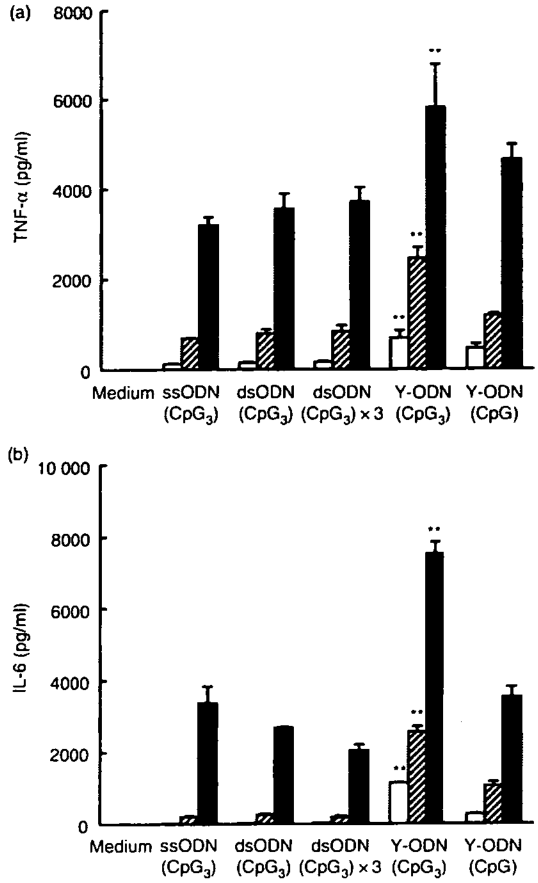


Figure 5. Secretion of cytokines from RAW264.7 cells after addition of CpG oligodeoxynucleotides (ODNs). Concentrations of (a) tumour necrosis factor-α (TNF-α) and (b) interleukin-6 (IL-6) in culture media were measured at 8 hr (TNF-α) or 24 hr (IL-6) after addition of each ODN to RAW264.7 cells at various concentrations: 2 μg/ml (open bars); 6 μg/ml (hatched bars); 18 μg/ml (closed bars). Results are expressed as the mean ± SD of three determinations. The experiment shown was representative of three experiments with similar results. $^{**}P < 0.01$, significantly different from double-stranded ODN($CpG_3$) 3 at the same concentration.

Table 2. Effect of polymyxin B on tumour necrosis factor-α (TNF-α) release from RAW264.7 cells after addition of oligodeoxynucleotides (ODNs) with or without lipopolysaccharide (LPS)

| | TNF-α (pg/ml) | |
|---|---|---|
| Compound | Control | + polymyxin B |
| Medium | 123 ± 11 | 394 ± 21 |
| Medium + LPS | 28 900 ± 700 | 448 ± 16 |
| ssODN | 278 ± 30 | 495 ± 23 |
| ssODN + LPS | 30 700 ± 300 | 557 ± 16 |
| Y-ODN($CpG_3$) | 31 200 ± 700 | 27 400 ± 700 |
| Y-ODN($CpG_3$) + LPS | 34 900 ± 1000 | 27 700 ± 1200 |

Polymyxin B, an inhibitor of LPS, was added to medium (Opti-MEM), single-stranded (ss) ODN or Y-ODN($CpG_3$) at a final concentration of 50 μg/ml. LPS was added to samples at a final concentration of 1 ng/ml. Each sample was added to cells, and the TNF-α concentration in supernatants was measured after an 8-hr incubation. Results are expressed as the mean ± SD of three determinations.

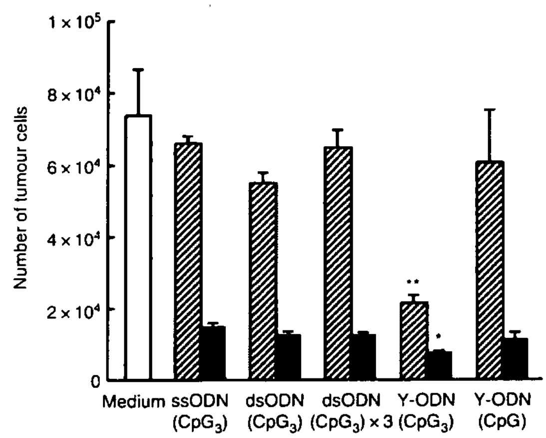


Figure 6. Growth inhibition of tumour cells by conditioned medium of RAW264.7 cells treated with oligodeoxynucleotides (ODN). Conditioned medium of ODN-treated RAW264.7 cells was added to B16-BL6/Luc cells, and the cells were cultured for additional 48 hr. Then, the number of cells was determined by measuring the luciferase activity of cell lysates. Results are expressed as the mean ± SD of three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from other conditions.

### Effect of conditioned medium of ODN-treated RAW264.7 cells on proliferation of B16-BL6/Luc cells

To examine whether Y-ODN($CpG_3$) is effective in inhibiting the proliferation of tumour cells, the conditioned medium of ODN-treated RAW264.7 cells was added to B16-BL6/Luc cells. The conditioned medium of Y-ODN($CpG_3$)-treated RAW264.7 cells significantly inhibited the proliferation of tumour cells compared with the media of other ODNs (Fig. 6).

## Discussion

CpG DNA activates immune cells expressing TLR9[1,2] and induces the cells to release a broad repertoire of chemokines and cytokines, including IL-6, IL-12, IFN-α/β, and TNF-α,[5,6] when it is sorted to endosomes after internalization via endocytosis.[24] CpG dinucleotide flanked by two purine bases on the 5′ side and two pyrimidine bases on the 3′ side, such as GACGTT, efficiently activates the murine immune system, whereas the optimal motif for humans is GTCGTT. Although the optimal sequences of CpG ODN for activating mouse or human immune cells have been identified by examining many possible base combinations, little is known about the structural requirements of CpG ODN for the recognition of TLR9 and following the activation of immune cells. Phosphodiester ODN is rapidly degraded by nucleases in body fluids, such as serum, and intracellular compartments, which greatly limits the use of such ODNs as immunostimulatory agents. However, a recent study has shown that polyguanosine at the 3′ end of ODNs can increase the immunostimulatory activity of phosphodiester ODN.[12] Furthermore, it has been reported that the immunostimulatory effect was enhanced by using self-stabilized CpG DNA.[25–27] In the present study, we prepared Y-shaped ODNs, with or without immunostimulatory CpG motifs, and found that the Y-shape formation increased the immunostimulatory responses of ODNs in RAW264.7 macrophage-line cells, even though the stability was not increased by the Y-shape formation.

Y-shaped ODN (Y-ODN), the element of dendrimer-like preparations of ODN, was prepared using three 30-base ODNs. As reported in a previous paper,[20] Y-ODN showed a single band on polyacrylamide gels, the mobility of which was less than that of the bands for ssODN or dsODN (Fig. 1b). The addition of Y-ODN to RAW264.7 cells resulted in a significant secretion of both TNF-α and IL-6 in a DNA concentration-dependent manner, even though it had no potent immunostimulatory CpG motifs. DNA sequences other than potent immunostimulatory CpG motifs may trigger weak, but detectable, cytokine responses, and these could be involved in the immunostimulatory responses to ODNs without any potent CpG motifs. Although all ODN preparations contained trace amounts of LPS, up to 2·5 EU/mg DNA, this level of LPS induced only a little TNF-α secretion from RAW264.7 cells (data not shown). Furthermore, the addition of polymyxin B, an inhibitor of LPS, had little effect on the release of TNF-α from the Y-ODN($CpG_3$)-treated RAW264.7 cells (Table 2). These results strongly support the idea that DNA is responsible for the cytokine response of cells to all ODN preparations under these experimental conditions, even though no potent CpG motifs were included in the sequences.

The RAW264.7 cells and other TLR9-positive cells secrete inflammatory cytokines on recognition of CpG DNA. TLR9 localizes in the intracellular compartments, such as the endoplasmic reticulum, and translocates to the lysosomal compartment when CpG DNA is taken up by the cells.[28] Therefore, CpG DNA should be transferred to such subcellular compartments to induce cytokine production. The level of cytokine release from TLR9-positive cells would be a function of variables, including the stability of DNA and the amount of DNA taken up by cells. Therefore, we examined whether the Y-shape formation of DNA increased the stability to nucleases and/or the cellular uptake. In the present study, we found that Y-ODN was less stable than dsODN. Y-ODNs have three terminals in one unit of the structure, whereas conventional dsODNs have two. This difference may explain the high susceptibility of Y-ODN to nuclease-mediated degradation.

In marked contrast, the Y-shape formation significantly increased the uptake of ODNs in RAW264.7 cells (Fig. 3b). A previous study demonstrated that increasing the length of ODN increases its endocytic uptake when ODNs with a length of 250 bp or less are used.[29] Because one unit of dsODN and Y-ODN contains 60 and 90 bases, respectively, this difference may be responsible for the greater uptake of Y-ODN compared with that of dsODN. However, the high immunostimulatory activity of Y-ODN could not be simply attributed to the increased uptake of Y-ODN because the increase in the level of cytokine release (three- to six-fold) by the Y-shape formation was greater than the increase in the uptake (about two-fold). Therefore, other factors, such as the affinity of ODN for TLR9 and intracellular localization, may also contribute to the increased immunostimulatory activity of Y-ODN.

