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Comparison of the Type of Liposome Involving Cytokine Production Induced by Non-CpG Lipoplex in Macrophages

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Abstract: To improve the transfection efficiency of plasmid DNA (pDNA) into cells, various types of cationic liposome have been used to prepare pDNA/cationic liposome complexes (lipoplexes). It is well-known that lipoplexes induce a large amount of proinflammatory cytokines because unmethylated CpG dinucleotides (CpG motifs) abundantly present in pDNA are recognized by Toll-like receptor-9 (TLR9) expressed in immune cells such as macrophages and dendritic cells. This nonspecific cytokine production is problematic in nonviral gene therapy. Moreover, recent studies have demonstrated that lipoplexes induce not only proinflammatory cytokines but also another type of cytokine, type I interferons (IFNs), irrespective of the frequency of CpG motifs in DNA and the expression of TLR9. To gain more insight into the CpG motif- and TLR9-independent induction of type I IFNs and proinflammatory cytokines by lipoplex, macrophage activation was evaluated *in vitro* using various cationic liposomes complexed with pDNA containing no CpG motifs. The production of IFN-β, TNF-α and IL-6 by lipoplex was confirmed to be induced independently of the interaction between CpG DNA and TLR9 in macrophages from TLR9-knockout mice. Then, the release of the cytokines, the mRNA expression of *Z-DNA binding protein-1* (*Zbp1*), a cytosolic double-stranded DNA sensor, and the cellular uptake of pDNA were examined in a macrophage-like cell line, RAW264.7. The level of cytokine production and the increase in the *Zbp1* mRNA varied depending on the type of cationic liposome used. A good correlation was observed between the cytokine level and the *Zbp1* mRNA. A confocal microscopic study using fluorescently labeled pDNA complexes showed that the complexes that released a lot of cytokines showed an enhanced distribution of pDNA-derived fluorescence into the cytosol. These results suggest that different intracellular trafficking derived from the type of liposomes determines the recognition of pDNA by ZBP1 after uptake of lipoplexes by the macrophages, followed by the release of type I IFNs and inflammatory cytokines. The present study demonstrates that cationic liposomes should be selected based on these findings for optimization of DNA-based therapies using lipoplexes.

Keywords: Cationic liposome; non-CpG plasmid DNA; transfection; inflammatory response; Z-DNA binding protein-1

Introduction

Plasmid DNA (pDNA) has become an important macromolecular agent suitable for nonviral gene therapy as well as DNA vaccination.[1] It is well-known that unmethylated CpG dinucleotides, or CpG motifs, which are abundant in bacterial DNA but not in vertebrate DNA, are recognized by the mammalian immune system as a danger signal and activate innate immunity.[2] Toll-like receptor-9 (TLR9) is a pattern recognition receptor recognizing CpG motifs,[3] and

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is present in the intracellular compartments of immune cells, such as macrophages and dendritic cells responsible for the innate immune response.[4−7] These cells secrete a large amount of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-12, upon uptake of the CpG motif-containing DNA. This nonspecific induction of proinflammatory cytokines is problematic because transgene expression from vectors is reduced due to their cytotoxicity to cells expressing the transgene or promoter attenuation activity.[8−10] In order to overcome this problem, CpG-reduced pDNA and CpG-deleted pDNA have been constructed, which offer an improvement in safety and gene expression through reduced CpG motif-dependent inflammatory cytokines induced by pDNA.[11,12]

To improve the transfection efficacy of pDNA, various types of cationic liposomes are often used to obtain pDNA/cationic liposome complexes (lipoplexes) and the resultant complexes have been used both *in vivo* and *in vitro*. However, we and others have demonstrated that DNA complexed with cationic liposomes induces innate immune responses via both TLR9-dependent and -independent pathways using primary cultured cells from *Tlr9*$^{-/-}$ mice.[13−15] Moreover, it has also been reported that, when a large quantity of DNA is introduced into the cells using cationic liposomes, double-stranded DNA (dsDNA) derived from either pathogens or the host, but not single-stranded DNA, activates both immune and nonimmune cells, such as thyroid cells and mouse embryonic fibroblasts (MEFs).[14,16] It has been reported that type I interferons (IFNs), most notably IFN-β, play a key role in the immunostimulatory cascade triggered by dsDNA.[17,18] Takaoka et al. have shown that Z-DNA binding protein-1 (ZBP1) is a candidate for a cytosolic dsDNA receptor leading to the induction of type I IFN signals and other immune responses, and renamed as a DNA-dependent activator of IFN-regulatory factors (DAI).[19] In addition, the authors of this previous study have demonstrated that a synthetic linear dsDNA containing no CpG motifs activates RAW264.7, murine macrophage-like cell lines, and L929 followed by not only the upregulation of *Ifn-β* and *Il-6* mRNA but also the production of their proteins via interaction with ZBP1.[19]

In addition to proinflammatory cytokines, type I IFNs also markedly affect various aspects of pDNA-based approaches. It has been reported that type I IFNs potently suppress gene expression from plasmid vector delivered in the form of lipoplex.[20] On the other hand, DNA-induced type I IFNs have been shown to be essential for the effect of DNA vaccination.[21] Therefore, it is extremely important to understand the mechanism of the pDNA-mediated production of both inflammatory

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cytokines and type I IFNs in pDNA-based therapies, such as gene therapy and DNA vaccination. In a series of basic *in vitro* studies on cytokine production induced by dsDNA, DNA has been complexed with various cationic reagents, such as Lipofectamine2000 (LA2000),[15,19,21–24] FuGENE 6,[17,18,25] 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP)[26] and LAplus.[13,27] However, only a single cationic reagent was used for efficient DNA delivery to the cells of interest in each study. To our knowledge, there is no report directly comparing the characteristics of immune responses to different types of lipoplexes.

