are also present in vertebrate DNA, but usually suppressed and most of them are silenced by methylation [11–13]. Interestingly, DNA in SLE patients' sera often has a high CG content [14–16], although this DNA is supposed to come from their own DNA released from dead cells. Thus, we hypothesized that DNA, especially CpG DNA, might be abnormally cleared by MΦs and DCs in SLE. This might result in a prolonged retention of circulating DNA and/or pathological Ag-presentation of DNA.

The clearance of ds- and ss-DNA in murine SLE models has been examined in detail in vivo, and it has been demonstrated that DNA is rapidly cleared from blood by the reticulo-endothelial system [17,18]. There is no difference in DNA clearance between mice with SLE and normal animals after the intravenous injection of DNA unless anti-DNA Ab is present [19]. However, autoantigens are mainly supplied by apoptotic cells and MΦs play key roles in the clearance of apoptotic cells [20]. In addition, MΦs excrete DNA from apoptotic cells after the digestion of these cells [21]. Furthermore, MΦs from patients with SLE have defective characteristics as far as phagocytosis of apoptotic debris is concerned [22]. Therefore, removal of autoantigens by MΦs and DCs must be a key process in the disease development. However, the processing of autoantigens, especially DNA, in MΦs and DCs in vitro is poorly understood in SLE.

Previously, we demonstrated that Kupffer cells (liver resident MΦs) play an important role in the uptake of plasmid DNA (pDNA) after intravenous injection into mice [23,24]. In vitro experiments, using mouse peritoneal MΦs, have also shown that primary cultured MΦs take up pDNA efficiently via a scavenger receptor-like mechanism in a specific manner [25–27]. In addition, we found that mouse DCs take up pDNA via a specific mechanism like mouse MΦs [28].

Here, we have examined whether MΦs and DCs from lupus-prone strains of New Zealand Black/White $F_1$ (NZB/W) mice have distinct characteristics in the uptake and degradation of DNA and found that MΦs and DCs from a murine lupus model display defective uptake and degradation of pDNA. We also show that the cellular binding defects of pDNA in MΦs and DCs from diseased, 6-month-old lupus mice may arise as a consequence of competitive inhibition by endogenous DNA. These findings characterize the cellular defects in NZB/W mice and provide novel insights into the pathogenesis of SLE.

## 2. Materials and methods

### 2.1. Chemicals

RPMI1640 medium and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Triton X-1 14 was purchased from Nacalai Tesque (Kyoto, Japan).

### 2.2. Isolation of peritoneal MΦs

Elicited peritoneal MΦs were isolated as previously descried [25]. Briefly, mice were injected i.p. with 1.0 ml 2.9% thioglycolate broth (Nissui Pharmaceutical, Tokyo, Japan). Four days later, the mice were euthanized, and peritoneal cavities were lavaged with RPMI 1640 medium (Nissui Pharmaceutical) supplemented with 1% L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin (all obtained from Invitrogen, Carlsbad, CA, USA). Peritoneal MΦs were allowed to adhere to 24-well tissue culture plates (Falcon, Becton-Dickinson, Lincoln Park, NJ) at a density of $1 \times 10^6$ cells/ml for 3 h in RPMI 1640 medium supplemented with 10% FBS, 1.2 μg/ml amphotericin B (Sigma, St. Louis, MO, USA), 1% L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin before washing to remove nonadherent cells.

### 2.3. Isolation of BM-derived DCs

Femurs were flushed with RPMI 1640 medium supplemented with 1% L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin to collect total bone marrow cells. Spicules and bone matrix were allowed to settle and were removed. Total bone marrow cells were washed once and resuspended in RPMI 1640 medium supplemented with 10% FBS, 20 ng/ml recombinant mouse GM-CSF (Nacalai Tesque, Kyoto, Japan), 1% L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells were fed every 2 days by adding 50% fresh medium. After 4 days in culture, nonadherent cells were removed and washed, and $CD11c^+$ cells were isolated by AUTO MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). These cell populations were then seeded on 24-well tissue culture plates at a density of $1 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FBS, 20 ng/ml recombinant mouse GM-CSF, 1% L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin.

### 2.4. Plasmid DNA

The vector pcDNA3 was purchased from Invitrogen. The vector pCMV-Luc, encoding the firefly luciferase gene, was constructed as described previously [29]. For the cellular association experiment, pCMV-Luc was radiolabelled with [α-$^{32}$P]dCTP by nick translation [30]. The reaction was performed as described previously [27].

### 2.5. Purification of DNA

To minimize the activation by contaminated LPS, DNA samples were extensively purified with Triton X-114 (Nacalai Tesque), a nonionic detergent. Extraction of endotoxin from pDNA samples was performed according to previously published methods with slight modifications [27,31,32]. The activity of LPS was measured by Limulus amebocyte lysate

(LAL) assay using the Limulus F Single Test kit (Wako, Tokyo, Japan). After purification using the Endo-free™ plasmid Giga kit, 1 μg/ml pDNA contained 0.01–0.05 EU/ml endotoxin. After Triton X-114 extraction, the endotoxin levels in DNA samples could be no longer be determined by LAL assay, i.e., 1 μg/ml DNA contained less than 0.001 EU/ml. Without extraction of endotoxin by Triton X-114, 100 μg/ml naked pDNA, which contains 1–5 EU/ml endotoxin, could release 521 ± 73 pg/ml TNF-α from mouse peritoneal MΦs over 24 h.

### 2.6. Cellular association experiments

Cells cultured on 24-well plates were pretreated with 0.5 ml Hanks' balanced salt solution (HBSS, Nissui Pharmaceutical) in the presence or absence of 10 U/ml DNase I for 1 h at 37 °C. Then, cells were washed with 0.5 ml HBSS and 0.5 ml HBSS containing 0.1 μg/ml naked $^{32}$P-labelled pDNA was added. After incubation at 37 °C or 4 °C for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1.0 ml 0.3 N NaOH with 0.1% Triton X-100. Cell lysates were taken for the determination of $^{32}$P radioactivity using an LAS-500 scintillation counter (Beckman, Tokyo, Japan).

### 2.7. TCA precipitation experiments

After the cellular association experiments, the medium containing radioactivity derived from $^{32}$P-labelled pDNA was subjected to TCA precipitation experiments to assess the degradation of pDNA by MΦs and DCs. A portion of the supernatant was directly subjected to radioactivity counting as described above. After extraction with 10 mM Tris–HCl, 1 mM EDTA (TE)-saturated phenol buffer (pH 7.8), aliquots of the supernatant were mixed with TCA to give a final concentration of 5% (w/v), kept on ice for 10 min, and then centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant (TCA-soluble fraction) was subjected to radioactivity counting and the amount of degradation products of $^{32}$P-labelled pDNA was calculated. The TCA-soluble degradation products will be small DNA fragments (short oligonucleotides) since 50% precipitation occurs with the 16-mer oligonucleotides for TCA [33].