To construct a new Y-ODN preparation with a potent immunostimulatory CpG motif, the GACGTT sequence, the most potent one in rodents, was inserted close to the 5′-terminal of Y0a, one of the components of Y-ODN, based on the information that TLR9 reads the CpG from the 5′-end of DNA.[30] Although the ssODN and dsODN with the CpG motifs were effective in inducing cytokines, such as TNF-α and IL-6, when added to RAW264.7 cells, the Y-ODN containing CpG motifs induced greater amounts of cytokines than these conventional CpG ODN preparations. Because all ODN preparations, i.e. ssODN($CpG_3$), dsODN($CpG_3$), dsODN($CpG_3$) × 3 and Y-ODN($CpG_3$), have identical numbers of potent CpG motifs, the Y-shape formation of ODN significantly increases the efficiency of cytokine production by CpG DNA. Furthermore, Y-ODN($CpG_3$), which contained three potent CpG motifs in one unit, was much more effective in inducing TNF-α and IL-6 compared with Y-ODN(CpG) containing only one potent CpG motif. In accordance with the levels of cytokines, the conditioned medium of Y-ODN($CpG_3$)-treated RAW264.7 cells showed a greater inhibitory effect on the growth of melanoma cells than other ODNs. Previous studies have demonstrated that CpG ODNs are a very strong activator of TLR9-positive cells, such as macrophages, B cells and dendritic cells, and have been considered as therapeutic agents against cancer, and infectious and allergic diseases.[1,31] As shown in the present study, cytokines possessing anti-proliferative activity of tumour cells, such as TNF-α, are induced by CpG ODNs. Therefore, cytokines released from CpG ODN-treated RAW264.7 cells would contribute to the growth inhibition of melanoma cells. These results indicate that highly potent immunostimulatory ODNs can be designed by increasing the number of CpG motifs in the sequences of Y-ODN.

The involvement of TLR9 in CpG DNA-mediated immune activation has been reported using TLR9$^{-/-}$ mice or cells isolated from those mice. Our preliminary experiments using TLR9$^{-/-}$ and wild-type mice suggested that Y-ODN($CpG_3$) induces TNF-α production in a TLR9-dependent manner, although the level obtained was lower than those obtained with DNA–cationic liposome complexes. The involvement of TLR9 in the immune activation by Y-ODNs was therefore evident, but it should be further investigated in future experiments.

In conclusion, the Y-shape formation of ODN has been shown to be effective in inducing greater amounts of cytokines, such as TNF-α and IL-6, in macrophage-like, TLR9-positive cells than conventional ssODN or dsODN. These enhanced immunostimulatory effects of Y-ODN are, at least partly, associated with an increase in the uptake by TLR9-positive cells, but not with stabilization of ODN. CpG DNA has been explored as a therapeutic agent for cancer, asthma, allergy, and infectious diseases and as an adjuvant in immunotherapy, but it generally requires phosphorothioate or other chemical modification. Such modification may have disadvantages associated with systemic toxicity, such as a transient anticoagulant effect, activation of complement cascade, and inhibition of basic fibroblast growth factor binding to surface receptors, because of non-specific protein binding.[32] The findings of the present study provide a novel strategy for the development of potent immunostimulatory CpG ODN preparations free from such modification problems.

## Acknowledgements

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# Effects of Inflammatory Response on *In Vivo* Transgene Expression by Plasmid DNA in Mice

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**ABSTRACT:** To examine the effects of inflammatory response to plasmid DNA (pDNA) on transgene expression, serum tumor necrosis factor-α (TNF-α) was measured after intravenous injection of pDNA or calf thymus DNA (CT DNA) in the naked or complexed form with cationic liposomes (lipoplex). pDNA with many CpG motifs induced TNF-α production regardless of the forms. No significant TNF-α production was detected when CT DNA or methylated pDNA was injected. Clodronate liposomes and dexamethasone were used to deplete phagocytes or to inhibit inflammatory responses, respectively. Transient depletion of phagocytes, such as liver Kupffer cells and splenic macrophages, by clodronate liposomes slightly altered the tissue distribution of $^{32}$P-pDNA lipoplex, but significantly reduced the TNF-α production and transgene expression. Dexamethasone significantly inhibited the initial transgene expression, but increased the duration of the expression slightly. Use of NF-κB activity-dependent plasmid vector suggested that the inhibition of NF-κB activation is involved in the reduced expression by these treatments. These findings indicate that tissue macrophages are closely involved in the CpG motif-dependent TNF-α production. It is also suggested that TNF-α activates NF-κB and increases transgene expression by pDNA having many NF-κB binding sites, but TNF-α also reduces transgene expression at later time periods, leading to short-term transgene expression. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

**Keywords:** plasmid DNA; gene delivery; lipids; lipoplexes; distribution; inflammatory cytokines; clodronate liposomes; dexamethasone; CpG motif

## INTRODUCTION

Plasmid DNA (pDNA) expressing a therapeutic protein shows great promise in applications to *in vivo* gene therapy. Compared with viral vectors, pDNA possesses several advantages, including an excellent safety profile, essentially unlimited DNA carrying capacity, and simple large-scale production. However, transfection efficiency needs to be greatly improved for therapeutic applications.

Another concern using pDNA is the abundant presence of unmethylated CpG dinucleotides, or CpG motifs in the sequence. They are recognized by the mammalian immune system as a danger signal through a pathogen recognition receptor, Toll-like receptor 9 (TLR9).[1] After TLR9-positive cells, such as macrophages and dendritic cells, take up CpG motif-containing DNA, they secrete inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-12. It has been previously reported by our laboratory that primary murine macrophages and dendritic cells efficiently take up and degrade DNA, and then release inflammatory cytokines depending not only on the presence of the motif, but also on the formulation of DNA.[2–5] Because these cytokines

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are cytotoxic, the excessive production of these cytokines is generally regarded as a side effect of nonviral vector administration and should be reduced as much as possible. Various approaches have been designed to reduce cytokine production, including reduction in the number of CpG motifs[6,7] and the use of PCR-amplified DNA fragments.[8,9]

On the other hand, biological responses, including cytokine production, may affect the level and duration of transgene expression after *in vivo* gene transfer. Of the cytokines induced by pDNA administration, TNF-α is a well-known activator of the transcription factor nuclear factor κB (NF-κB) that is present in the cytoplasm of a variety of cells.[10–12] Following activation, NF-κB translocates and accumulates in the nucleus, binds to DNA elements containing NF-κB biding sequence, and participates in the activation of transcription of various genes.[13] Genes activated by NF-κB include cell-surface molecules, such as immunoglobulin κ light chain, class I and II major histocompatibility complexes, and various cytokines. In addition, some viruses including cytomegalovirus (CMV) have also NF-κB binding sites in their enhancers, and viral production is stimulated by agents that activate NF-κB.[14]

These pieces of evidence have led us to form the following hypothesis: (1) TNF-α is secreted primarily by macrophages that recognize the CpG motifs in pDNA, (2) pDNA-induced TNF-α activates NF-κB in target cells, which, in turn, increases the transgene expression by pDNA in which any NF-κB responsive element is incorporated, and (3) pDNA-induced TNF-α plays a major role in cytokine-mediated cytotoxicity and suppresses transgene expression. In a previous study,[15] we have proved that NF-κB activation by pDNA-cationic liposome complex, or lipoplex, can be used to enhance lipoplex-mediated transgene expression by inserting NF-κB binding sites into a conventional pDNA. Although nonviral vectors could induce the release of TNF-α and other cytokines upon administration, depending on their physicochemical and biological properties, the overall effects of such inflammatory response on transgene expression are not yet fully understood. To develop a strategy for optimizing *in vivo* gene therapy using pDNA, it is important to investigate the correlation between inflammatory cytokine production and transgene expression after pDNA administration.

In the present study, therefore, we first investigated the cellular basis for the secretion of TNF-α in mice following administration of naked pDNA or its lipoplex. Then, the effects of TNF-α production on transgene expression were examined in mice treated with clodronate liposomes or dexamethasone to inhibit cytokine production. Correlation between NF-κB activation and transgene expression was also examined using an NF-κB activity-dependent pDNA.

## MATERIALS AND METHODS

### Chemicals

*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol (Chol) was purchased from Nacalai Tesque (Kyoto, Japan). Lipopolysaccharide (LPS), dichloromethylenediphosphonic acid disodium salt (clodronate), and dexamethasone were purchased from Sigma (St. Louis, MO). [α-$^{32}$P]dCTP was purchased from Amersham (Tokyo, Japan). All other chemicals used were of the highest purity available.

