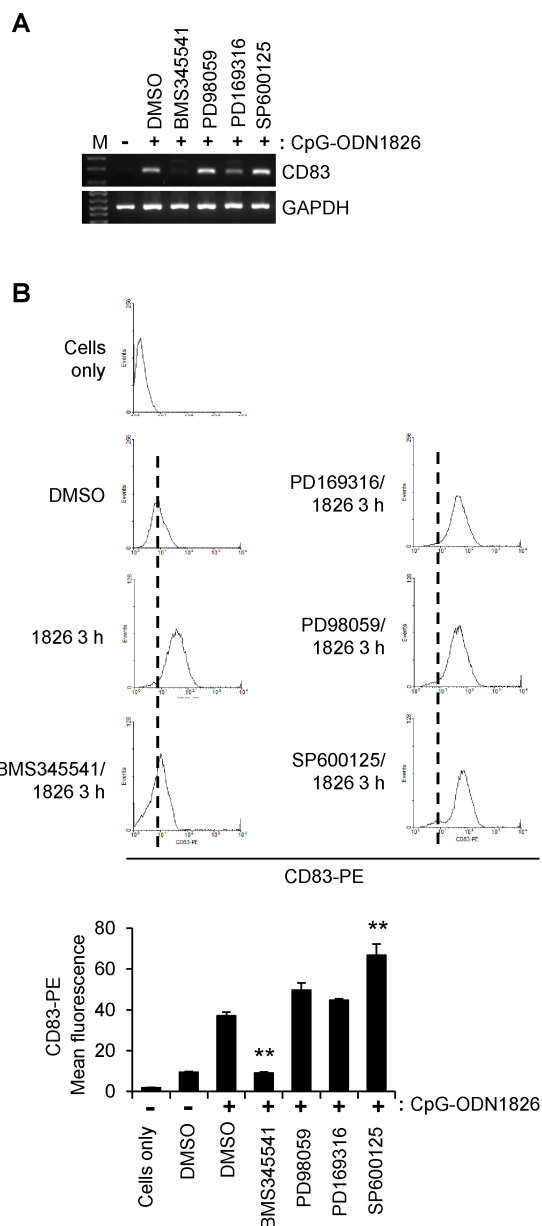
stimulation (Fig. 2A). However, the expression of CD83 mRNA was not induced by control non-CpG-DNA (Fig. 2B). Therefore, we can conclude that induction of CD83 by CpG-DNA is regulated at a transcriptional level in the cells of the macrophage cell line RAW 264.7.

### NF- $\kappa$ B-dependent expression of CD83 induced by CpG-DNA treatment

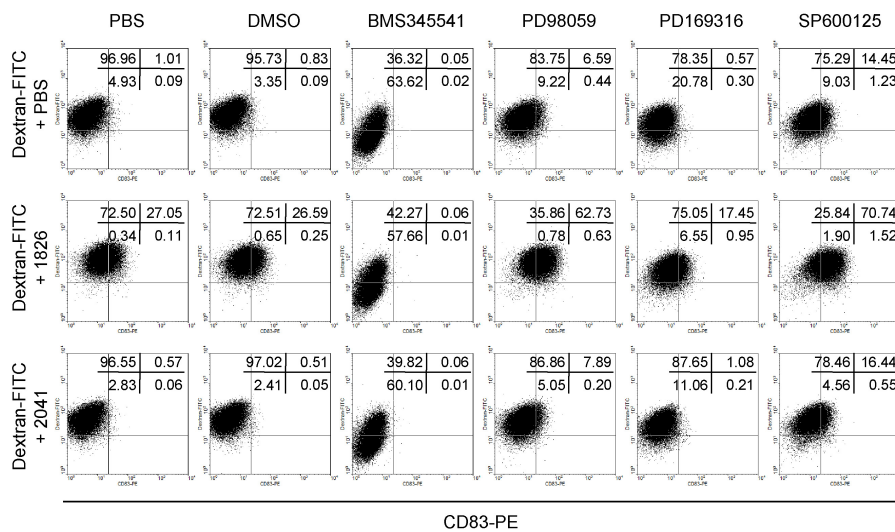
CpG-DNA is known to activate NF- $\kappa$ B and MAP kinase pathways in DCs and macrophages (3, 7, 8). To estimate the involvement of these signal transduction pathways in regard to induction of CD83 by CpG-DNA, we stimulated RAW 264.7 cells with CpG-DNA in the presence of signal transduction pathway inhibitors. As shown in Fig. 3A, the expression of CD83 mRNA was abolished in the presence of BMS345541, a NF- $\kappa$ B inhibitor, and reduced in the presence of PD169316, a p38 MAP kinase inhibitor. In contrast, the expression levels of CD83 mRNA were increased in the presence of PD98059, a MEK1 inhibitor, and SP600125, a stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) inhibitor. The FACS analysis confirmed that inhibition of the NF- $\kappa$ B pathway clearly suppressed CD83 expression on the cell surface (Fig. 3B). In contrast, inhibition of other MAP kinase pathways did not induce down-regulation but induced slightly upregulated CD83 expression. These results suggest that CD83 gene expression appears to be critically regulated by the NF- $\kappa$ B activation pathway in the CpG-DNA-stimulated RAW 264.7 cells.