## 3. Results

### 3.1. Establishment of the evaluation method for the apparent total pDNA uptake by MΦs and DCs

To estimate the apparent pDNA uptake, we measured the amount of DNA cellular association and that of degraded DNA detected in culture medium as the consequence of excretion. Prior to the experiments using the cells from NZB/W mice, we carried out cellular association experiments

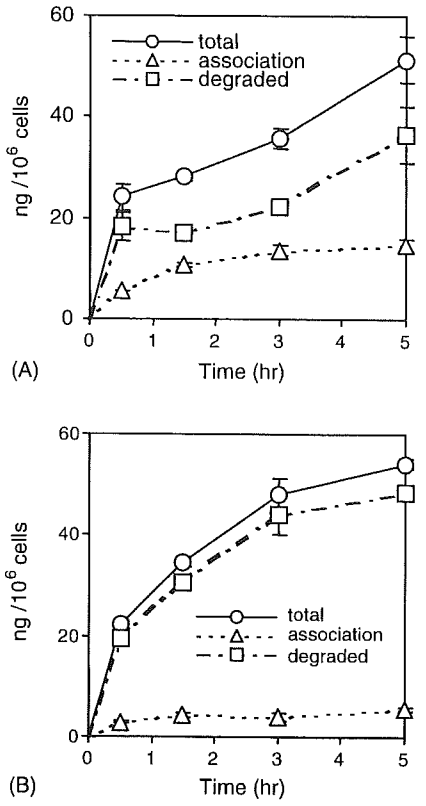


Fig. 1. The uptake and degradation of $^{32}$P-labelled pDNA in MΦs and DCs. Elicited peritoneal MΦs (A) and bone marrow-derived DCs (B) from ICR mice were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 37 °C. At the time-point indicated, culture supernatants and cell lysates were collected and the cellular association and degradation of pDNA was determined as described in Section 2. The amount of apparent $^{32}$P-labelled pDNA uptake was calculated by the addition of the amount of cellular association and degradation. Each point represents the mean ± standard deviation ($n$ = 3).

at 37 °C using MΦs and DCs from control ICR mice. MΦs and DCs digested $^{32}$P-labelled pDNA very rapidly and a large part of the degraded pDNA was detected in the medium (Fig. 1A and B). Culture medium alone or culture supernatant of these cells after 5 h incubation could not digest the $^{32}$P-labelled pDNA (data not shown). Furthermore, the generation of degraded pDNA in the medium was significantly reduced by endosomal acidification inhibitors, such as bafilomycin $A_1$ and chloroquine (data not shown), suggesting that DNA digestion occurs mainly in the lysosomal compartment. Therefore, we can exclude the possibility that $^{32}$P-labelled pDNA is digested simply by nucleases secreted from MΦs and DCs in the culture medium. Thus, we defined the apparent pDNA uptake as the following equation:

(Apparent pDNA uptake)
= (pDNA remaining in the cellular compartment)
+(degraded pDNA detected in the medium)

### 3.2. pDNA binding to MΦs and DCs from 6-month-old NZB/W mice

To evaluate the apparent DNA uptake in the cells from active SLE, MΦs and DCs were collected from 6-month-old NZB/W mice with SLE. First of all, we examined the binding of $^{32}$P-labelled pDNA to MΦs and DCs. Cellular binding in these cells was examined by incubation with $^{32}$P-labelled pDNA at 4 °C for up to 5 h. MΦs and DCs from 6-month-old NZB/W mice showed significantly lower DNA binding compared with that of control mice (Fig. 2 A and B). To determine the cause of this low DNA binding, we studied the effects of pretreatment with DNase. Since SLE mice have a relatively high concentration of circulating DNA [34], we hypothesized that this DNA could remain on the cellular surface and could inhibit the DNA binding. MΦs and DCs were incubated with HBSS containing 10 U/ml of DNase I for 1 h before interaction with $^{32}$P-labelled pDNA. This procedure could completely digest the surface-bound DNA after a 5 h incubation with $^{32}$P-labelled pDNA at 4 °C (data not shown). Therefore, pretreatment in this condition can exclude the disturbance of putative endogenous DNA. The treatment itself did not affect the DNA binding to control cells (Fig. 2C and D). In contrast, MΦs and DCs from 6-month-old NZB/W mice showed significantly enhanced DNA binding after the treatment with DNase and the DNase-treated cells exhibited almost the same DNA binding as the control cells. These results suggest that DNA binding is inhibited by endogenous DNA in MΦs and DCs from diseased NZB/W mice.

### 3.3. pDNA uptake and subsequent degradation by MΦs and DCs from 6-month-old NZB/W mice

Next, we examined the time-course of apparent pDNA uptake in MΦs and DCs for up to 5 h at 37 °C. MΦs and DCs from 6-month-old NZB/W mice showed significantly reduced DNA uptake and subsequent degradation compared with those of control ICR mice (Fig. 3A and B). The impairment was apparent only 30 min after the incubation and last for all the time-points examined. Although SLE preferentially affects females, the impairment was not affected by gender. To further examine the impairment, we next compared the distribution of pDNA. In MΦs from NZB/W mice, both the cellular association and degradation of pDNA were reduced, although there was no statistically significant difference in the amount of degradation (Fig. 3C). On the other hand, degradation of pDNA was significantly reduced in DCs from NZB/W mice, although the cellular association of pDNA was slightly enhanced rather than reduced in the same cells (Fig. 3D). These results suggest that both internalization and degradation/excretion processes are

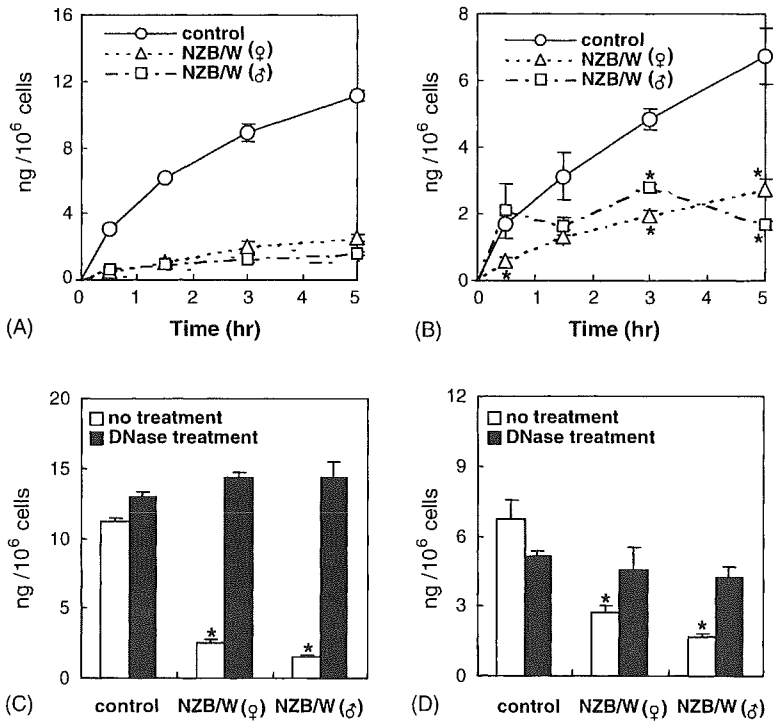


Fig. 2. Impaired $^{32}$P-labelled pDNA binding to MΦs and DCs from 6-month-old NZB/W mice. Elicited peritoneal MΦs (A) and bone marrow-derived DCs (B) from 6-month-old control ICR mice and NZB/W mice were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 4 °C. Each point represents the mean ± standard deviation ($n = 3$). Elicited peritoneal MΦs (C) and bone marrow-derived DCs (D) from 6-month-old control ICR mice and NZB/W mice were incubated in HBSS in the presence or absence of DNase (10 U/ml) for 1 h at 37 °C. Then, the cells were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 4 °C for 5 h. Each result represents the mean ± standard deviation ($n = 3$). $^{*}p < 0.01$ vs. control.