### DNA

pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). pGL3-control vector was purchase from Promega (Madison, WI). PathDetect® NF-κB *cis*-reporting pNF-κB-Luc and pLuc-mcs plasmids were purchased from Stratagene (La Jolla, CA, USA). pCMV-Luc encoding firefly *luciferase* gene was constructed based on pcDNA3 as described previously.[16] pcDNA3 contains 26 Pur-Pur-CpG-Pyr-Pyr sequences including two GACGTT that have been reported to be the most potent CpG motif in mice.[17] pGZB vector,[18] a CpG-reduced pDNA that has a backbone different from pCMV vectors, was kindly provided by Dr. Yew (Genzyme Corporation, Cambridge, MA). To construct pGZB-Luc, the firefly *luciferase* cDNA fragment amplified from pCMV-Luc was inserted into the *Sfi*I/*Eco*RI site of pGZB vector. pCpG-Luc was constructed by inserting the firefly *luciferase* cDNA fragment amplified from pCMV-Luc into the *Bgl*II/*Nhe*I site of pCpG-mcs vector (Invivogen, San Diego, CA). pCpG-Luc was amplified in *E. coli* GT115 and the other pDNA were amplified in *E. coli* DH5α and then isolated and purified using a Qiagen Endofree™ Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The frequency of CpG dinucleotides was 6.0% and 3.1% for pCMV-Luc and pGZB-Luc, respectively, and these


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pDNA were used as CpG motif-containing DNA. pCMV-Luc was treated with *Sss*I CpG methylase (New England Biolabs, Ipswich, MA), which methylates only the cytosine of CpG dinucleotides, to obtain methylated pCMV-Luc.[3] The methylation was confirmed by digesting the DNA with *Hpa*II. Calf thymus DNA (CT DNA; Sigma) and methylated pCMV-Luc were used as DNA with few or no CpG motifs.

### Preparation of Cationic Liposomes and Lipoplex

Cationic liposomes containing DOTMA and cholesterol in a 1:1 molar ratio were prepared by allowing the lipids to dry as a thin film in a round-bottomed flask using a rotary evaporator, and then hydrating in 5% w/v dextrose by gentle vortexing. After hydration, the dispersions were sonicated for 3 min and passed through a MILLEX®-HV 0.45 μm filter unit (Millipore, Bedford, MA). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako kit (Wako Pure Chemical, Osaka, Japan). Cationic liposomes and DNA in 5% dextrose were mixed at a charge ratio +2.24 and the mixture was left for at least 30 min before use as lipoplex.

### Animals

Male ICR and male C3H/HeJ mice were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and maintained on a standard food and water diet under conventional housing conditions. All animal experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocols for animal experiments were approved by the Institutional Animal Experimentation Committee, Kyoto University.

### Tissue Distribution of [$^{32}$P]pDNA

pDNA (pCMV-Luc) was radiolabeled with [$\alpha$-$^{32}$P] dCTP by nick translation, and diluted with non-radiolabeled pDNA for injection. ICR mice received naked or lipoplexed [$^{32}$P]pDNA by tail vein injection at indicated doses. Under anesthesia, blood was withdrawn from the vena cava and urine was collected from the urinary bladder. Then mice were euthanized at 3, 10, and 60 min after injection and the heart, lung, liver, spleen, and kidney were harvested, rinsed with saline, and weighed. Samples of blood, urine and small pieces of tissue were dissolved in 0.7 mL Solene-350 (Packard, Netherlands) at 55°C, and 0.2 mL isopropanol, 0.2 mL $H_2O_2$, 0.1 mL 5 M HCl, and 5 mL Clear-sol I (Nacalai Tesque) were added to each sample. The radioactivity was counted in an LSC-5000 liquid scintillation counter (Beckman, Tokyo, Japan).

### Transgene Expression

Male ICR mice received pDNA at a dose of 25 μg pDNA/mouse in 1.6 mL saline (the so-called hydrodynamics-based procedure) or pDNA lipoplex at a dose of 25 μg pDNA/mouse in 300 μL 5% dextrose by tail vein injection. In addition, naked pDNA in 50 μL saline was injected into the gastrocnemius muscle at a dose of 25 μg pDNA/mouse. At indicated times after injection, tissues including the heart, lung, liver, spleen, kidney, and gastrocnemius muscle were harvested, washed with ice-cold saline, and homogenized in 5 mL/g (liver) or 4 mL/g (other tissues) of lysis buffer (0.1 M Tris, 0.05% Triton X-100, 2 mM EDTA, pH 7.8), and subjected to three cycles of freezing in liquid nitrogen for 3 min and thawing in a water bath at 37°C for 3 min. The homogenates were centrifuged at 10000*g* for 10 min at 4°C. Then, 10 μL of the supernatant was mixed with 100 μL luciferase assay buffer (Pikkagene, Toyo Ink, Tokyo, Japan) and the chemiluminescence was measured with a luminometer (Lumat LB 9507, EG&G Bethhold, Bad Wildbad, Germany). The protein concentration of each tissue extract was determined using a protein quantification kit (Dojindo Laboratories, Kumamoto, Japan). Luciferase activity in each organ was normalized to relative light units (RLU) per milligram extracted protein. Luciferase activities in lungs of untreated or 5% dextrose-injected mice were 15 RLU/mg protein or lower.

### Cytokine Assay

Naked DNA was injected into the tail vein by a normal procedure or the hydrodynamics-based procedure, or into the gastrocnemius muscle. Lipoplex was injected into the tail vein. Blood was collected in plastic tubes from the vena cava of mice under anesthesia, and allowed to stand for 3 h at 4°C. Then the samples were centrifuged at

3000$g$ for 20 min at 4°C and the serum obtained was used for cytokine assay. Organs such as heart, lung, liver, spleen, and kidney were isolated at 1.5 h after injection of DNA, washed with ice-cold saline, blotted dry and stored at −80°C until use. The frozen organs were slowly thawed on ice, then homogenized in 2 mL phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors for assay. The cytoplasmic fractions were isolated as the supernatant following centrifugation at 15000$g$ for 10 min at 4°C. The levels of TNF-α in serum and cytoplasmic fractions of organs were measured using an ELISA kit (AN'ALYZA™, Genzyme). Experiments were performed at least in duplicate, and a typical set of data was indicated.

### Clodronate Liposomes

Phosphatidylcholine and cholesterol were dissolved in chloroform and a thin lipid film was formed by low-vacuum rotary evaporation. This film was dispersed in 10 mL PBS in which clodronate was dissolved. The suspension was maintained at room temperature for 2 h followed by sonication for 3 min. After another 2 h at room temperature, the suspension was centrifuged at 22000$g$ for 1 h at 10°C to remove free clodronate, and then washed four times using centrifugation at 22000$g$ for 25 min at 10°C. The liposomes were then resuspended in 4 mL PBS and stored at 4°C until use. Clodronate liposomes were used without removal of free clodronate.

### Transient Depletion of Macrophages

Macrophages were transiently depleted by intravenous injection of clodronate liposomes as previously reported.[19] Clodronate liposomes or PBS (200 μL) was injected into the tail vein 24 h prior to the injection of naked DNA or pDNA lipoplex. This treatment of mice with clodronate liposomes was reported to transiently deplete phagocytes, including Kupffer cells, because clodronate induces apoptosis of cells taking up the liposomes. To avoid possible effects of empty liposomes on macrophage functions, PBS was injected to the control group of mice.

### Inhibition of NF-κB Activation

NF-κB activation was inhibited by pre-administration of dexamethasone.[20] Dexamethasone was dissolved in PBS, and injected via the tail vein 1 h prior to the injection of lipoplex at a dose of 5 mg/kg body weight. The dosing and timing of dexamethasone for inhibition of the lipoplex-induced NF-κB activation were determined by according to a previous paper.[21] Control mice received PBS instead of dexamethasone.

### Statistical Analysis

Differences were statistically evaluated by Student's $t$-test. $p$-value of less than 0.05 was considered to be statistically significant.

## RESULTS

### TNF-α Production by CpG Replete DNA

Lipoplex consisting of pCMV-Luc induced a large amount of TNF-α in serum after intravenous injection at a dose of 25 μg DNA/mouse (Fig. 1A). No detectable TNF-α was found in serum after intravenous (Fig. 1B) or intramuscular (data not shown) injection of naked pCMV-Luc at the same dose. Increasing the dose of naked pCMV-Luc to 250 μg induced TNF-α production in serum after intravenous injection (Fig. 1B). To show that the TNF-α production was not due to contaminated lipopolysaccharide (LPS) in DNA samples, similar experiments were carried out in C3H/HeJ mice, a mouse strain lacking a response to LPS. Naked pCMV-Luc at a high dose of 250 μg induced TNF-α even in C3H/HeJ mice (104 ± 22 pg/mL), indicating that the DNA, pCMV-Luc, is the source of the TNF-α production even in the naked form. In both cases of the pCMV-Luc lipoplex and the high-dose naked pCMV-Luc, serum TNF-α levels increased rapidly and reached a peak at 1.5 h, then gradually decreased and had almost disappeared at 12 h post injection (data not shown). These results indicate that pCMV-Luc, a conventional CpG replete pDNA, induces TNF-α production regardless of its form, naked, or lipoplex. In addition, it was found that the immunostimulatory activity of pDNA is dependent on the method of administration.