In the present study, in order to gain more insight into the production of type I IFNs and inflammatory cytokines induced by lipoplex, we examined the production of IFN-β, TNF-α and IL-6 in macrophages upon stimulation with lipoplexes composed of various cationic liposomes focusing on ZBP1. From therapeutic point of view, it would be very important to reveal the molecular mechanisms underlying the induction of these cytokines by lipoplex, a representative pDNA delivery system, because they could affect the outcome of DNA-based therapies, gene therapy and DNA vaccination. We used pCpG-ΔLuc, a pDNA containing no CpG motifs, for lipoplex preparation to clearly analyze the cytokine production independent of the interaction of CpG motifs and TLR9. Here, we show that the non-CpG pDNA/cationic liposome complex (non-CpG lipoplex) activates macrophages in a TLR9-independent manner and that ZBP1 is significantly involved in the cytokine production. We also demonstrate the importance of the type of cationic liposome in the cytokine production induced by non-CpG lipoplexes, because it markedly influences ZBP1 expression and the amount of pDNA delivered to the cytosol.

Experimental Section

Chemicals. Lipofectamine 2000 (LA2000), Lipofectamine (LA), and Plus reagent were purchased from Invitrogen (Carlsbad, CA). According to the manufacturer's information, LA is composed of 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA)/dioleoylphosphatidylethanolamine (DOPE), and LAplus was prepared by adding the Plus reagent to LA. LA2000 contained various components although no details were given. *N*-[1-(2,3-Dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was obtained from Tokyo Kasei (Tokyo, Japan). DOPE was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol and Triton X-114 were obtained from Nacalai Tesque (Kyoto, Japan). LPS, polymyxin B sulfate salt, and poly(dA) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IFN-β was obtained from PBL InterferonSource (Piscataway, NJ).

Cell Cultures. Male C57BL/6 mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). *Tlr9*$^{-/-}$ mice with a C57BL/6 genetic background were used at 9−11 weeks of age. Peritoneal macrophages were collected and cultured on 24-well culture plates as previously reported.[28] The murine macrophage-like cell line, RAW264.7 cells, was cultured in RPMI-1640 supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (292 μg/mL). The cells were plated on 96- or 24-well culture plates at a density of 5×10^4 cells/well or 2.5×10^5 cells/well, respectively, and cultured for 24 h. Colon26/NF-κB-Luc, a clone of murine colon carcinoma colon26 cells stably expressing NF-κB responsive firefly *luciferase* reporter gene,[29] were cultured in DMEM supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (292 μg/mL). They were then plated on 96-well culture plates at a density of 2.5×10^4 cells/well and cultured for 24 h.

Plasmid DNA. pcDNA3.1 vector (Invitrogen) has 27 5′-Pur-Pur-CpG-Pyr-Pyr-3′ sequences including two GACGTT sequences reported to be the most potent CpG motifs for

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mice.[30] pCpG-ΔLuc vector was constructed by inserting the *Bgl*II/*Nhe*I firefly *luciferase* cDNA fragment from pORF-Luc::Sh-ΔCpG (InvivoGen, San Diego, CA), which contains no CpG, into the *Bgl*II/*Nhe*I site of the pCpG-mcs vector (InvivoGen). pNF-κB-Luc containing five tandem repeats of an NF-κB-binding site was purchased from Stratagene (La Jolla, CA). pCMV-Luc was amplified in the *Escherichia coli* strain DH5α and pCpG-ΔLuc was amplified in the *E. coli* strain GT115 and then isolated and purified using a JET-STAR 2.0 Plasmid GIGA Purification Kit (Genomed GmbH, Bad Oyenhausen, Germany). Purified pCpG-ΔLuc was sequenced by the Shimadzu Genomic Research Laboratory (Shimadzu, Kyoto, Japan), and it was confirmed that the vector had no CpG dinucleotides.

Purification of DNA. After purification using the JET-STAR 2.0 Plasmid GIGA Purification Kit, DNA samples were extensively purified with Triton X-114 to minimize cellular activation by contaminated LPS as previously reported.[28,31,32] The level of contaminated LPS was checked by a Limulus amebocyte lysate assay using the Limulus F Single Test kit (Wako, Tokyo, Japan) and was found to be reduced below the detection limit of 0.001 EU/μg pDNA by the Triton X-114 extraction.

Preparation of Cationic Liposomes and Lipoplexes. Cationic liposomes were prepared as previously reported.[33] In brief, DOTMA and neutral helper lipid (DOPE or cholesterol) were mixed in chloroform at a molar ratio of 1:1, then the mixture was dried, vacuum-desiccated, and resuspended in 5% dextrose solution in sterile test tubes. After hydration for 30 min at room temperature, the dispersion was sonicated for 2.5 min in a tip sonicator to form liposomes, and then three cycles of freezing and thawing treatment were performed. Finally, the dispersion was sterilized by passing through a Ministart 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). DOTMA/DOPE liposomes or DOTMA/cholesterol liposomes and DNA were mixed at a charge ratio of +2.0 and left at room temperature for 30 min to form a lipoplex. The DNA/LA2000 complex and DNA/LAplus complex were prepared according to the manufacturer's instructions. LA2000, LA, and Plus reagent were added at a concentration of 2, 1, and 1.2 μL volume per 1 μg of DNA, respectively. The size and zeta-potential of lipoplexes were measured using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) (Table 1). Condensation of pDNA and positively charged lipoplex formation were confirmed.