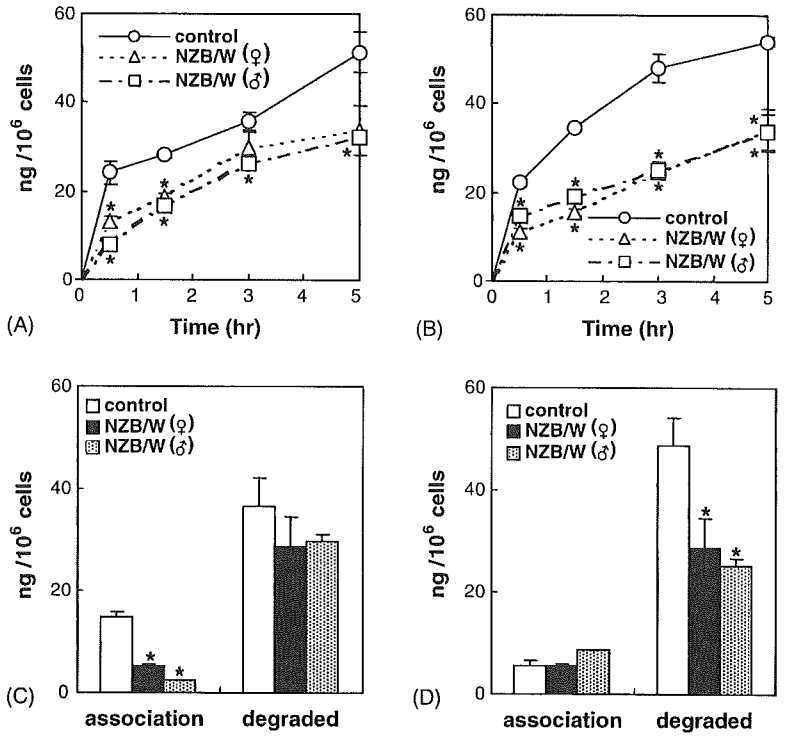


Fig. 3. Impaired $^{32}$P-labelled pDNA uptake and degradation in MΦs and DCs from 6-month-old NZB/W mice. Elicited peritoneal MΦs (A) and bone marrow-derived DCs (B) from 6-month-old control ICR mice and NZB/W mice were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 37 °C. Each point represents the mean ± standard deviation ($n = 3$). (C, D) Impaired DNA degradation contributing to the impaired DNA uptake. Elicited peritoneal MΦs (C) and bone marrow-derived DCs (D) from 6-month-old control ICR mice and NZB/W mice were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 37 °C for 5 h. Then, pDNA cellular association and degradation were determined as descried in Section 2. Each point represents the mean ± standard deviation ($n = 3$). $^{*}p < 0.01$ vs. control.

impaired in MΦs and DCs from NZB/W mice but degradation/excretion process is more strongly afflicted in DCs from NZB/W mice. Enhanced pDNA cellular association in DCs from diseased NZB/W mice could be the consequent of deficient DNA degradation/excretion that would cause prolonged pDNA retention in the cells. To examine whether the impaired pDNA uptake was caused by reduced (inhibited) binding, pretreatment with DNase was carried out. MΦs and DCs were treated with DNase and then incubated with $^{32}$P-labelled pDNA at 37 °C for 5 h. Surprisingly, although MΦs and DCs from 6-month-old NZB/W mice showed recovered DNA binding after DNase treatment at 4 °C (Fig. 2C and D), this treatment had little effect on the uptake and degradation of pDNA at 37 °C (Fig. 4). However, MΦs from 6-month-old NZB/W mice exhibited enhanced cellular association and reduced degradation of pDNA compared with cells without DNase treatment (data not shown). These results suggest that reduced DNA binding, probably caused by competitive inhibitions by endogenous DNA, was not the only reason for the impaired DNA uptake. These cells would also deficient in DNA internalization and the deficiency might mask the enhanced DNA binding by DNase treatment at 37 °C. Taken together, impaired DNA uptake in MΦs and DCs from NZB/W mice suggests that these cells have some deficiencies in some processes in the removal of DNA.

### 3.4. DNA uptake in MΦs and DCs from 5-week-old NZB/W mice

As MΦs and DCs from 6-month-old NZB/W mice showed impaired DNA uptake, we next examined whether the impairment is the consequence of disease progression or not. To address this question, DNA uptake and degradation in MΦs from 5-week-old, prediseased NZB/W mice was examined. In contrast to MΦs from 6-month-old NZB/W mice, MΦs from 5-week-old NZB/W mice showed almost identical characteristics for the binding of $^{32}$P-labelled pDNA as those of control ICR mice at 4 °C (Fig. 5A). DNase treatment had no effect on $^{32}$P-labelled pDNA binding to MΦs from 5-week-old NZB/W mice. This result suggests that the circulating DNA in active SLE mice might be the main competitive inhibitor of DNA binding since prediseased NZB/W mice have little circulating DNA. Interestingly, although MΦs from 5-week-old NZB/W mice exhibited normal DNA binding, these MΦs showed impaired DNA uptake and degradation compared with normal cells at 37 °C (Fig. 5B). Furthermore, the impairment

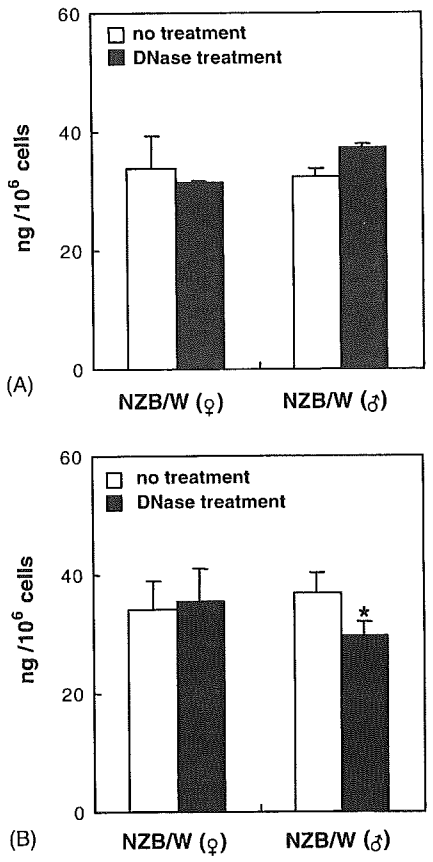


Fig. 4. DNase had little effect on $^{32}$P-labelled pDNA uptake and degradation in the cells from 6-month-old NZB/W mice. Elicited peritoneal MΦs (A) and bone marrow-derived DCs (B) from 6-month-old NZB/W mice were incubated in HBSS in the presence or absence of DNase (10 U/ml) for 1 h at 37 °C. Then, the cells were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 37 °C for 5 h. Each result represents the mean ± standard deviation ($n = 3$). $^{*}p < 0.01$ vs. control.

was mainly observed in the degradation of pDNA (Fig. 5C). DNase treatment had no effect on pDNA uptake and degradation in MΦs from 5-week-old NZB/W mice and control ICR mice (data not shown). Therefore, these results suggest that MΦs from NZB/W mice have impaired characteristics in both uptake and degradation processes in spite of their age or the development of the disease.