### Effect of CpG Motifs on TNF-α Production

Methylated pCMV-Luc, pGZB-Luc (CpG reduced pDNA), and CT DNA (linear mammalian DNA) were injected into the tail vein of mice in the naked or lipoplex form. pGZB-Luc lipoplex


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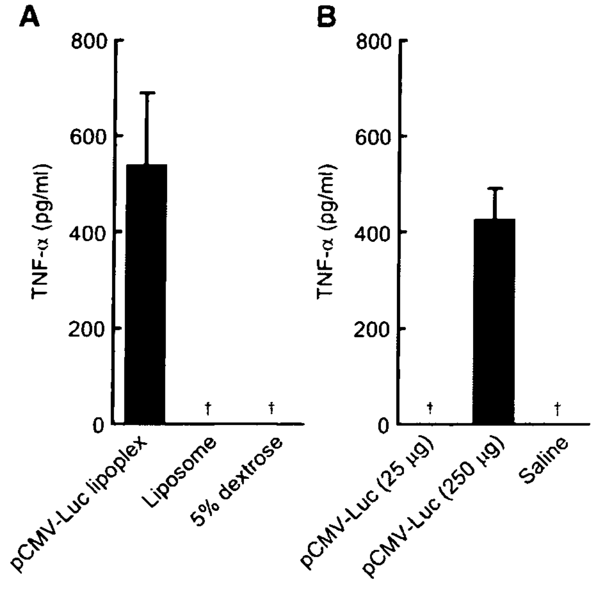


**Figure 1.** TNF-α level in serum after injection of naked pCMV-Luc or pCMV-Luc lipoplex. Mice were intravenously injected with (A) pCMV-Luc lipoplex at a dose of 25 μg DNA/mouse, (B) naked pCMV-Luc at indicated doses. Vehicles, 5% dextrose and saline, were used as controls. At 1.5 h after injection, serum was collected and the TNF-α level in serum was measured by ELISA. The results are expressed as the mean ± SD of three mice. †, not detected.

induced TNF-α production in serum, but the level was almost half that of pCMV-Luc lipoplex (Fig. 2A). Methylated pCMV-Luc lipoplex or CT DNA lipoplex induced hardly any TNF-α production. Naked DNA-induced TNF-α production also showed similar results to those with DNA lipoplex (Fig. 2B). Naked CT DNA induced no detectable TNF-α production even at the high dose of 250 μg. These results indicate that CpG motifs in DNA are essential for the induction of inflammatory cytokines in mice, and the level of cytokines is proportional to the number of CpG motifs in DNA.

## Effects of Macrophage Depletion on Tissue Distribution of DNA and TNF-α Production

As reported previously, clodronate liposomes were injected into the tail vein of mice to deplete macrophages and other phagocytes. Figure 3 shows the effects of macrophage depletion on the tissue distribution of [$^{32}$P]pCMV-Luc lipoplex after intravenous injection. In untreated mice, $^{32}$P-radioactivity rapidly disappeared from the

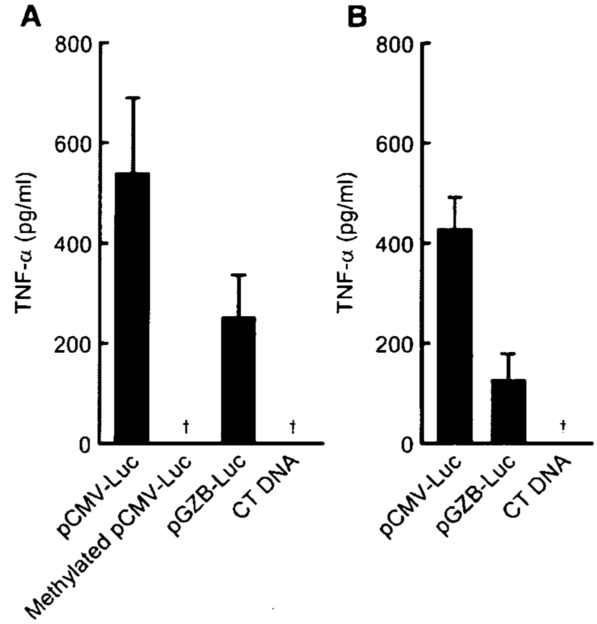


**Figure 2.** TNF-α level in serum after intravenous injection of naked DNA and DNA lipoplex. Mice were injected with (A) DNA lipoplex at a dose of 25 μg DNA/mouse, or (B) naked DNA at a dose of 250 μg DNA/mouse. At 1.5 h after injection, serum was collected and the TNF-α level in serum was determined by ELISA. The results are expressed as the mean ± SD of three mice. †, not detected.

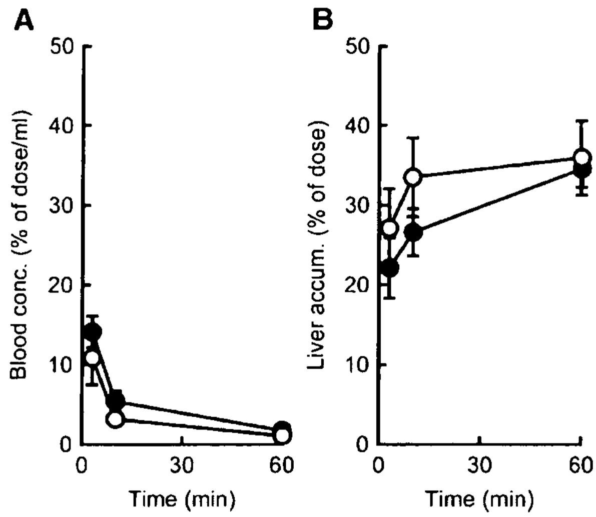


**Figure 3.** Blood concentration (A) and liver accumulation (B) of $^{32}$P-radioactivity after intravenous injection of [$^{32}$P]pCMV-Luc lipoplex into mice pretreated with clodronate liposomes or PBS. Mice were injected with [$^{32}$P]pCMV-Luc lipoplex at a dose of 25 μg DNA/mouse 24 h after intravenous injection of clodronate liposomes (closed circle) or PBS (open circle). The results are expressed as the mean ± SD of three mice.

systemic circulation, was trapped in the lung and then was gradually delivered to the liver. Pre-administration of clodronate liposomes slightly altered the distribution of radioactivity after intravenous injection of [$^{32}$P]pCMV-Luc lipoplex. In marked contrast, DNA lipoplex-induced TNF-α in serum and organs were greatly reduced by the pre-administration of clodronate liposomes (Fig. 4). Taking into account the fact that clodronate liposomes transiently deplete phagocytes, such as liver Kupffer cells and splenic macrophages, these results indicate that these cells are the major contributor to the TNF-α production by pDNA lipoplex.

### Effects of Macrophage Depletion on Transgene Expression

As reported previously, intravenous injection of pCMV-Luc lipoplex resulted in transgene expression in various organs, with the highest in the lung (Fig. 5A). Pre-administration of clodronate liposomes significantly reduced the levels of transgene expression by the lipoplex. The transgene expression in the lung by pNF-κB-Luc lipoplex was also reduced by the clodronate liposome treatment (Fig. 5B). Although a similar level of TNF-α was produced by administration of pGL3-control vector lipoplex, the expression by the pGL3-control vector lipoplex was hardly affected by clodronate liposomes (Fig. 5C). As listed in Table 1, there are two NF-κB binding sites in the enhancer region of pGL3-control, whereas pCMV-Luc and pNF-κB-Luc have 4 and 5 NF-κB binding sites, respectively. TNF-α is one of the most potent stimulators of NF-κB, so it is possible that pDNA-induced TNF-α activates

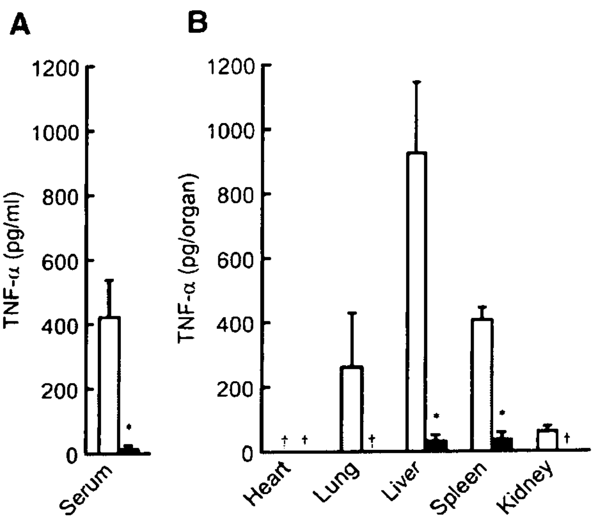


**Figure 4.** Effect of clodronate liposomes on TNF-α production after intravenous injection of pCMV-Luc lipoplex at a dose of 25 μg DNA/mouse. Mice were injected with pCMV-Luc lipoplex 24 h after intravenous injection of clodronate liposomes (closed bars) or PBS (open bars). At 1.5 h after injection of pCMV-Luc lipoplex, (A) serum and (B) organs were collected and the TNF-α levels were determined by ELISA. The results are expressed as the mean ± SD of three mice. †, not detected. *Significantly different ($p < 0.05$) from the PBS-treated mice.