Table 1. Physicochemical Properties of pDNA and Lipoplexes Used in This Study

	particle size (nm)	zeta-potential (mV)
naked pCpG-ΔLuc	1870 ± 476	
lipoplex		
LA2000	107.8 ± 26.5	35.1 ± 2.6
LAplus	78.7 ± 40.1	33.3 ± 3.6
DOTMA/DOPE	69.4 ± 15.8	32.7 ± 3.7
DOTMA/cholesterol	72.1 ± 3.9	29.6 ± 2.0

Cytokine Release from Macrophages. Cells were incubated with polyI:C, LPS, or pcDNA3.1 for 8 h, and the supernatants were collected for ELISA and kept at −80 °C. In the case of polyI:C, 5 μg/mL polymyxin B was added to avoid activation by contaminated LPS. Separately, cells were incubated with lipoplex for 2 h. Then, cells were washed with RPMI 1640 and incubated with each growth medium for an additional 6 h, and the supernatants were collected for ELISA and kept at −80 °C. The incubation time of lipoplex was slightly shorter than the standard transfection time described in the manufacturer's instruction of LA2000 and LAplus to avoid the cytotoxicity induced by the high concentration of lipoplexes. The level of TNF-α and IL-6 in the supernatants was determined by ELISA using the OptEIA set (BD Biosciences Pharmingen, San Diego, CA). The IFN-β level in the supernatants was measured by ELISA as described previously.[20] In the IFN-β pretreatment experiment, cells were incubated with IFN-β at the indicated concentration for 6 h, and the IFN-β in the medium was washed out before addition of the lipoplex. In our preliminary experiments, 1000 U/mL IFN-β was approximately equal to 20 ng/mL IFN-β. Preapplied IFN-β was negligible in the ELISA assay because no IFN-β was detected in medium-treated cells.

NF-κB Activation in Colon26/NF-κB-Luc Cells. Colon26/NF-κB-Luc cells were incubated with the lipoplex for 2 h. Then, cells were washed and incubated with growth medium for an additional 6 h. The activation of NF-κB was determined by measuring the luciferase activity of cell lysates in a luminometer (Lumat LB9507; EG & G Berthold, Bad Wildbad, Germany) as previously reported.[29]

Uptake of Lipoplex in RAW264.7 Cells. pDNA was labeled using a LabelIT Cy3 Labeling Kit (Mirus Bio, Madison, MI) according to the manufacturer's instructions. Cy3-labeled pDNA was used for the preparation of the lipoplex. RAW264.7 cells were incubated with naked Cy3-labeled pDNA or Cy3-labeled lipoplex for 30 min or 2 h at 4 or 37 °C and washed three times with phosphate-buffered saline. Then, the intensity of the cell fluorescence was analyzed by flow cytometry (FACScan; BD Biosciences, San

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Jose, CA) using CellQuest software (version 3.1; BD Biosciences).

mRNA Quantification. Total RNA was isolated using MagExtractor MFX-2100 and a MagExtractor RNA kit (TOYOBO, Osaka, Japan) according to the manufacturer's protocol. Before reverse transcription, the total RNA was treated with DNase I (Takara Bio, Shiga, Japan). Reverse transcription was performed using a SuperScript II (Invitrogen) and dT-primer following the manufacturer's protocol. For quantitative mRNA expression analysis, real-time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The oligodeoxynucleotide primers used for amplification were as follows: *Zbp1*-sense 5′-GAC GAC AGC CAA AGA AGT GA-3′, *Zbp1*-antisense 5′-GAG CTA TGT CTT GGC CTT CC-3′; and *Gapdh*-sense 5′-CTG CCA AGT ATG ATG ACA TCA AGA A-3′, *Gapdh*-antisense 5′-ACC AGG AAA TGA GCT TGA CA-3′. Amplification products were detected online via intercalation of the fluorescent dye SYBR green (SYBR *Premix Ex Taq*; Takara Bio).

Confocal Microscopy. pCpG-ΔLuc was fluorescently labeled with YOYO-1 iodide (Molecular Probes, Eugene, OR) or using a LabelIT Fluorescein Labeling Kit (Mirus Bio) according to the manufacturer's instructions. Cells were incubated with fluorescently labeled (FL-pDNA) complexed with LA2000 or DOTMA/cholesterol. After a 15 min or 2 h incubation at 37 °C, cells were washed and incubated with the medium containing 10 μg/mL of transferrin-Alexa Fluor 594 (Molecular Probes) or 100 nM LysoTracker Red DND-99 (Molecular Probes), for 15 min. Cells were washed and fixed with 4% paraformaldehyde for 15 min at room temperature. The images of the cells were captured digitally using a laser scanning confocal microscope (LSM5 Pascal; Zeiss, Jena, Germany) and Pascal LSM software (Zeiss).

Statistical Analysis. Differences were statistically evaluated by Student's *t* test. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

TLR9- and CpG Motif-Independent Cytokine Production Induced by Lipoplexes in Peritoneal Macrophages from *Tlr9*$^{-/-}$ Mice. To confirm the TLR9- and CpG motif-independent cytokine production by pDNA, the production of IFN-β and IL-6 was examined in peritoneal macrophages from *Tlr9*$^{-/-}$ mice. A CpG replete pDNA, pcDNA3.1, or a non-CpG pDNA, pCpG-ΔLuc, was mixed with LA2000, one of the most widely used cationic liposomes for *in vitro* transfection, to obtain lipoplexes. Both pcDNA3.1/LA2000 and pCpG-ΔLuc/LA2000 complexes induced IFN-β (Figure 1A) and IL-6 (Figure 1B) production in peritoneal macrophages. The presence of CpG motifs in pDNA did not increase the production of these cytokines induced by the pDNA/LA2000 complex, probably because the cells did not express TLR9. Naked CpG ODN 1668, a single-stranded phosphorothioate DNA and a typical ligand for TLR9, was a weak inducer of cytokines in *Tlr9*$^{-/-}$ macrophages. Therefore, the cytokine production in *Tlr9*$^{-/-}$ macrophages can be considered to be mediated by mechanisms