## 4. Discussion

In the present study, we evaluated the capacity of apparent DNA uptake (uptake and subsequent secretion after the degradation) in MΦs and DCs by measuring the amount of radioactivity associated with these cells and released from the cells in degraded form following incubation with $^{32}$P-labelled pDNA. We assumed that the degradation occurred intracellularly after internalization probably via the endosomal/lysosomal pathway based on the finding that the degradation was significantly reduced by endosomal acidification inhibitors. This assumption was also supported by our previous observation that both MΦs and DCs can efficiently internalize FL-labelled pDNA [25,28]. Therefore, we speculate that lysosomal enzymes, such as DNase II, are responsible for the DNA digestion after internalization. However, we cannot completely exclude the possibility that the degradation might occur before internalization, i.e., in some compartments other than endosome/lysosomes because these cells may have DNases expressed on their plasma membrane. Emlen and Mannik have speculated about the presence of membrane-bound DNase in liver MΦs [35]. The membrane-bound DNase, if it exists, could digest DNA more rapidly than lysosomal DNases because membrane DNase does not require internalization. However, even if the membrane DNases might be involved, at least in part, in the pDNA degradation in this study, the capacities of

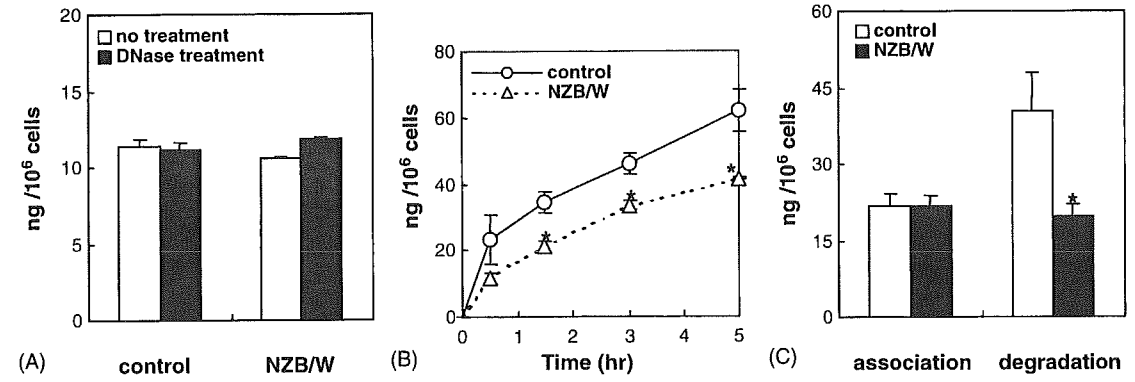


Fig. 5. Impaired DNA uptake and degradation in MΦs from 5-week-old NZB/W mice. (A) DNase treatment cannot influence the $^{32}$P-labelled pDNA binding to MΦs from 5-week-old NZB/W mice. Elicited peritoneal MΦs from 5-week-old control ICR mice and NZB/W mice were incubated in HBSS in the presence or absence of DNase (10 U/ml) for 1 h at 37 °C. Then, the cells were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 4 °C for 5 h. Each result represents the mean ± standard deviation ($n = 3$). (B) Impaired $^{32}$P-labelled pDNA uptake in MΦs from 5-week-old NZB/W mice. Elicited peritoneal MΦs from 5-week-old control ICR mice and NZB/W mice were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 37 °C. Each point represents the mean ± standard deviation ($n = 3$). (C) Impaired DNA digestion contributes to the impaired DNA uptake. Elicited peritoneal MΦs from 5-week-old control ICR mice and NZB/W mice were incubated with $^{32}$P-labelled pDNA (0.1 μg/ml) at 37 °C for 5 h. Then, pDNA cellular association and degradation were determined as descried in Section 2. Each point represents the mean ± standard deviation ($n = 3$). $^{*}p < 0.01$ vs. control.

apparent DNA uptake by MΦs and DCs from NZB/W and control mice can be compared in a quantitative manner.

In this study, we have demonstrated impaired DNA uptake and degradation in MΦs and DCs from NZB/W mice. The impairment largely resulted from the deficient degradation of DNA, so dysfunction and/or underexpression of DNases, such as DNase II or cell surface DNase if it exists, would be suspected. Defective DNA digestion could prolong the intracellular retention of DNA and might lead to an overestimation of the DNA uptake. We observed a significant reduction in DNA cellular association only in MΦs from 6-month-old NZB/W mice but the association was not enhanced in DCs from 6-month-old NZB/W mice or MΦs from 5-week-old NZB/W mice. However, we have possibly overestimated the cellular association as the result of prolonged retention by deficient degradation. Therefore, MΦs and DCs could have reduced ability for DNA uptake as well as DNA degradation.

Previous studies have shown that there are low serum and urine DNase activities in both SLE patients and SLE-prone NZB/W mice [36,37]. Moreover, DNase I-deficient mice produced by gene targeting show the classical symptoms of SLE, which are the presence of anti-nuclear antibodies and glomerulonephritis [38]. Mutation of *DNase I* gene has also been found in some SLE patients [39], although not all SLE patients have defects in DNase genes [40–42]. Therefore, multiple factors are probably involved in causing the reduced DNase activity in SLE. The impaired DNA uptake and degradation in MΦs and DCs from NZB/W mice observed in this study is one of these factors.

In our previous studies, we showed that pDNA uptake by mouse peritoneal MΦs and DCs is mediated by a mechanism specific to some polyanions [25,26,28]. The MΦ class A scavenger receptor (SRA) was a likely candidate responsible for the pDNA uptake in MΦs, since SRA was able to bind its polyanionic ligands in a divalent cation-independent manner by forming a specific direct bond between the positively charged lysine cluster in SRA and the negatively charged ligands. However, further studies using cultured Chinese hamster ovary cells expressing SRA and peritoneal MΦs from SRA-knockout mice have shown that SRA is not involved in pDNA uptake by MΦs [26]. In addition, there is no difference between bacterial DNA and vertebrate DNA in the phase of surface binding to MΦs because DNA recognition is based on the negative charges of the phosphate groups of DNA [25].