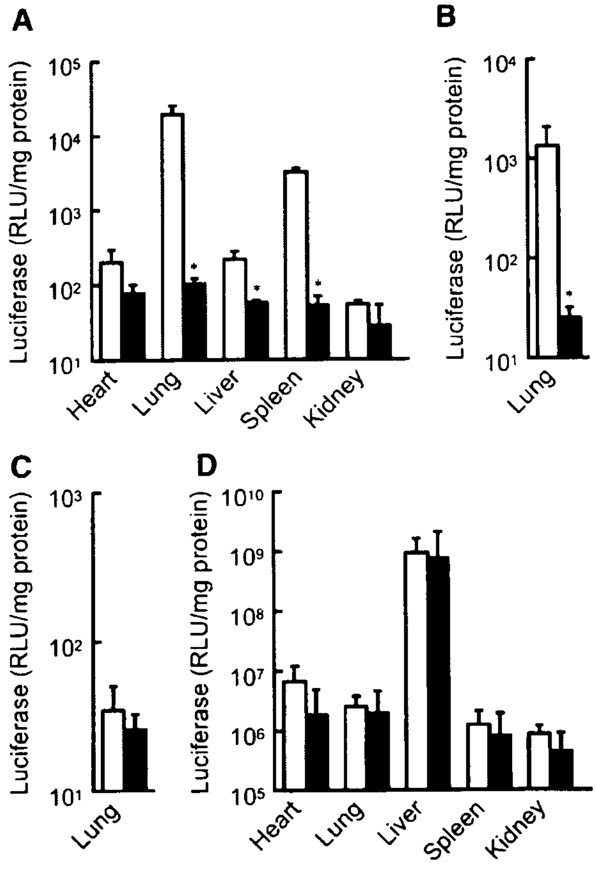


**Figure 5.** Effect of clodronate liposomes on transgene expression after intravenous injection of pDNA lipoplex. Mice were injected with lipoplex containing 25 μg (A) pCMV-Luc, (B) pNF-κB-Luc, (C) pGL3-control vector, or (D) 25 μg naked pCMV-Luc by the hydrodynamics-based procedure 24 h after intravenous injection of clodronate liposomes (closed bars) or PBS (open bars). At 6 h after injection of pDNA lipoplex, mice were euthanized and the luciferase activities in organs were measured. The results are expressed as the mean ± SD of three mice. * Significantly different ($p < 0.05$) from the PBS-treated mice.


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**Table 1.** Properties of pDNA Used in This Study

| Plasmid | Length (kbp) | Number of CpG (% to pCMV-Luc) | Promoter | Number of NF-κB Binding Sites |
|---|---|---|---|---|
| pCMV-Luc | 7.1 | 846 (100) | CMV | 4 |
| pGZB-Luc | 4.5 | 282 (53) | CMV | 4 |
| pGL3-control | 5.1 | 604 (100) | SV40 | 2 |
| pNF-κB-Luc | 5.7 | Undisclosed | TATA box + NF-κB binding sites | 5 |
| pCpG-Luc | 4.8 | 192 (34) | EF1α | 8 |
| pLuc-mcs | 5.7 | 500 (68) | TATA box | 0 |

NF-κB which, in turn, increases the level of transgene expression from pDNA containing many NF-κB binding sites. The relationship between the number of the NF-κB binding sites and TNF-α-induced increase in transgene expression was further examined using two additional pDNA: pCpG-Luc, a plasmid containing 8 NF-κB binding sites, and pLuc-mcs with no binding sites. Macrophage depletion significantly reduced the peak level of transgene expression in the lung 6 h after administration of pCpG-Luc lipoplex ($10100 \pm 1600$ and $1950 \pm 830$ RLU/mg protein in the PBS-treated-, and clodronate liposome-treated mice, respectively). No significant transgene expression was observed in both the PBS- and clodronate liposome-treated mice when pLuc-mcs lipoplex was injected, because of the very weak promoter activity of the plasmid. Then, to confirm the importance of TNF-α, not of macrophage depletion, on transgene expression, pCMV-Luc was injected into the tail vein of mice by the hydrodynamics-based procedure (Fig. 5D), which induced little TNF-α.[22] Pre-administration of clodronate liposomes hardly changed the levels of transgene expression. These findings strongly support the hypothesis that TNF-α produced by pDNA lipoplex increases transgene expression from the lipoplex through the activation of NF-κB.

### Inhibition of NF-κB Activation by Dexamethasone

Dexamethasone inhibits NF-κB activation by inducing the NF-κB inhibitor, IκB.[20] Mice receiving dexamethasone 1 h prior to the injection of pCMV-Luc or pNF-κB-Luc lipoplex exhibited a significantly lower level of transgene expression at 6 h than those pre-injected with PBS (Fig. 6A and B). These findings suggest a positive correlation between TNF-α production and NF-κB activation. Although transgene expression at later time points did not show impressive increases, it was slightly greater in dexamethasone-treated mice than in control mice. A dexamethasone-induced increase in transgene expression was observed in mice receiving pGL3-control plasmid lipoplex (Fig. 6C), or naked pCMV-Luc by the hydrodynamics-based procedure (Fig. 6D). As reported in previous studies, these results suggest that TNF-α reduces transgene expression at later time points regardless of the type of pDNA.

## DISCUSSION

Recent studies have demonstrated that pDNA administration induces significant inflammatory responses. The inflammatory properties of bacteria-derived pDNA limit many current pDNA-based gene therapy strategies. However, the cellular basis for the inflammatory response to pDNA and the effects of the inflammatory response on pDNA-induced transgene expression are poorly understood.

In the present study, we focused on TNF-α as the inflammatory cytokine induced by pDNA administration, because this cytokine is initially secreted by macrophages recognizing a foreign substrate. We confirmed that a significant level of TNF-α was detected in the serum after intravenous injection of pCMV-Luc lipoplex or a high dose of naked pCMV-Luc (Fig. 1). These results indicate that pDNA containing CpG motifs induces TNF-α production regardless of the naked or complexed form. The inflammatory responses observed in mammalian cells have been shown to arise in part from the recognition of the CpG motifs present in bacterial DNA or pDNA.[1] The importance of the CpG motif on TNF-α production was confirmed by using CpG-reduced pGZB-Luc, CpG-methylated pCMV-Luc, and CT DNA. In many nonviral *in vivo* gene transfer studies using mice, several tens of micrograms pDNA are generally administered. Because of the less toxic nature of naked pDNA than lipoplex, the dose for naked pDNA can be as high as a few hundreds

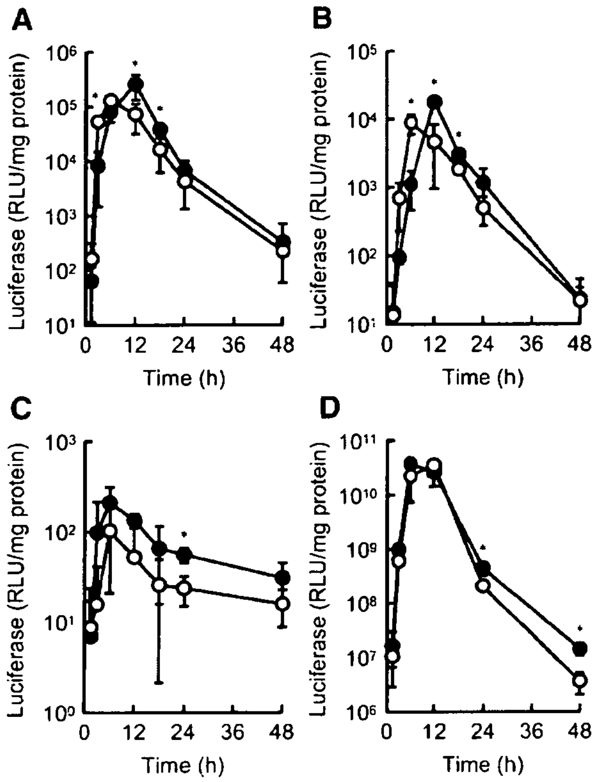


**Figure 6.** Effect of dexamethasone on transgene expression after intravenous injection of pDNA lipoplex. Mice were injected with pDNA lipoplex consisting of 25 μg (A) pCMV-Luc, (B) pNF-κB-Luc, (C) pGL3-control vector, or (D) 25 μg naked pCMV-Luc by the hydrodynamics-based procedure 1 h after intravenous injection of dexamethasone (closed circle) or PBS (open circle). At the indicated times after injection, mice were euthanized and the luciferase activities in lung were measured. The results are expressed as the mean ± SD of three mice. *Significantly different ($p < 0.05$) from the PBS-treated mice.

of micrograms. Thus, we selected the dose of lipoplex to 25 μg DNA per mouse, and those of naked DNA to 25 and 250 μg per mouse. The results of the present study clearly demonstrated that more naked pDNA is required to induce TNF-α production than lipoplex, suggesting that complexation with cationic liposomes greatly enhances the pDNA-induced TNF-α production.