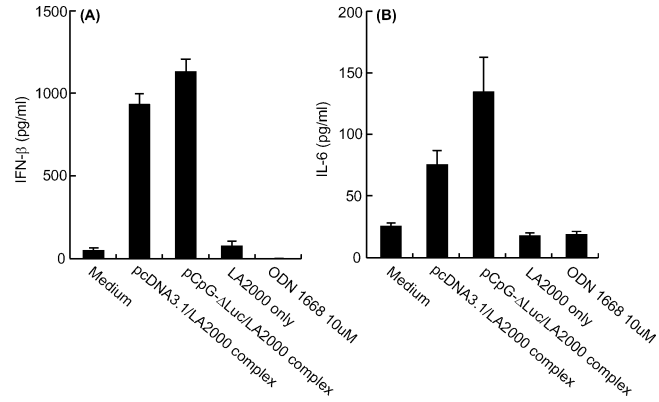

Figure 1. Cytokine production induced by pDNA/LA2000 complexes in peritoneal macrophages from *Tlr9*$^{-/-}$ mice. The pDNA/LA2000 complex (5:10 μg/mL) was added to the cells. After an 8 h incubation, supernatants were collected and the level of IFN-β (A) or IL-6 (B) was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

other than the interaction of CpG DNA and TLR9. These results clearly demonstrate that pDNA complexed with LA2000 induces the production of IFN-β and IL-6 in macrophages in a TLR9- and CpG motif-independent manner.

Cytokine Production Induced by Non-CpG Lipoplexes Composed of Various Cationic Liposomes in RAW264.7 Cells. In the following experiments, RAW264.7, a murine macrophage cell line, was selected, and the CpG motif-independent immune response was examined using non-CpG lipoplexes. Figure 2 shows the cytokine production in RAW264.7 cells upon addition of non-CpG lipoplexes composed of various cationic liposomes. The incubation with

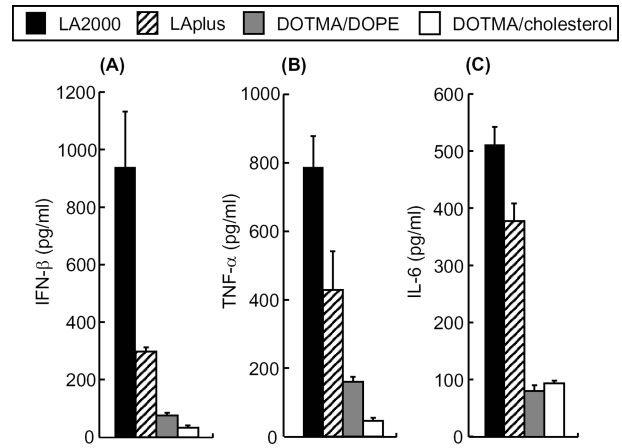

Figure 2. Cytokine production induced by non-CpG lipoplexes composed of various cationic liposomes in RAW264.7 cells. The cells were incubated with pCpG-ΔLuc complexed with LA2000 (black bars) (5:10 μg/mL), LAplus (hatched bars) (5:10 μg/mL), DOTMA/DOPE (gray bars) (5: 42.7 μg/mL) or DOTMA/cholesterol (white bars) (5: 31.9 μg/mL). After an 8 h incubation, the supernatants were collected, and the concentration of IFN-β (A), TNF-α (B) or IL-6 (C) was determined by ELISA. Each result represents the mean ± SD ($n = 3$).

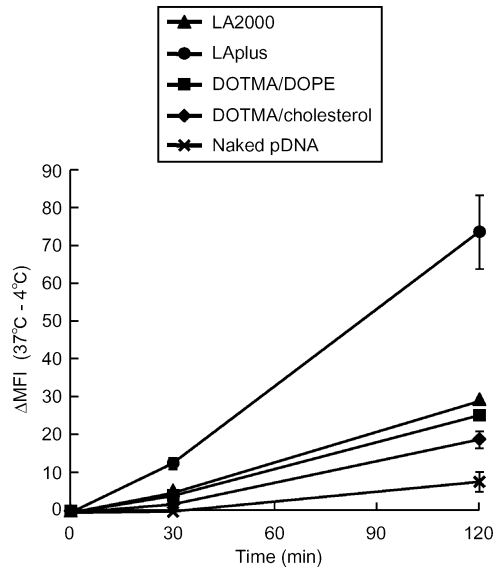

Figure 3. Uptake of Cy3-labeled pCpG-ΔLuc/cationic liposome complexes by RAW264.7 cells. The cells were incubated with naked Cy3-labeled pDNA (cross) or the Cy3-labeled pCpG-ΔLuc complexed with LA2000 (triangle) (5:10 μg/mL), LAplus (circle) (5:10 μg/mL), DOTMA/DOPE (square) (5: 42.7 μg/mL), DOTMA/cholesterol (diamond) (5: 31.9 μg/mL) for 30 min or 2 h at 4 °C or 37 °C and the amount of pDNA associated with the cells was measured by flow cytometry. The MFI is expressed as the mean ± SD ($n = 2$).