In contrast, in the phase of DNA uptake, there is the possibility that bacterial DNA is cleared differently from vertebrate DNA by MΦs. McCoy et al. reported that unmethylated CpG motifs in bacterial DNA activate murine MΦ cell-line RAW264.7 cells and the activated cells take up bacterial DNA more efficiently [43]. In addition, Blander has demonstrated that TLR signaling can induce the maturation of endosomes and facilitate the digestion of their contents [44]. Thus, these findings imply that efficient DNA uptake requires appropriate cellular activation and defective or inappropriate activation may cause dysfunction in the removal of DNA. Therefore, impaired pDNA removal observed in this study might be one of the consequences of deficient or inappropriate activation of MΦs and DCs from NZB/W mice. Indeed, abnormal activation in PBMCs from SLE patients has been demonstrated using a CpG motif containing oligonucleotides [45]. In addition, MΦs and DCs from NZB/W mice have been shown to abnormally respond to DNA (our unpublished observation). Although mammalian DNA are usually suppressed and highly methylated [11–13], they also contain CpG sequences and can often activate immune cells via TLR9-dependent pathway [46,47]. Therefore, cellular activation of MΦs and DCs by CpG motifs present in genome DNA would critically contribute to the efficient DNA removal by accelerating the DNA uptake in physiological conditions. Taken together, impaired DNA removal in MΦs and DCs reported in this study, although not so dramatic, could delay removal of DNA in vivo and be involved in the development of SLE. Studies are in progress to characterize further the effects of activation via TLR signaling on DNA uptake in MΦs and DCs.

Various MΦ dysfunctions in SLE have been reported. For example, deficient Fc-dependent phagocytosis of antibody-coated erythrocytes has been demonstrated in peritoneal MΦs from NZB/W mice [48] and impaired phagocytosis of apoptotic cells has also been found [49]. These dysfunctions might also be relevant to the deficient or inappropriate cellular activation of MΦs since engulfment of macromolecules could induce cellular activation in many cases. Alleva has demonstrated that peritoneal MΦs from young NZB/W mice are abnormally activated by LPS [50]. So, MΦs in SLE might generally have the deficient characteristics in activation itself or merely in clearing macromolecules.

In conclusion, the present study has demonstrated that MΦs and DCs from NZB/W mice have an impaired ability to clear DNA. This impaired DNA removal may promote the development of anti-DNA Ab by disrupting Ag clearance. Finally, these findings provide valuable insights into the intrinsic functional defects in MΦs and DCs associated with SLE.

## Acknowledgements

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# Cellular uptake and activation characteristics of naked plasmid DNA and its cationic liposome complex in human macrophages

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**Abstract**

Plasmid DNA (pDNA) is an important macromolecular therapeutic agent suitable for DNA-based therapies, such as non-viral gene therapy and DNA vaccination. Unmethylated CpG motifs abundant in bacterial DNA, but not in vertebrate DNA, are known to trigger an inflammatory response, which inhibits transgene expression, while modulating immunological consequences following vaccination. We studied cellular uptake and activation characteristics of naked pDNA and its cationic liposome complex in human macrophage-like cells. The present study has demonstrated that naked pDNA was recognized by human macrophage-like cells via specific mechanisms for polyanions. Moreover, it has shown that pDNA complexed with cationic liposomes activates human macrophage-like cells to induce the production of tumor necrosis factor-α (TNF-α) in a CpG motif-independent manner, while any types of naked DNA could not induce TNF-α production from these cells, regardless of the presence of CpG motifs in pDNA or oligonucleotide (ODN). These findings form an important basis for DNA-based therapies including gene therapy and DNA vaccination.


*Keywords:* Macrophages; Plasmid DNA; CpG motif; Cationic liposome; Tumor necrosis factor-α

## 1. Introduction

Plasmid DNA (pDNA) has become an important macromolecular therapeutic agent suitable for DNA-based therapies, such as non-viral gene therapy and DNA vaccination (Leitner et al., 1999; Nishikawa and Huang, 2001). However, unmethylated CpG motifs in pDNA or bacterial DNA, but not in vertebrate DNA, are recognized by immune cells as a danger signal (Krieg, 2002). When macrophages or dendritic cells (DCs) take up CpG DNA, inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and IL-12 are secreted. These cytokines significantly influence DNA-based therapies in different ways. In gene therapy, the cytokine production generally seems inappropriate because these inflammatory cytokines sig-

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nificantly reduce transgene expression of therapeutic proteins in target cells through their direct cytotoxic and promoter attenuation effects (Ghazizadeh et al., 1997; Qin et al., 1997). On the other hand, these inflammatory cytokines are essential for the efficacy of DNA vaccination because these cytokines can enhance the immune responses and the balance of these cytokines profoundly affects the nature of these responses (Leitner et al., 1999; Krieg and Kline, 2000).

CpG motifs in bacterial DNA or synthetic ODNs are recognized by Toll-like receptor 9 (TLR9) and trigger an immune cascade, resulting in improved antigen uptake and presentation by antigen-presenting cells and the secretion of polyreactive Ig, chemokines and cytokines by B cells, natural killer (NK) cells, DCs and monocytes. TLR9 is abundantly expressed in immunocompetent cells in mice, but the receptor is expressed only in primarily B cells and plasmacytoid dendritic cells (pDCs) in humans (Hornung et al., 2002). In addition, some CpG ODN can induce cell type-specific immune responses. For example, CpG-A ODN produces marked induction of interferon-α (IFN-α) production from pDCs and CpG-B ODN can prominently induce the proliferation of B cells. Moreover, some ODNs containing non-CpG sequences are able to activate these cells (Bauer et al., 1999; Pasquini et al., 1999; Vollmer et al., 2002). Thus, there seems to be various dangerous sequences and, perhaps, various molecules that could recognize those signals. These possibilities could lead to problems involving the application of pDNA because bacterial DNA and pDNA contain various CpG sequences. However, most of the earlier studies have examined the effects of CpG DNA using single stranded ODN containing ~20 phosphorothioate-stabilized nucleotides and the effects of CpG DNA in bacterial DNA or pDNA are poorly understood.

We have investigated the in vivo disposition characteristics of naked pDNA in mice and found that liver non-parenchymal, probably, Kupffer (liver resident macrophages) cells play an important role (Yoshida et al., 1996; Takagi et al., 1998). Further, in vitro studies using cultured mouse peritoneal macrophages have suggested that a specific receptor, like the class A scavenger receptor, may be involved in the endocytic uptake of pDNA by macrophages (Takagi et al., 1998). We also examined the pDNA uptake by the murine DC cell-line, DC2.4 cells, and found that DCs take up pDNA by a specific mechanism more efficiently and rapidly than macrophages (Yoshinaga et al., 2002). Moreover, we previously demonstrated that DNA complexed with cationic liposomes could activate murine peritoneal macrophages in a CpG-independent manner, while naked pDNA could not (Yasuda et al., 2002). Our recent study has demonstrated that the macrophage activation by DNA/cationic liposome complex requires endosomal acidification and both TLR9-dependent and -independent pathways (Yasuda et al., 2005).