The liver and spleen are known to play central roles in the removal of foreign particles and invasive microorganisms from the circulation, mainly via Kupffer cells and splenic macrophages.[23,24] Kupffer cells are liver macrophages and their functions are activated by a variety of particles, viruses, LPS, and TNF-α.[25] The phagocytosis of parasites by Kupffer cells is accompanied by the release of TNF-α. Splenic macrophages are also activated in addition to liver Kupffer cells. We and other groups have also reported that lipoplex accumulates in the liver and spleen, as well as in the lung after intravenous injection, and liver Kupffer cells and splenic macrophages are directly involved in the uptake of pDNA lipoplex and inflammatory cytokine production.[26–30] We functionally depleted these cells by injecting clodronate liposomes[19] and examined the tissue distribution and TNF-α production after intravenous injection of pDNA. Pretreatment with clodronate liposomes slightly reduced the liver accumulation of pDNA lipoplex, but significantly reduced TNF-α production. These results suggest that, even when liver Kupffer cells are depleted, pDNA lipoplex is largely delivered to the liver, but where it induces hardly any inflammatory responses. Other liver-constituting cells, such as hepatocytes and sinusoidal endothelial cells, are responsible for the hepatic uptake of the lipoplex.

To examine the effects of TNF-α on transgene expression, we compared the level of transgene expression in mice, with or without pretreatment of clodronate liposomes. Pretreatment resulted in a significant reduction in the level of transgene expression by pCMV-Luc lipoplex and pNF-κB-Luc lipoplex (Fig. 5A and B). Clodronate liposomes hardly affected the level of transgene expression by hydrodynamically delivered naked pCMV-Luc (Fig. 5D), which excludes the possibility that the treatment reduces transgene expression in a nonspecific manner. pCMV-Luc and pNF-κB-Luc contain 4 and 5 NF-κB binding sites in their promoter/enhancer regions,[31] whereas pGL3-control vector has only two NF-κB binding sequences in the enhancer region. The results of the present study using several types of pDNA with different properties suggest that the number and/or position of NF-κB binding sites affect the responsibility of pDNA to NF-κB. A recent study by Kuramoto et al.[15] showed that the TNF-α-induced increase in transgene expression correlated with the number of NF-κB binding sites in pDNA. Therefore, the fewer numbers of the binding sites in the pGL3-control vector may explain why the expression by the pGL3-control lipoplex was not significantly affected by the treatment.

To investigate the relationship between the transgene expression and NF-κB activation, dexamethasone, an inhibitor of NF-κB activation,[20] was injected prior to pDNA administration. Again, pretreatment with dexamethasone signifi-


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cantly reduced transgene expression by pCMV-Luc and pNF-κB-Luc lipoplex during the early period of injection. However, transgene expression by pGL3-control vector lipoplex was slightly increased by dexamethasone at any time point examined (Fig. 6C). Pretreatment with dexamethasone also significantly increased transgene expression by naked pCMV-Luc and pNF-κB-Luc at later time periods. These findings suggest that there is a positive correlation between TNF-α production and NF-κB activity-dependent transgene expression when pDNA contains effective NF-κB binding sites. Other studies also support these findings. CMV promoter activity was increased by inflammation in a rat arthritis model, where a positive correlation between transgene expression and the dose of LPS was obtained.[32] Reactivation of the previously silenced CMV promoter was observed after administration of LPS in mouse liver.[33] It is possible that pDNA containing NF-κB binding sites can be translocated into the nucleus through NF-κB activation. Mesika et al. reported an NF-κB-assisted import of pDNA into the nuclei of mammalian cells *in vitro*.[34] At the same time, however, the results of dexamethasone treatment also suggested that TNF-α reduces transgene expression in the long-term even at low concentrations. Tan et al.[35] reported that dexamethasone increased the pDNA complex-induced transgene expression in mouse lung via the suppression of TNF-α production. They also demonstrated that co-delivery of an NF-κB decoy increased the pDNA complex-mediated transgene expression. pDNA lipoplex has been shown to induce apoptosis of lung endothelial cells via TNF-α.[36] Thus, it is possible that TNF-α induces apoptosis of the transfected target cells and the expression is eventually lost.

In conclusion, we have demonstrated that tissue macrophages involving liver Kupffer cells and splenic macrophages are closely involved in TNF-α production after pDNA administration. TNF-α production was dependent on the number of CpG motifs in DNA regardless of the form of the DNA administered. TNF-α can increase transgene expression through NF-κB activation during the early phase after gene transfer, but it reduces the expression, probably through the apoptosis of transfected target cells or other mechanisms. These findings provide useful basic information for the development of more efficient *in vivo* gene delivery systems.

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# Cellular Activation by Plasmid DNA in Various Macrophages in Primary Culture

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**ABSTRACT:** Macrophages are an important group of cells responsible for the inflammatory response to unmethylated CpG dinucleotide (CpG motif) in plasmid DNA (pDNA) via Toll-like receptor 9 (TLR9). This finding is primarily based on *in vitro* studies. Previous *in vivo* studies also have suggested that tissue macrophages are involved in inflammatory cytokine release in the circulation following intravenous administration of pDNA to mice. However, the relationship between the *in vitro* and *in vivo* studies has not been sufficiently clarified. To gain insight into which types of cells are responsible for the production of cytokines upon interaction with pDNA, peritoneal macrophages, splenic macrophages, hepatic nonparenchymal cells (NPCs) including Kupffer cells and mesangial cells were isolated from mice. All types of primary cultured cells, except for mesangial cells, express TLR9 at varying levels. Splenic macrophages and hepatic NPCs were activated to produce tumor necrosis factor-α (TNF-α) by naked pDNA, whereas peritoneal macrophages and mesangial cells were not. pDNA complexed with *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium chloride/cholesterol liposome induced TNF-α in the splenic macrophages but not in the other cell types. These results indicate that splenic macrophages and hepatic NPCs are closely involved in TNF-α production in response to pDNA. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci

**Keywords:** plasmid DNA; lipoplexes; liposomes; DNA delivery; immunology; tissue macrophage; naked DNA; inflammatory cytokine; CpG DNA

## 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, in bacterial DNA, but not in vertebrate DNA, are recognized by the mammalian immune system as a danger signal.[2] Toll-like receptor 9 (TLR9) is a pattern recognition receptor recognizing CpG motifs,[3] and is present in the intracellular compartments of immune cells, such as macrophages and dendritic cells.[4] These cells secrete inflammatory cytokines, such as TNF-α, interleukin-6 (IL-6), and IL-12, upon uptake of CpG motif-containing DNA. These cytokines reduce transgene expression in target cells through direct cytotoxicity and/or promoter attenuation.[5,6] Consequently, reduction or prevention of pDNA-induced cytokine production is important for increasing the efficiency of *in vivo* gene transfer.

To improve the transfection efficacy with pDNA, DNA/cationic liposome complexes are often used both *in vivo* and *in vitro*. Several

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recent studies have shown that intravenous (i.v.) administration of pDNA/cationic liposome complexes leads to effective gene expression although such complexes also induce high amounts of inflammatory cytokines.[5–10]

In a series of investigations on the tissue disposition of naked pDNA we have shown that the hepatic nonparenchymal cells (NPCs), such as Kupffer cells and sinusoidal endothelial cells, play important roles in the clearance of pDNA.[11,12] In addition, it has been suggested that tissue macrophages, such as Kupffer cells (liver resident macrophages) and splenic macrophages, are responsible for cytokine production following intravenous injection of DNA/cationic liposome complexes.[13] On the other hand, there is very little production of cytokines by cultured peritoneal macrophages following stimulation with naked pDNA,[14] even although they exhibit extensive DNA uptake via scavenger receptorlike mechanisms.[15,16] Surprisingly, peritoneal macrophages produce inflammatory cytokines upon incubation with DNA/cationic liposome complexes irrespective of the presence of the CpG motif.[17] Moreover, we found that this response was induced via a TLR9-independent pathway, because primary cultured cells from TLR9 knockout mice also released cytokines upon incubation with such complexes.[18,19]

In spite of extensive studies on the cellular activation induced by CpG DNA including pDNA, the relationship between *in vitro* and *in vivo* studies requires further investigation because specific types of immune cells including cell lines were generally used in these *in vitro* studies. In order to understand the pDNA-mediated cellular activation *in vivo*, it is necessary to evaluate the capability of various types of macrophages and macrophage-like cells to induce inflammatory cytokines because pDNA is distributed to some organs after i.v. administration. In the present study, we examined whether primary cultured cells from mouse organs, including peritoneal macrophages, are activated by naked DNA or DNA/cationic liposome complexes in order to identify the roles of these cells in the immune response at the whole-body level.

## MATERIALS AND METHODS

### Chemicals

*N*-[1-(2,3-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was purchased from Tokyo Kasei (Tokyo, Japan). Cholesterol and Triton X-114 was purchased from Nacalai Tesque (Kyoto, Japan). pcDNA3 vector was purchased from Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS), a cocktail of protease inhibitors, calf thymus (CT) DNA and type I-A collagenase were purchased from Sigma (St. Louis, MO). ITS™ was purchased from BD Bioscience (San Diego, CA). Monoclonal mouse anti-mouse TLR9 antibody was purchased from InvivoGen (San Diego, CA) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody was purchased from Amersham Biosciences Inc. (Piscataway, NJ).