### Association of CD83 expression and phagocytic capacity

To investigate the contribution of CD83 to the functional activity of RAW 264.7 cells, we stimulated the cells with CpG-DNA in the presence or absence of signal transduction pathway inhibitors and examined their phagocytic capacity by measuring dextran uptake activity. As expected, dextran-FITC uptake was increased by stimulation with CpG-DNA but not by non-CpG-DNA serving as a negative control (Supplementary Fig. 2). Inhibition of the NF- $\kappa$ B pathway reduced basal dextran uptake activity and stimulation of CpG-DNA did not induce further activation (Supplementary Fig. 2). Next, we treated RAW 264.7 cells with dextran-FITC and simultaneously stained the cells with anti-CD83 antibodies and analyzed the cell population in the context of CD83 expression and dextran uptake activity. As shown in Fig. 4, stimulation of RAW 264.7 cells with CpG-DNA induced expression of CD83 and increased dextran uptake activity. However, neither CD83 expression nor dextran uptake activity was enhanced by CpG-DNA in the presence of BMS345541. The NF- $\kappa$ B pathway activated by CpG-DNA is known to induce several cytokines and chemokines involved in inflammation and immunostimulation, which many investigators, including us, have previously reported (3, 7, 8). The results of this study suggest that the NF- $\kappa$ B pathway activated by CpG-DNA also contributes to the induction of CD83 expression and that CD83 expression may contribute to the functional activity of acti-



**Fig. 3.** Involvement of NF- $\kappa$ B and AP-1 in the expression of CD83. RAW 264.7 cells were treated with CpG-ODN1826 in the presence or absence of signaling pathway inhibitors, and the expression of CD83 at the mRNA and protein level was analyzed. (A) The cells were treated with CpG-ODN1826 for 1 h and the expression of CD83 mRNA was analyzed by RT-PCR. (B) The cells were treated with CpG-ODN1826 for 3 h and the surface expression of CD83 was analyzed by FACS. BMS345541, an IKK-2 inhibitor. PD169316, a p38 inhibitor. SP600125, a SAPK/JNK inhibitor. PD98059, a MEK1 inhibitor. One representative set of data of three independent experiments is shown. \*\*P < 0.05 (vs DMSO+CpG-ODN1826).



**Fig. 4.** Association of CD83 expression and phagocytic activity. RAW 264.7 cells were treated with CpG- ODN1826 and non-CpG-ODN2041 for 6 h and dextran-FITC uptake and CD83 expression were analyzed by FACS. Percentages of the cell population in each quadrant are indicated. These results are representative of three experiments.

vated macrophage cells, at least in part.

CD83 is a type-1 surface glycoprotein of the Ig superfamily with a molecular weight of 45 kDa (19). Reportedly, CD83 has been illustrated as a specific maturation marker for DCs and is expressed on mature dendritic cells (DCs) including Langerhan's cells in the skin, interdigitating reticulum cells in the T cell zones of lymphoid organs, and monocyte derived DCs (19, 20). Further, CD83 is upregulated after stimulation with LPS, TNF- $\alpha$ , CD40L, IL-4, GM-CSF on monocyte derived DCs, monocytes, and myelocytes (21, 22).

It has been described that CD83 plays a role in the development of CD4<sup>+</sup> T cells. CD83 knockout mice showed a significantly reduced amount of CD4<sup>+</sup> cells in the thymus. The reduction of CD4<sup>+</sup> T cells in the thymus resulted in a decrease of CD4<sup>+</sup> T helper cells in the periphery. However, normal development of CD4<sup>+</sup> T cells was induced when CD83 knockout thymocytes were transferred to wild type mice, proving that the expression of CD83 on the thymic epithelium is required (23).

It was also reported that CD83 has a role in the regulation of B cell function (24, 25). The expression of CD83 is increased on activated B cells in *Leishmania major*-infected mice. Over-expression of CD83 leads to a reduction of antigen-specific antibodies in thymus-independent and thymus-dependent models *in vivo* (24). Furthermore, Lüthje *et al.* showed that CD83 negatively regulates B cell maturation in mouse spleens (25).

While the function of CD83 has been widely studied in DCs, T cells, and B cells as described above, no study has yet been undertaken to understand the function of CD83 in macrophages. In this study, we observed that CD83 expression and dextran uptake activity are regulated by CpG-DNA in a NF- $\kappa$ B-dependent manner in the cells of the macrophage cell line RAW 264.7.