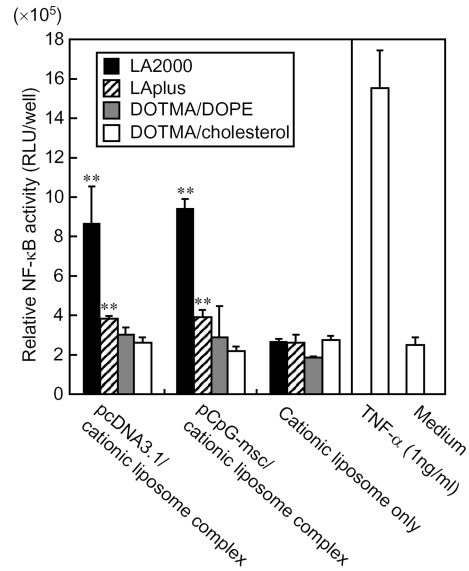

Figure 4. NF-κB activation induced by lipoplexes composed of various cationic liposomes in colon26/NF-κB-Luc cells. The cells were incubated with the pDNA complexed with LA2000 (black bars) (5:10 μg/mL), LAplus (hatched bars) (5:10 μg/mL), DOTMA/DOPE (gray bars) (5:42.7 μg/mL) or DOTMA/cholesterol (white bars) (5:31.9 μg/mL). After an 8 h incubation, the activity of NF-κB was determined by measuring the luciferase activity of cell lysates. Each result represents the mean ± SD ($n = 3$). Significantly different (**$P < 0.01$) from medium-treated cells.

liposomes alone induced no cytokine production, suggesting that cationic liposomes are immunologically inert in the cells (data not shown). pCpG-ΔLuc/LA2000 complex induced the production of IFN-β (Figure 2A) and inflammatory cytokines, TNF-α (Figure 2B) and IL-6 (Figure 2C), in RAW264.7 cells, indicating that CpG motif-independent cytokine production can also be observed in the cells. To examine whether the type of cationic liposome affects this CpG motif-independent cytokine production from macrophages, lipoplexes were prepared using various cationic liposomes: LA2000, LAplus, DOTMA/DOPE, and DOTMA/cholesterol liposomes. All the non-CpG lipoplexes prepared induced the production of IFN-β (Figure 2A), TNF-α (Figure 2B) and IL-6 (Figure 2C). However, the level of cytokine production was markedly different among the four types of lipoplexes composed of different cationic liposomes. The highest level of cytokines was observed in the LA2000 complex-treated cells, followed by the LAplus complex and the DOTMA/DOPE complexes, and the DOTMA/cholesterol complex scarcely induced any production of cytokines in the cells. In a different set of experiments, we measured the *luciferase* gene expression in RAW264.7 cells following incubation with lipoplexes under the same conditions. The luciferase activities in the cells treated with LA2000 lipoplex and LAplus lipoplex were significantly higher than those of DOTMA/DOPE lipoplex and DOTMA/cholesterol lipoplex (data not shown). Therefore, there was a similar trend in the *luciferase* gene expression to the cytokine production by lipoplex.

Uptake of pCpG-ΔLuc Complexed with Various Cationic Liposomes by RAW264.7 Cells. To investigate whether the cytokine level induced by non-CpG lipoplexes is dependent on the amount of DNA taken up by cells, the uptake of Cy3-labeled pCpG-ΔLuc/cationic liposome complexes was examined in RAW264.7 cells by measuring the fluorescence intensity of the cells using a flow cytometer, and the difference in the mean fluorescence intensity (MFI) at 4 and 37 °C (ΔMFI) was plotted. Figure 3 shows the time courses of the ΔMFI of cells incubated with naked pDNA and various non-CpG lipoplexes. For all lipoplexes examined, the uptake of pDNA was higher than that of naked pDNA and increased with time up to 2 h of incubation with DNA samples. The cellular uptake of pDNA was the highest when LAplus complex was added to cells. The ΔMFI values were almost identical for the complex using LA2000, DOTMA/DOPE and DOTMA/cholesterol. These results indicate that the cytokine production by non-CpG lipoplex is not proportional to the apparent amount of DNA taken up by cells.

NF-κB Activation by Lipoplexes Composed of Various Cationic Liposomes in Colon26/NF-κB-Luc Cells. It has been reported that dsDNA-induced cytokine production is accompanied by the activation of NF-κB, which is a key molecule leading to the production of proinflammatory cytokines. The determination of NF-κB activity can be used as one of the valuable tools for the evaluation of the immune activation mechanism at a molecular level.[19] Therefore, we investigated whether NF-κB is activated by lipoplexes by