### Plasmid DNA

Plasmid vector pCMV-Luc, which encodes firefly *luciferase* gene, was constructed based on pcDNA3 as described previously.[20] pCMV-Luc has 33 Pur-Pur-CpG-Pyr-Pyr sequences including two GACGTT, one of the most potent CpG motifs for mice.[21] pCMV-Luc was amplified in the *E. coli* strain DH5α and then isolated and purified using a Qiagen Endofree™ Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany).

### Purification of DNA

To minimize the activation by contaminated LPS, DNA samples were extensively purified with Triton X-114, a nonionic detergent. Extraction of LPS from pDNA and CT DNA samples was performed according to previously published methods.[14,22,23] The level of contaminated LPS was checked by a Limulus amebocyte lysate assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free™ plasmid Giga kit, the level of LPS was found to be 0.01–0.05 EU/μg pDNA, which was reduced below the detection limit of 0.001 EU/μg pDNA by the Triton X-114 extraction.

### Preparation of Cationic Liposomes and DNA/Cationic Liposome Complexes

In the present study, DOTMA/cholesterol liposomes were used to prepare the DNA complexes because these complexes are widely used in *in vivo* studies due to their high *in vivo* transfection efficacy.[24,25] Cationic liposomes consisting of DOTMA and cholesterol in a 1:1 molar ratio were prepared by allowing the lipids to dry as a thin


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film in a round-bottomed flask using a rotary evaporator, and then hydrating in 5% (w/v) dextrose by gentle mixing. After hydration, the dispersions were sonicated for 3 min and passed through a Minisart® 0.45 μm filter unit (Sartorius K.K., Tokyo, Japan). The lipid concentrations of cationic liposomes were determined by the Cholesterol E-Test Wako (Wako Pure Chemical, Osaka, Japan). Cationic liposomes and DNA in 5% dextrose were mixed at a charge ratio of +2.24 and left for 30 min at 37°C to form DNA lipoplex.

## Isolation of Primary Cultured Cells

Male ICR (5–6-week-old) mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan).

### *Resident Peritoneal Macrophages*

Peritoneal macrophages were collected aseptically from the peritoneal cavity of unstimulated mice with cold phosphate-buffered saline (PBS). Cells were washed, suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (1.2 μg/mL), and then plated on 24-well culture plates (Falcon®, Becton-Dickinson, Franklin Lakes, NJ) at a density of $5 \times 10^5$ cells/well for the activation experiments. After a 2 h incubation at 37°C in 5% $CO_2$-95% air, adherent macrophages were washed twice with RPMI-1640 medium to remove nonadherent cells and then cultured for 24 h.

### *Splenic Macrophages*

Spleens were removed aseptically from ICR mice, divided up with a spatula and filtered through a cell-strainer (mesh size 100 μm, Falcon®, Becton-Dickinson, Franklin Lakes, NJ) to obtain single-cell suspensions. Red blood cells were lysed by exposure to a hypo-osmotic solution of ammonium chloride 0.1% for 5 min at 4°C. After washing three times, splenocytes were suspended in RPMI-1640 medium supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL), $10^{-5}$ M 2-mercaptoethanol, and plated in 10 cm culture dishes. After a 3 h incubation at 37°C in 5% $CO_2$–95% air, adherent macrophages were washed twice with RPMI-1640 medium to remove nonadherent cells and plated again on 24-well culture plates at a density of $5 \times 10^6$ cells/well for the activation experiments.

### *Hepatic Nonparenchymal Cells*

Isolation of hepatic NPCs was performed as previously described[26] with slight modifications. Briefly, mouse liver was perfused under anesthesia via the portal vein with $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS) at 37°C for 5 min at a flow rate of 4–5 mL/min. Then, the liver was perfused with HBSS containing 5 mM $Ca^{2+}$ and 0.05% (w/v) collagenase for 7 min. The digested liver was minced and filtered through a cotton gauze and cell-strainer (mesh size 100 μm) and then fractioned into hepatocytes and hepatic NPCs by differential centrifugation. Hepatic NPCs were suspended in RPMI-1640 medium supplemented with 10% FBS, penicillin G (100 U/mL), streptomycin (100 μg/mL) and amphotericin B (2.5 μg/mL), and then plated on 24-well culture plates at a density of $5 \times 10^5$ cells/well. After a 3 h incubation at 37°C in 5% $CO_2$–95% air, adherent hepatic NPCs were washed twice with RPMI-1640 medium to remove nonadherent cells and then cultured for 24 h for the activation experiments.

### *Mesangial Cells*

Isolation of glomerular cores was performed by the method of Mori et al.[27] with a slight modification. Briefly, the kidneys of ICR mice were removed aseptically and cut into slices. Each slice was pressed through a 425 μm pore-size metal sieve. The resulting material was rinsed three times in HBSS and pellets were centrifuged at 1700*g* for 3 min. The pellets were then incubated for 27 min at 37°C with 750 units/mL collagenase in 10 mL HBSS, followed by washing three times with HBSS and passing through two sieves with pore-sizes of 100 and 40 μm. The glomerular cores remaining on the finest sieve were cultured at 37°C for 2 weeks in RPMI-1640 medium with 20% FBS, penicillin G (100 units/mL), streptomycin (100 μg/mL) and ITS in 75 $cm^2$ plastic tissue culture flasks in 5% $CO_2$–95% air. The mesangial cells obtained from the 6th to 10th passage were plated on 24-well culture plates at a density of $5 \times 10^5$ cells/well and cultured for 24 h for the activation experiments.

### *Cell Line*

Murine macrophagelike cell lines, RAW264.7 cells, were cultured in RPMI-1640 supplemented with 10% FBS, penicillin G (100 U/mL) and streptomycin (100 μg/mL). They were plated on 24-well culture plates at a density of $2.5 \times 10^5$ cells/well and cultured for 24 h.

## mRNA Quantification

Total RNA was extracted from RAW264.7 cells and primary cultured cells using a MagExtractor MFX-2100 and 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). Reverse transcription was performed using a SuperScript II (Invitrogen) and dT-primer according to the manufacturer's protocol. For quantitative mRNA expression analysis, real time PCR was carried out with total cDNA using a LightCycler instrument (Roche Diagnostics, Basle, Switzerland). The oligodeoxynucleotide primers used for amplification were TLR9-sense: 5′-AGC TCA ACC TGT-3′, TLR9-antisense: 5′-ATG CCG TTC ATG TTC AGC TCC TGC-3′, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-sense: 5′-CTG CCA AGT ATG ATG ACA TCA AG AA-3′, GAPDH-antisense: 5′-ACC AGG AAA TGA GCT TGA CA-3′. The size of the TLR9 and GAPDH products was 313 and 186 bp, respectively. Amplification products were detected online via intercalation of the fluorescent dye SYBR green (LightCycler-FastStart DNA Master SYBR Green I kit, Roche Diagnostics, Basle, Switzerland). The cycling conditions were as follows: initial enzyme activation at 95°C for 10 min, followed by 55 cycles at 95°C for 10 s, 58°C for 5 s, and 72°C for 10 s for TLR9 and GAPDH. All cycling reactions were performed in the presence of 3.5 mM $MgCl_2$. Gene specific fluorescence was detected at 72°C.

## Western Blotting

Western blot analysis of TLR9 was performed by a standard method. Briefly, cells were lysed with RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 0.2% deoxycholic acid, 0.2% NP-40, 1 mM phenylmethylsulfonyl fluoride) containing a cocktail of protease inhibitors. Fifty micrograms of protein were dissolved in a loading buffer, denatured at 95°C for 3 min, and loaded on 4.75 or 9% SDS–polyacrylamide gels, respectively. After electrophoresis, proteins were transferred onto a polyvinylidene fluoride microporous membrane (Immobilon-P; Millipore Corp., Bedford, MA) by semidry-blotting with Transblot SD (Bio-Rad, Hercules, CA). Membrane was blocked with 5% skimmed milk in Tris buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature, washed, and incubated with primary anti-mouse TLR9 monoclonal antibody (1/1000 dilution) overnight at 4°C. Then, the membrane was washed with TBS-T and incubated with the HRP-conjugated secondary antibody (1/1000 dilution) for 1 h at 37°C. Detection of antigen was performed using chemiluminescent HRP substrate (Immobilon Western; Millipore Corp., Bedford, MA).

## Cytokine Secretion

Cells plated on 24-well culture plates were washed twice with 0.5 mL RPMI-1640 before use. Cells were incubated with naked DNA or LPS for 8 hr, and the supernatants were colleted for ELISA and kept at −80°C. Separately, cells were incubated with DNA/DOTMA/cholesterol complex 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 level of TNF-α in the supernatant was determined by the OptEIA™ set (BD Biosciences, San Diego, CA).