Thus, the immune activation mediated by CpG-motif is well characterized in mice, but there is insufficient information about the effects of CpG on human immune cells. A better understanding of the effects of pDNA in humans is required in order to apply the knowledge obtained in mouse studies to human therapeutic strategies. In the present study, the uptake of pDNA by human macrophage-like cells is examined as well as the activation of human macrophages stimulated with DNA in both naked and complexed form. These findings will be an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

## 2. Materials and methods

### 2.1. Reagents

RPMI 1640 medium and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). [α-$^{32}$P]dCTP (3000 Ci/mmol) was obtained from Amersham (Buckinghamshire, England). pCMV-Luc encoding firefly luciferase gene constructed previously was used as a model pDNA. Calf thymus DNA (CTDNA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). LipofectAMINE 2000 (LA) and Opti-MEM were purchased from Lifetechnologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). All other chemicals used were of the highest purity available.

### 2.2. Cell cultures

Human monocytic cell-lines, U937 cells and THP-1 cells were obtained from the Cell Resource

Center for Biomedical Research, Tohoku University, and maintained in RPMI 1640 supplemented with 10% FBS, penicillin G (100 U/mL) and streptomycin (100 μg/mL). For experiments, cells were plated on a 24-well culture plate at a density of $1 \times 10^6$ cells/mL in the presence of 100 ng/mL PMA at 37 °C in 5% $CO_2$–95% air for 48 h, to differentiate to macrophage-like cells.

### 2.3. Plasmid DNA

For cellular association experiments, pCMV-Luc was radiolabeled using [α-$^{32}$P]dCTP by nick translation (Rigby et al., 1977). For synthesis of methylated pDNA, cytosine residues in CpG sequences of pDNA were methylated by SssI methylase (New England Biolabs, Beverly, MA, USA). The methylated pDNA was tested for digestion with *Hpa*II (Takara, Kyoto, Japan) to confirm methylation. pDNA mobility was analyzed by 1% agarose gel electrophoresis. DNA/LA complexes were prepared according to manufacturer's instructions.

### 2.4. Oligodeoxynucleotides

Phosphorothioate ODN were purchased from Hokkaido System Science Co. Ltd. (Sapporo, Japan). The sequences of CpG S-ODN 2006 are 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′, a proven activator of human immune cells as previously described (Kerkmann et al., 2003). Phosphorothioate non-CpG ODN 2006GC (5′-TGCTGCTTTTGTGCTTTTGTGCTT-3′) was used as a control.

### 2.5. Purification of DNA

To minimize the activation by contaminated lipopolysaccharide (LPS), DNA samples were used following thorough purification with Triton X-114, a non-ionic detergent. Extraction of endotoxin from pDNA, methylated pDNA, and CTDNA samples was performed according to previously published methods (Cotten et al., 1994; Hartmann and Krieg, 1999) with slight modifications as previously described (Yasuda et al., 2002). DNA samples were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitation. DNA (10 mg) was diluted with 20 mL pyrogen-free water, and then 200 μL Triton X-114 was added followed by mixing. The solution was placed on ice for 15 min and incubated for 15 min at 55 °C. Subsequently, the solution was centrifuged for 20 min at 25 °C, $600 \times g$. The upper phase was transferred to a new tube, 200 μL Triton X-114 was added, and the previous steps were repeated three or more times. The activity of LPS was measured by limulus amebocyte lysate (LAL) assay using the Limulus *F*-Single Test kit (Wako, Tokyo, Japan). After Triton X-114 extraction, the endotoxin levels of DNA samples could no longer be determined by LAL assay, i.e., 1 μg/mL DNA contained less than 0.001 EU/mL. Without extraction of endotoxin by Triton X-114, 100 μg/mL naked pDNA contained 1–5 EU/mL endotoxin.

### 2.6. Cellular association experiments

Cells were washed with 0.5 mL HBSS without phenol red and 0.5 mL HBSS containing 0.1 μg/mL naked [$^{32}$P]pDNA or pDNA/LA complex was added. After incubation at 37 or 4 °C for a specified time, the HBSS was removed and the cells were washed five times with ice-cold HBSS and then solubilized with 1 mL 0.3 M NaOH with 0.1% Triton X-100. Aliquots of the cell lysate were taken for the determination of $^{32}$P radioactivity using an LAS-500 scintillation counter (Beckman, Tokyo, Japan) and the protein content was measured using the modified Lowry method with bovine serum albumin as a standard. To examine the competition in binding, unlabeled macromolecules, such as polyinosinic acid (poly[I], M.W. 103.3 kDa), polycytidylic acid (poly[C], M.W. 99.5 kDa), dextran (M.W. 70 kDa) and dextran sulfate (M.W. 150 kDa) were added to the incubation wells concomitantly with [$^{32}$P]pDNA.

### 2.7. TCA precipitation experiments

After the cellular association experiments, the medium and cell lysates containing radioactivity derived from [$^{32}$P]pDNA were subjected to trichloroacetic acid (TCA) precipitation experiments to assess the degradation of pDNA by human macrophage-like cells as previously described (Yoshinaga et al., 2002).

### 2.8. Confocal microscopy

pCMV-Luc was labeled with fluorescein using a Fasttag FL labeling kit according to the manufacture's instructions (Vector Laboratories, Burlinghame, CA, USA). Cells were washed three times and incubated with medium containing fluorescein–pDNA or fluorescein–pDNA/LA complex. After an 8 h incubation at 4 or 37 °C, the cells were washed four times and fixed with 1% paraformaldehyde for 10 min. The cells were then scanned by confocal microscopy (MRC-1024, BIO-RAD, CA, USA).

### 2.9. Cytokine secretion

PMA-treated U937 cells were washed with 0.5 mL RPMI 1640 before stimulation. Naked DNA and DNA/LA complex were diluted in 0.5 mL Opti-MEM. The cells were incubated with the DNA-containing solution continuously for 8 or 24 h. In the case of DNA/LA complexes, cells were incubated for 2 h with the solution containing the complexes and then the solution was removed and the cells were incubated with Opti-MEM continuously for specified periods up to 24 h. After incubation, the conditioned medium was collected to assess cytokines and kept at −80 °C. The levels of TNF-α in the conditioned medium were quantitated by human TNF-α ELISA set (eBioscience, San Diego, CA) or OptEIA mouse TNF-α set (BD PharMingen, San Diego, CA), following the manufacturer's instructions.

## 3. Results

### 3.1. Cellular association of $^{32}P$ or fluorescein-labeled pDNA with naked or complexed form in human macrophage-like cells

A time-dependent increase in the cellular association of naked [$^{32}$P]pDNA with U937 cells was observed at 37 °C. The cellular association significantly decreased at 4 °C (Fig. 1A), where only binding to the cellular surface occurred. On the other hand, when pDNA was complexed with cationic liposomes, the amount of pDNA binding to the cell surface increased compared with naked pDNA (Fig. 1B). However, the degree of total cellular association at 37 °C was similar

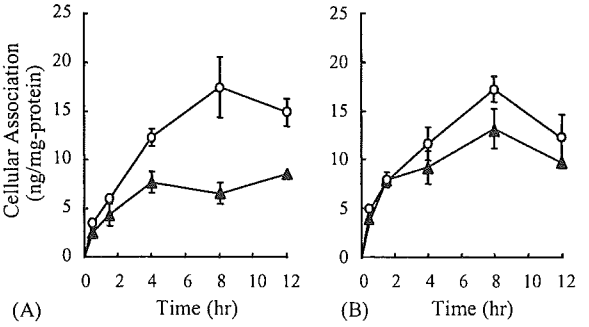


Fig. 1. Cellular association time-courses of naked [$^{32}$P]pDNA (A) or [$^{32}$P] pDNA/cationic liposome complex (B) in U937 cells. These cells were incubated with [$^{32}$P]pDNA (0.1 μg/mL) or [$^{32}$P]pDNA/LA complex (0.1 μg/mL: 0.2 μg/mL) at 37 °C (open circle) or 4 °C (closed triangle). Each point represents the mean ± S.D. ($n$ = 3).

to that at 4 °C. This tendency was also observed in other human macrophage-like cells, PMA-treated THP-1 cells (Fig. 2). The degradation of pDNA by human macrophage-like cells was investigated by the TCA precipitation method, which can detect degraded DNA smaller than about 16mer. In both PMA-treated U937 cells and THP-1 cells, little of the pDNA was fragmented to units smaller than 16mer (data not shown).