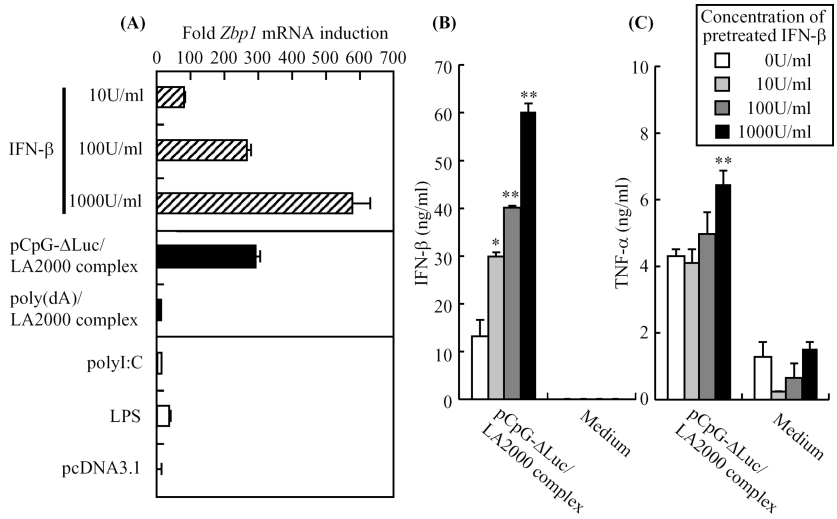

Figure 5. Involvement of *Zbp1* mRNA expression in type I IFN and inflammatory cytokine production induced by non-CpG lipoplex in RAW264.7 cells. (A) *Zbp1* mRNA expression was induced by IFN-β, DNA/LA2000 complexes, and TLR ligands in RAW264.7 cells. The cells were incubated with IFN-β (indicated concentration), DNA/LA2000 complexes (5:10 μg/mL), and TLR ligands, polyI:C (TLR3 ligand, 5 μg/mL), LPS (TLR4 ligand, 1 ng/mL), and pcDNA3.1 (TLR9 ligand, 10 μg/mL). *Zbp1* mRNA levels were measured 6 h later by real-time PCR. Each result represents the mean $\pm$ SD ($n = 3$) in *Zbp1*/*Gapdh* mRNA levels compared with untreated cells. (B, C) Cytokine production induced by the pCpG-ΔLuc/LA2000 complex in IFN-β-pretreated RAW264.7 cells. The cells were pretreated with IFN-β at indicated concentrations. After a 6 h incubation, IFN-β was washed out, and then the cells were incubated with the pCpG-ΔLuc/LA2000 complex (5:10 μg/mL). After an incubation of more than 8 h, supernatants were collected, and the concentration of IFN-β (B) or TNF-α (C) was determined by ELISA. Each result represents the mean $\pm$ SD ($n = 3$). Significantly different (*$P < 0.01$, **$P < 0.001$) from 0 U/mL of IFN-β pretreated cells.

using colon26/NF-κB-Luc cells. The cells did not express TLR9 but were stably transfected with pNF-κB-Luc encoding firefly *luciferase* cDNA driven by a basic element plus five tandem copies of NF-κB binding elements [(TGGGGACTTTCCGC)$_5$],[34] so NF-κB activation induced by lipoplexes can easily be evaluated by measuring the luciferase activity in the cells. To avoid the expression of firefly luciferase from pDNA added as lipoplexes, empty pDNAs, i.e., pcDNA3.1 (CpG-replete pDNA) and pCpG-mcs (non-CpG pDNA), were used. Figure 4 shows the luciferase activity of the lysates of colon26/NF-κB-Luc cells 8 h after addition of CpG or non-CpG lipoplex. Cationic liposome alone did not increase the luciferase activity. The addition of pCpG-mcs/LA2000 complexes as well as TNF-α, a well-known activator of NF-κB, greatly increased the luciferase activity of the cells, indicating that NF-κB was activated by pCpG-mcs/LA2000 complexes. pCpG-mcs/LAplus complex showed only weak activation, and no significant activation was observed for DOTMA-based complexes. On the other hand, lipoplexes containing pcDNA3.1 increased the luciferase activity of the cells as much as lipoplexes containing pCpG-mcs did, which can be explained by the fact that TLR9 is not expressed in the cells. Therefore, CpG lipoplexes and non-CpG lipoplexes equally activate NF-κB through mechanisms different from the TLR9 pathway, when pDNA is added to cells in the complexed form with the same cationic liposomes.

Involvement of *Zbp1* mRNA Expression in Cytokine Production Induced by Non-CpG Lipoplex in RAW264.7 Cells. As one of the DNA receptors other than TLR9, ZBP1 was recently identified to be a candidate molecule for a cytosolic dsDNA receptor contributing to cytokine production.[19,35] Therefore, we examined the relationship between the cytokine production and ZBP1 expression. The expression of ZBP1 is known to be modulated by cytosolic dsDNA and IFN-β treatment. At first, we confirmed the effect of these treatments on the *Zbp1* mRNA expression in RAW264.7 cells (Figure 5A). RAW264.7 cells were treated with different concentrations of IFN-β, DNA/LA2000 complex or TLR ligands. The mRNA expression of *Zbp1* in RAW264.7 cells was significantly increased by IFN-β treatment in a concentration-dependent manner (Figure 5A, hatched bar), which was in good agreement with the previous report using MEFs.[19] Furthermore, the *Zbp1* mRNA expression was also greatly increased by pCpG-ΔLuc/LA2000 complex (Figure 5A, black bar). On the other hand, poly(dA)/LA2000, a complex consisting of a single-stranded DNA, induced hardly

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any increase in the *Zbp1* mRNA expression, suggesting the importance of dsDNA for the induction of *Zbp1* mRNA expression. Other TLR ligands, such as polyI:C (TLR3 ligand), LPS (TLR4 ligand) and pcDNA3.1 (TLR9 ligand), were much less effective in increasing the *Zbp1* mRNA expression compared with pCpG-ΔLuc/LA2000 complex.

Based on these results, we examined the cytokine production in RAW264.7 cells with elevated levels of the *Zbp1* mRNA expression by pretreatment with IFN-β. Figure 5B shows the IFN-β concentration in culture medium of RAW264.7 cells pretreated with different concentrations of IFN-β followed by the addition of non-CpG lipoplex. Pretreatment of IFN-β followed by the incubation with the medium alone, the negative control group, induced hardly any IFN-β production in the culture media (Figure 5B), indicating that contamination of the preapplied IFN-β was negligible in the ELISA assay for IFN-β in the cells. The IFN-β production induced by the addition of non-CpG lipoplexes increased depending on the concentration of pretreated IFN-β. On the other hand, the effect of pretreatment with IFN-β on the TNF-α production was relatively minor: only the highest concentration (1000 U/mL) significantly increased the level of TNF-α (Figure 5C). Taken together with the results showing that pretreatment with IFN-β increased the mRNA expression of *Zbp1* in an IFN-β concentration-dependent manner (Figure 5A), these results suggest that cytokine production induced by non-CpG lipoplexes correlates well with the level of mRNA expression of *Zbp1*.

Correlation of *Zbp1* mRNA Expression with Cytokine Production Induced by Non-CpG Lipoplexes Composed of Various Cationic Liposomes in RAW264.7 Cells. Next, we investigated whether the *Zbp1* mRNA expression is induced by the addition of non-CpG lipoplexes composed of different cationic liposomes. The expression of *Zbp1* mRNA in RAW264.7 cells was increased by the addition of any of the four types of complex (Figure 6A), although the level was very dependent on the type of liposome. In Figure 6B, IFN-β and TNF-α production induced by non-CpG lipoplexes in RAW264.7 cells shown in Figures 2A and 2B were plotted against the *Zbp1* mRNA expression shown in Figure 6A. A good correlation was observed between the production of these cytokines and the level of *Zbp1* mRNA expression among the four cationic liposomes.