# RESULTS

## All Primary Cultured Cells, Except for Mesangial Cells, Express TLR9

Figure 1A shows the level of TLR9 mRNA expression in primary cultured cells and RAW264.7 cells. TLR9 mRNA was detected in peritoneal macrophages, splenic macrophages, hepatic NPCs and RAW264.7 cells, but not in mesangial cells. Splenic macrophages expressed at least thirty times more TLR9 mRNA than the other cells. The amount of TLR9 protein correlated well with the level of the mRNA (Fig. 1B). Splenic macrophages showed the highest level of TLR9 protein, followed by peritoneal macrophages and hepatic NPCs. No TLR9 protein was detected in RAW264.7 cells.

## Splenic Macrophages Secrete TNF-α Upon Stimulation with DNA in Both Naked and Complexed Form in a CpG Motif-Dependent Manner

When highly purified naked plasmid DNA (<0.001 EU LPS/μg pDNA), CpG-replete DNA, was added to splenic macrophages, TNF-α was secreted from splenic macrophages in a


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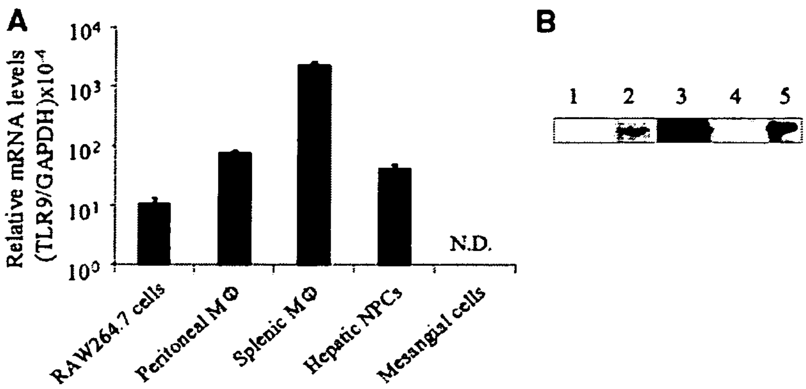


**Figure 1.** Expression of TLR9 in primary cultured cells and RAW264.7 cells. (A) Expression of TLR9 mRNA was examined by real-time polymerase chain reaction (real-time PCR) using extracted total RNA. Each result represents the mean and standard deviation of three measurements. (B) TLR9 protein expression was evaluated by Western blotting on cell lysate as described in Materials and methods. Lane 1, RAW264.7 cells; lane 2, peritoneal macrophages; lane 3, splenic macrophages; lane 4, hepatic NPCs; lane 5, size marker representing 120 kDa.

dose-dependent manner. A very low level of cytokines was detected by CT DNA, a mammalian DNA containing many fewer CpG motifs (Fig. 2A). TNF-α was also secreted from splenic macrophages when DNA complexed with DOTMA/cholesterol liposome was added. However, with DOTMA/cholesterol liposomes, only pDNA induced TNF-α production and CT DNA did not (Fig. 2B). LPS, a TLR4 ligand, induced significant TNF-α in the splenic macrophages in primary culture. These results suggest that splenic macrophages are able to produce TNF-α by addition of pDNA which contains many CpG motifs.

### Hepatic NPCs Secrete TNF-α Upon Stimulation Only with Naked pDNA

Next, we used hepatic NPCs in which Kupffer cells and sinusoidal endothelial cells are the major components. Similar to the results using splenic

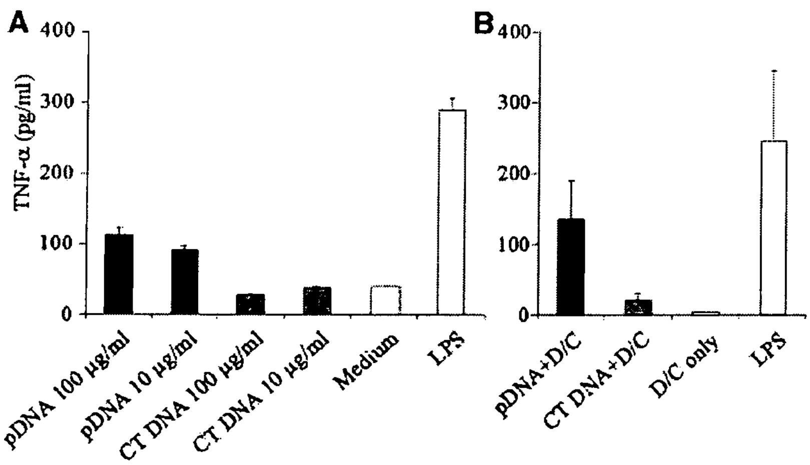


**Figure 2.** TNF-α secretion by DNA from splenic macrophages. Cells were incubated with naked DNA (100 or 10 μg/mL) (A) or DNA/cationic liposome complex (10:70.18 μg/mL) (B). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 1000 ng/mL. The concentration of TNF-α secreted from splenic macrophages was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

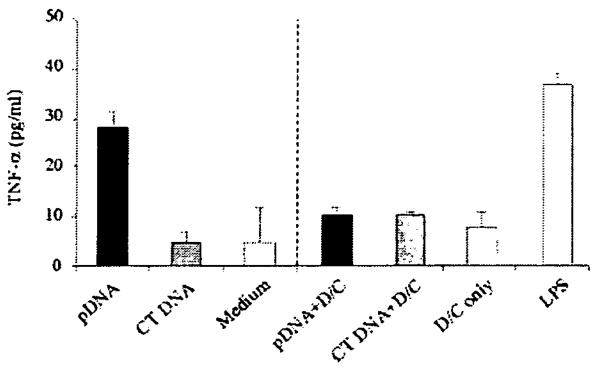


**Figure 3.** TNF-α secretion by DNA from hepatic NPCs. Cells were incubated with naked DNA (100 μg/mL) or DNA/cationic liposome complex (10:70.18 μg/mL). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 1000 ng/mL. The concentration of TNF-α secreted from NPCs was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

macrophages, hepatic NPCs secreted TNF-α when incubated with naked pDNA, but not with CT DNA (Fig. 3). Addition of the DNA/cationic liposome complexes produced very little TNF-α release from hepatic NPCs irrespective of the type of DNA used. TNF-α production was observed following LPS stimulation, but the level was very low.

### Peritoneal Macrophages and Mesangial Cells Do Not Secret TNF-α Following Addition of DNA

Peritoneal macrophages did not secrete any TNF-α following addition of pDNA (Fig. 4A), which was in a good agreement with our previous findings. Moreover, the pDNA/cationic liposome complexes also did not induce TNF-α production in the peritoneal macrophages. The responsiveness of the macrophages to LPS was confirmed by TNF-α secretion. Mesangial cells, which expressed no detectable TLR9 mRNA, did not induce TNF-α following DNA addition to the cells in naked or complex form (Fig. 4B). LPS stimulation also failed to induce TNF-α secretion in the cells.

### RAW264.7 Cells Secrete Large Amounts of TNF-α Following Addition of Naked DNA and DNA/Cationic Liposome Complexes

Finally, we investigated the cellular response of a murine macrophagelike cell line, RAW264.7, to DNA. In spite of the low expression of TLR9 (Fig. 1), a large amount of TNF-α was produced by naked DNA in a CpG motif-dependent manner from RAW264.7 cells (Fig. 5A). Naked CT DNA hardly induced any TNF-α. On the other hand, TNF-α was induced in a CpG motif-independent manner when DNA/DOTMA/cholesterol liposome complexes were added to the cells (Fig. 5B).

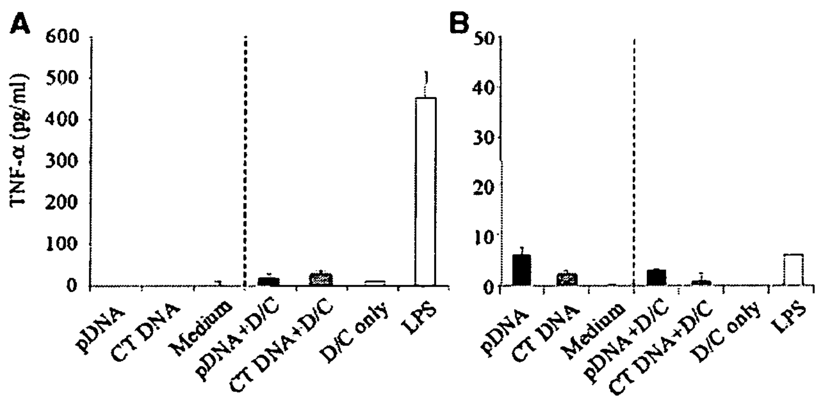


**Figure 4.** TNF-α secretion by DNA from peritoneal macrophages (A), and mesangial cells (B). Cells were incubated with naked DNA (100 μg/mL) or DNA/cationic liposome complex (10:70.18 μg/mL). After a 2-h incubation, the complexes were removed and fresh growth medium was added to the cells. The supernatants were collected 8 h after addition of naked DNA or DNA complex. LPS was used at a concentration of 100 ng/mL. The concentration of TNF-α secreted from the cells was quantified by ELISA. Each result represents the mean and standard deviation of triplicate values.

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