In order to examine the intracellular localization of pDNA, the cellular association of fluorescein-labeled pDNA by U937 cells was investigated using confocal microscopy (Fig. 3). Localization of both naked

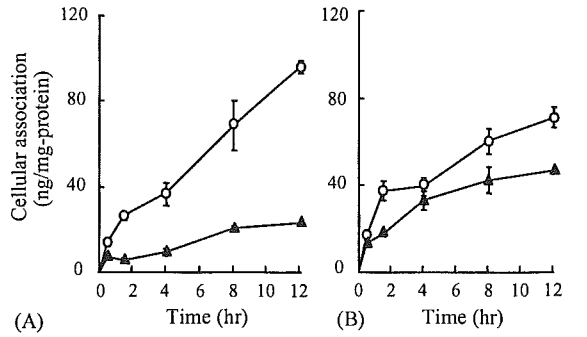


Fig. 2. Cellular association time-courses of naked [$^{32}$P]pDNA (A) or [$^{32}$P] pDNA/cationic liposome complex (B) in THP-1 cells. These cells were incubated with [$^{32}$P]pDNA (0.1 μg/mL) or [$^{32}$P]pDNA/LA complex (0.1 μg/mL: 0.2 μg/mL) at 37 °C (open circle) or 4 °C (closed triangle). Each point represents the mean ± S.D. ($n$ = 3).

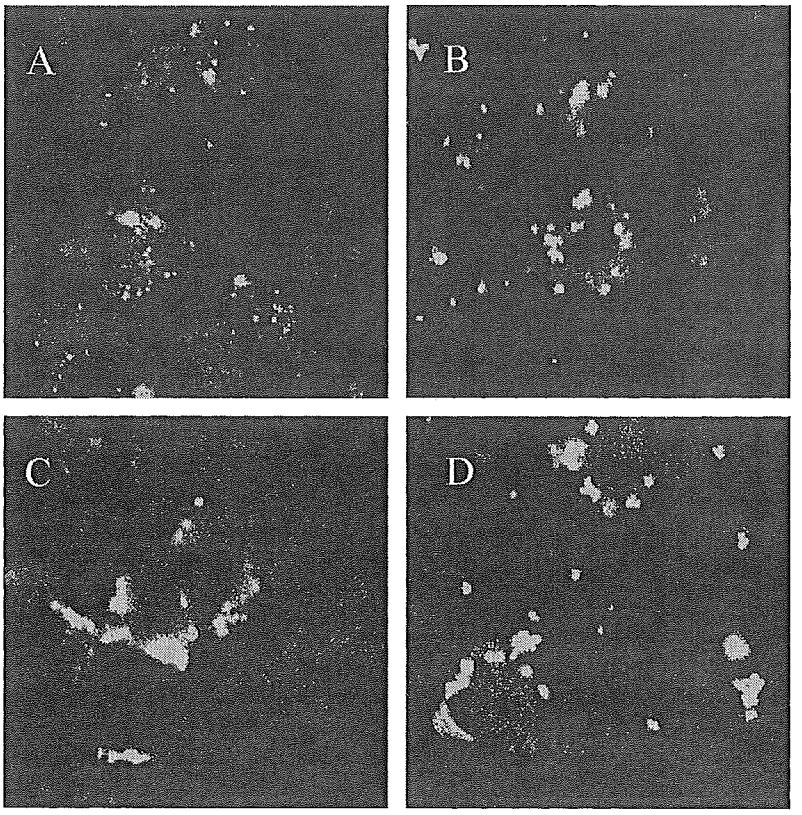


Fig. 3. Intracellular localization of naked fluorescein–pDNA or fluorescein–pDNA/LA complex in U937 Cells. The cells were incubated with naked fluorescein–pDNA (5 μg/well, A and C) or fluorescein–pDNA/LA complex (2.5 μg: 5 mL/well, B and D) for 8 h at 37 °C (A and B) or 4 °C (C and D).

and complexed pDNA at 4 °C was restricted to the cell surface (Fig. 3C and D) and both were taken up by the cells at 37 °C (Fig. 3A and B). However, the localization of naked and complexed pDNA at 37 °C was different. Naked pDNA was internalized in the cytosolic compartment (Fig. 3A), while complexed pDNA was localized in the vicinity of the cell membrane (Fig. 3B). This more pronounced temperature-dependence in naked pDNA uptake by U937 cells was consistent with the results obtained with [$^{32}$P]pDNA (Fig. 1).

### 3.2. *Effect of polyanions on pDNA binding to human macrophage-like cells*

We have already demonstrated that in mouse macrophages and DCs, pDNA was recognized by specific mechanisms for some polyanions. However, the recognition of pDNA in human macrophages is not yet fully understood.

The specificity of the binding of naked [$^{32}$P]pDNA was examined by cross-competition experiments using various macromolecules. As shown in Fig. 4, the cellular association of naked [$^{32}$P]pDNA was significantly inhibited by the presence of poly[I] and dextran sulfate, but not by poly[C] and dextran at 4 °C. These results suggest that a specific mechanism may be also involved in the recognition of pDNA by human macrophage-like cells.

### 3.3. *Immune activation stimulated by naked DNA in human macrophage-like cells*

In human immune cells, it is known that pDCs and B cells, but not monocytes or macrophages, express TLR9 and mainly recognize CpG motifs in DNA,

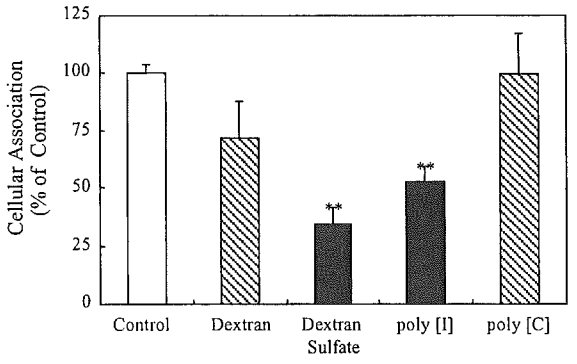


Fig. 4. Inhibition of cellular association of [$^{32}$P]pDNA with U937 Cells at 4 °C. The cells were incubated with [$^{32}$P]pDNA (0.1 μg/mL) for 8 h in the presence or absence various polyanions. Each point represents the mean ± S.D. ($n = 3$). $^{**}p < 0.01$ vs. control.

obtained using immunostimulatory CpG ODN. However, pDNA and ODN differ structurally. To examine whether human macrophage-like cells could be activated by pDNA, cells were stimulated with various DNA-containing unmethylated CpG motifs or a limited number of unmethylated CpG motifs. When PMA-treated U937 cells were cultured with ODN2006, a proven activator of human immune cells (Kerkmann et al., 2003), these cells could not be activated, nor could control ODN 2006GC. Naked pDNA even at a high concentration did not induce a significant amount of TNF-α in the cells nor did methylated pDNA or CTDNA containing few unmethylated CpG motifs (Fig. 5). In RAW264.7 cells, which are a mouse macrophage cell-line and express TLR9, the same concentration of ODN or pDNA was able to induce a significant amount of TNF-α in a CpG motif-dependent manner.