Intracellular Localization of pDNA Complexed with LA2000 or DOTMA/Cholesterol in RAW264.7 Cells. Based on the above results, we assumed that the difference in the cytokine production by each non-CpG lipoplex would be due to the difference in the intracellular distribution of pDNA because ZBP1 is localized in the cytosolic compartment of cells. To test this hypothesis, confocal microscopic studies were carried out using the FL-pCpG-Luc complexed with LA2000 or DOTMA/cholesterol in RAW264.7 cells. At 15 min after addition of pDNA complexes, FL-pCpG-ΔLuc complexed with LA2000 and DOTMA/cholesterol were colocalized with the early endosomal marker transferrin-Alexa Fluor 594 (Figure 7A,B). After a 2 h incubation, while FL-pCpG-ΔLuc complexed with DOTMA/cholesterol was colocalized with LysoTracker, a marker for the lysosomes (Figure 7C), colocalization of FL-pCpG-ΔLuc/LA2000 with LysoTracker was significantly less (Figure 7D), suggesting that pDNA complexed with DOTMA/cholesterol, but not with LA2000, was efficiently sorted to the late endosomes or the lysosomes.

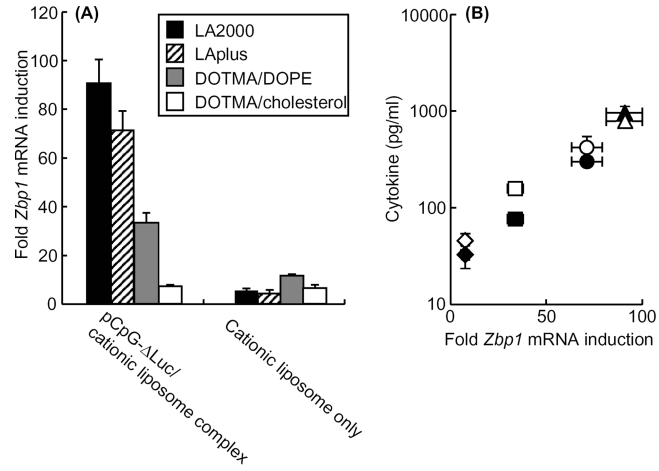

Figure 6. Correlation of *Zbp1* mRNA expression with cytokine production induced by non-CpG lipoplexes in RAW264.7 cells. (A) RAW264.7 cells were incubated with the pCpG-ΔLuc complexed with LA2000 (black bars) (5:10 μg/mL), LAplus (hatched bars) (5:10 μg/mL), DOTMA/DOPE (gray bars) (5: 42.7 μg/mL) or DOTMA/cholesterol (white bars) (5: 31.9 μg /mL). After a 12 h incubation, the *Zbp1* mRNA level in the cells was measured by real-time PCR. Each result represents the mean ± SD (n = 3) in *Zbp1*/*Gapdh* mRNA levels compared with untreated cells. (B) IFN-β (closed mark) and TNF-α (open mark) in Figure 2 were plotted against the level of *Zbp1* mRNA expression in RAW264.7 cells upon stimulation of pCpG-ΔLuc complexed with LA2000 (triangle), LAplus (circle), DOTMA/DOPE (square) and DOTMA/cholesterol (diamond).

Discussion

A number of studies using lipoplexes have reported that the efficiency of gene expression varies depending on the type of cationic liposome used for complex formation with pDNA. Cationic liposomes containing DOPE as a neutral helper lipid, which has a high ability to destabilize lipid bilayers, exhibit a high transfection activity *in vitro*.[36,37] It is suggested that cationic liposomes which contain DOPE destabilize the endosomal membrane and allow the release of DNA into the cytosol. On the other hand, cationic

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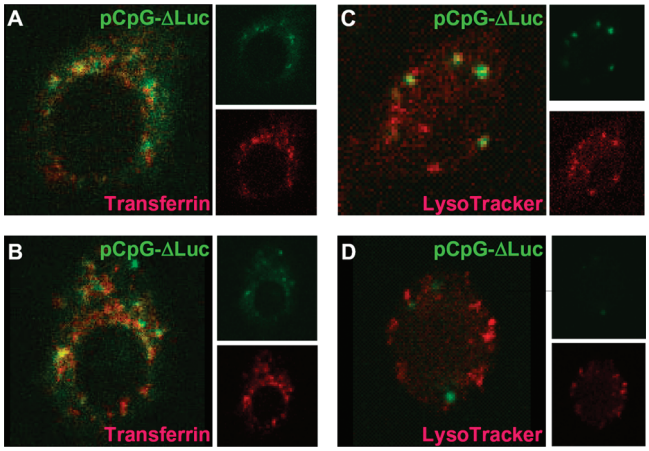

Figure 7. Confocal images of the FL-pCpG-ΔLuc complexed with LA2000 or DOTMA/cholesterol in RAW264.7 cells. The FL-pCpG-ΔLuc complexed with DOTMA/cholesterol (A, C) (1:6.4 μg/mL) or LA2000 (B, D) (1:2 μg/mL) was incubated with the cells for 15 min (A, B) or 2 h (C, D) and washed, and the medium containing transferrin-Alexa Fluor 594 (10 μg/mL) or LysoTracker Red (100 nM), respectively, was added. After a 15 min incubation, the cells were fixed and scanned by confocal microscopy.