## MATERIALS AND METHODS

### CpG-DNA

CpG-DNA was synthesized by GenoTech (Daejeon, Korea). The CpG-ODN 1826 consisted of 20 bases containing two CpG motifs (underlined): TCCATGACGTTCTCTGACGTT. The non-CpG ODN 2041 (CTGGTCTTCTGGTTTTTCTGG) served as a negative control. The sequences of CpG-DNA and non-CpG-DNA used in this study were phosphorothioate-modified. The endotoxin content was <1 ng/mg of CpG-ODN 1826 and non-CpG ODN 2041, as measured by a *Limulus ameobocyte* assay (Whittaker Bioproducts, Walkersville, MD, USA).

### Cell culture and reagents

We obtained the RAW 264.7 mouse macrophage cell line from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C under a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cell cultures were maintained until passage 20 and then discarded. Cells were treated with CpG-DNA (5  $\mu$ g/ml) at 37°C with 5% CO<sub>2</sub> for the indicated time periods. The IKK-2 inhibitor BMS-345541 and the stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) inhibitor SP600125 were purchased from Calbiochem (San Diego, CA, USA). The MAPK/ERK kinase (MEK) inhibitor PD98059 and the p38 inhibitor PD169316 were purchased from A.G. Scientific, Inc. (San Diego, CA, USA). For the analysis of the signaling pathway, RAW 264.7 cells were preincubated with SP 600125 for 10 min and with BMS-345541, PD 98059, or PD 169316 for 1 h before stimulation with CpG-DNA. DMSO was used as a vehicle control.

### Reverse-transcription PCR analysis

We performed a RT-PCR analysis after cells were treated with CpG-ODN 1826 or non-CpG-ODN 2041 (3 µg/ml) in the presence or absence of pathway-specific inhibitors for the indicated periods as described elsewhere (26). Total RNAs were extracted from the cells with an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Five micrograms of total RNA was reverse-transcribed in the first-strand buffer containing 6 µg/ml oligo (dT) primers, 50 U StrataScript reverse transcriptase, 2 mM dNTP, and 40 U RNase inhibitor. The reaction was performed at 42°C for 1 h. One microliter of the cDNA solution was subjected to the standard PCR reaction. The primer sequences are as follows: Mouse CD83, 5'-CGG AGAGCAAGCAAAACAGC-3' (sense) and 5'-TGTAGCTTCCT TGGGGCATC-3' (anti-sense); mouse GAPDH, 5'-ATGGTGAA GGTCGGTGTGAACG-3' (sense), and 5'-GTTGTCATGGATGA TCTTGCC-3' (anti-sense). PCR products were resolved on a 1% agarose gel and visualized with UV light after being stained by ethidium bromide.

### FACS analysis

The expression of MHC class II and costimulatory molecules (CD80, CD83, and CD86) was analyzed with a FACS Aria II flow cytometer (BD Biosciences, San Diego, CA, USA). FITC-conjugated anti-MHC class II antibodies, PE-conjugated anti-CD80 antibodies, PE-conjugated anti-CD83 antibodies, and PE-conjugated anti-CD86 antibodies were purchased from BD Biosciences. RAW 264.7 cells were washed with PBS containing 0.1% bovine serum albumin and incubated for 20 min at 4°C with 10 µg/ml of anti-FcγRII/III antibody (BD Biosciences) to block Fc receptors. After blocking, the cells were incubated with the indicated antibodies for 1 h at 4°C. FACS data were analyzed with the aid of WinMDI 2.8 FACS software.

### Dextran uptake assay

FITC-conjugated dextran (150 kDa) was obtained from TdB Consultancy AB (Uppsala, Sweden). RAW 264.7 cells were stimulated with non-CpG ODN 2041 (5 µg/ml) or CpG-ODN 1826 (5 µg/ml) in the presence or absence of pathway-specific inhibitors for 6 h and then cultured with FITC-conjugated dextran (25 µg/ml) for 2 h at 37°C. After incubation, cells were washed three times with PBS containing 0.1% bovine serum albumin to remove excess dextran and fixed with cold 1% formalin. The cells were washed with PBS containing 0.1% bovine serum albumin and incubated for 20 min at 4°C with 10 µg/ml of anti-FcγRII/III antibody (BD Biosciences) to block Fc receptors. After blocking, the cells were incubated with the PE-conjugated anti-CD83 antibodies for 1 h at 4°C. FACS data were analyzed with the aid of WinMDI 2.8 FACS software. All experiments were repeated at least 3 times with similar results. Data are expressed as the mean ± SD. Statistical analysis was conducted using the student's t-test (\*\*P < 0.05).

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