liposomes containing cholesterol, which is known to stabilize lipid bilayers, exhibit high transfection efficiency *in vivo* because they produce hardly any fusion with erythrocytes within the blood circulation.[38] However, in the basic studies of cytokine production induced by pDNA complexed with cationic liposome, only a single cationic liposome was used for efficient DNA delivery into the cells. Therefore, we examined macrophage activation characteristics by stimulation with non-CpG pDNA complexed with four kinds of cationic liposome, i.e., two types of LA-based cationic liposomes (LA2000 and LAplus) and two types of DOTMA-based cationic liposomes (DOTMA/DOPE and DOTMA/cholesterol liposome). In the present study, lipoplexes have a tendency to induce IFN-β, IL-6 and TNF-α production dependent on the complexes in RAW264.7 cells; the complexes that induced large amounts of IFN-β exhibited a significant release of proinflammatory cytokines. pDNA complexed with LA2000 or LAplus showed more potent immunostimulatory activity than pDNA complexed with DOTMA/DOPE or DOTMA/cholesterol (Figure 2). In addition, using the TLR9-lacking cell line, colon26/NF-κB-Luc cells, we have demonstrated that pDNA complexed with LA2000 or LAplus strongly activated NF-κB in a TLR9-independent manner (Figure 4). These findings suggest that cellular activation by lipoplex is highly dependent on the type of cationic liposome complexed with pDNA. In the *in vivo* study using cholesterol-based liposomes, Sakurai et al.[39] have reported that inflammatory cytokine production was hardly induced after intravenous injection of non-CpG lipoplex composed of DOTAP/cholesterol into mice. Our very recent *in vivo* study has also demonstrated that non-CpG lipoplex composed of DOTMA/cholesterol does not produce any significant induction of IFN-β or IL-6 after intravenous injection into mice.[40] These *in vivo* results are in good agreement with the results of the present *in vitro* study using cholesterol-based liposomes and suggest that the predominant immune responses induced by lipoplexes are dependent on both the CpG motif and TLR9. However, our present study has shown that CpG motif- and TLR9-independent cytokine production may occur *in vivo* when different cationic liposomes are used. Further *in vivo* studies will be required to clarify the cytokine production by other types of lipoplexes.

Cytosolic dsDNA has recently been reported to be an immunostimulatory molecule which triggers the induction of type I IFNs and other genes involved in innate immunity through a cytosolic DNA sensor, ZBP1.[14,16,19,25] Moreover, a very recent report demonstrated that innate immune activation by cytosolic dsDNA is also observed in MEFs and bone marrow-derived dendritic cells from *Zbp1*$^{-/-}$ mice.[21] Furthermore, the existence of other DNA-sensing molecules for recognition of cytosolic dsDNA other than ZBP1 is implied, and the relative contribution of ZBP1 to the immune response induced by dsDNA depends on the type of cell.[35] In our previous studies, lipoplexes were mainly taken up by phagocytic cells such as resident macrophages after intravenous administration into mice.[40,41] Therefore, we have evaluated the cytokine production induced by non-CpG lipoplex using macrophages and clearly indicated that non-CpG lipoplex-induced cytokine production is closely related to the expression of ZBP1 in RAW264.7 cells (Figure 6B). However, taking other recent studies into consideration, DNA-sensing molecules other than ZBP1 could also be involved in the cellular activation by pDNA in RAW264.7 cells. In addition, very recent studies have identified another novel cytosolic DNA sensor involved in different innate

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immune responses in macrophages.[42−44] Further studies using various types of immune and nonimmune cells of the immune responses to cytosolic DNA are needed to obtain a better understanding of dsDNA-induced innate immunity.

Among the four types of complexes, pDNA/LA2000 complex and pDNA/LAplus complex were strong inducers of cytokine production and *Zbp1* mRNA expression. The cellular uptake of pDNA/LA2000 complex was almost equal to that of pDNA/DOTMA/DOPE complex and pDNA/DOTMA/cholesterol complex (Figure 3), indicating that the apparent amount of pDNA taken up by the cells is not a determinant of cellular activation. Therefore, we focused on the intracellular trafficking of pDNA. Confocal microscopic analysis showed that intracellular localization of pDNA complexed with LA2000 was different from that of pDNA complexed with DOTMA/cholesterol. In the latter case, fluorescent signals were observed in the late endosomal or lysosomal compartment after being localized in the early endosomes, indicating that most pDNA complexed with DOTMA/cholesterol follows the classical endosome−lysosome pathway, and then the DNA is efficiently degraded in the lysosomes. On the other hand, pDNA complexed with LA2000 was not clearly detected in the lysosomal compartment after localization in the early endosomes. These results imply that pDNA complexed with LA2000 escaped from the lysosomal compartment, perhaps into the cytosol, more efficiently than that complexed with DOTMA/cholesterol. This speculation is supported by the results showing that the LA2000 complex exhibited higher reporter gene expression than the DOTMA/cholesterol complex. More efficient delivery to the cytosol may be one of the reasons why the pDNA/LA2000 complex induces potent cellular activation through the recognition of DNA by ZBP1 and other dsDNA receptors present in the cytosol.

In conclusion, we have investigated the cytokine production induced by non-CpG lipoplex in macrophages and found that the types of cationic liposome affect cytokine production, indicating that cationic liposomes should be selected taking this into consideration. Moreover, we have shown the possibility of the involvement of ZBP1 in the cytosolic compartment in the cellular activation induced by non-CpG pDNA efficiently escaping from the lysosomal compartment with the help of cationic liposomes. The present studies suggest that the efficient trafficking of pDNA to the cytosolic compartment is generally important for effective gene expression, although this results in CpG motif-independent cytokine production by dsDNA. The results of the present study provide useful information about DNA-induced innate immune response for the achievement of optimal DNA-based therapies using nonviral plasmid vectors.

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