### *3.4. Immune activation by DNA/cationic liposome complex in human macrophage-like cells*

We carried out the same experiments using pDNA complexed with cationic liposomes, LipofectAMINE2000 (LA), which are taken up by macrophages via a non-specific mechanism based on electrostatic interaction. The pDNA/LA complex stimulated the cells to produce TNF-α (Fig. 6B). The secreted amount of TNF-α slightly decreased at 24 h presumably due to degradation and cellular uptake. Since liposomes alone are unable to stimulate

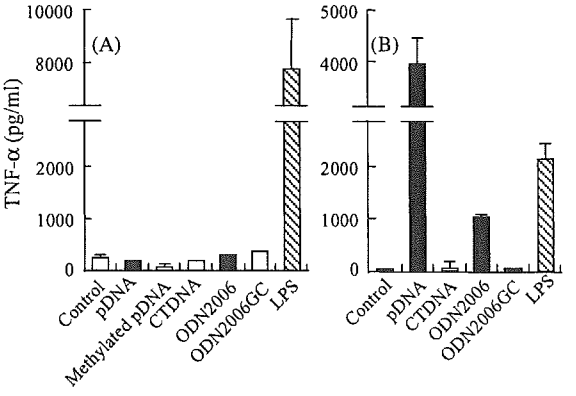


Fig. 5. Cellular activation by pDNA and other DNAs in U937 cells (A) and RAW264.7 cells (B). Cells were incubated with pDNA (100 μg/mL), methylated pDNA (100 μg/mL), CTDNA (100 μg/mL), ODN2006 (10 μM) or ODN2006GC (10 μM) at 37 °C. After 24 h (A) or 8 h (B), the supernatants were collected. The amount of TNF-α release was determined by ELISA. Each point represents the mean ± S.D. ($n = 3$).

the macrophages sufficiently to release TNF-α, these results show that pDNA is indispensable for TNF-α production by the liposome formulation.

To explore whether the unmethylated CpG motif in pDNA complexed with LA is required for TNF-α induction from macrophages, we prepared methylated-CpG pDNA. The methylated-CpG pDNA/LA complex induced a similar amount of TNF-α compared with the

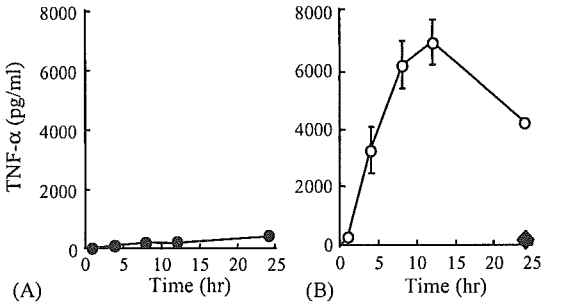


Fig. 6. Time-courses of cytokine release induced by naked pDNA (A) or pDNA/LA complex (B) from U937 cells. (A) Cells were incubated with naked pDNA (50 μg/well, closed circle) at 37 °C. (B) Cells were incubated with pDNA/LA complex (2.5 μg:5 μg/well, open circle) or LA only (5 μg/well, closed diamond) at 37 °C. After 2 h incubation, the cells were washed and incubated with growth medium. At the times indicated, the supernatants were collected. The amount of TNF-α release was determined by ELISA. Each point represents the mean ± S.D. ($n = 3$).

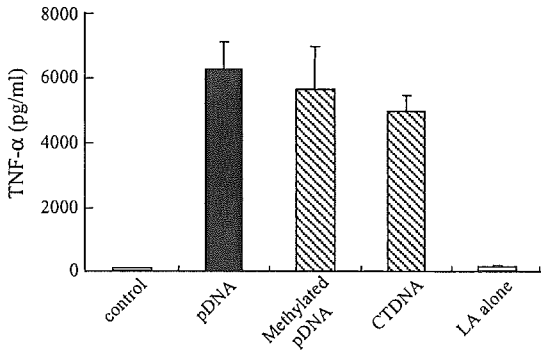


Fig. 7. TNF-α release by DNA/LA complex from U937 cells. DNA/LA complex was added to the cells. After 2 h incubation, the complex was removed and fresh growth medium was added to the cells. The supernatants were collected after 24 h incubation. The amount of TNF-α released from the cells was determined by ELISA. Each point represents the mean ± S.D. ($n = 3$).

pDNA/LA complex. Vertebrate DNA, CTDNA, also induce a significant amount of TNF-α when complexed with cationic liposomes (Fig. 7). These results indicate that the DNA/cationic liposome complex is able to activate human macrophages in a CpG motif-independent manner.

## 4. Discussion

Although the important role of the immunostimulatory effects mediated by CpG motifs in gene therapy and DNA vaccination is well understood, most in vitro studies focusing on the mechanisms of activation mediated by CpG DNA have been carried out using CpG ODN and bacterial genomic DNA in mouse macrophages, DCs and human peripheral blood mononuclear cells (PBMCs). The present study shows that pDNA is recognized by human macrophage-like cells via a specific mechanism for polyanions and DNA complexed with cationic liposomes is able to activate human macrophage-like cells in a CpG motif-independent manner, while the cells are not activated by naked CpG DNA.

In some reports using CpG ODN, it has been shown that CpG ODN is internalized to human PBMCs by endocytosis and subsequent endosomal acidification is necessary for signal transduction via TLR9 similar to murine macrophages or DCs (Macfarlane and Manzel, 1998; Bauer et al., 1999).

There are differences between humans and mice as far as the active CpG motifs are concerned. CpG ODNs that activate PBMC from humans were initially classified into two types (Verthelyi et al., 2001). The B (also known as K) type ODNs have phosphorothioate backbones, encode multiple TCG motifs and primarily stimulate B cell and monocyte proliferation, and IgM, interleukin-10 (IL-10) and IL-6 secretion. By contrast, the A (also known as D) type ODN have mixed phosphodiester-phosphorothioate backbones and contain a single hexameric purine-pyrimidine-CG-purine-pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3′-end by a poly-G tail. However, pDNA and immunostimulatory CpG ODN differ structurally. CpG ODNs are single-stranded with phosphorothioate bonds, including a single CpG motif. On the other hand, pDNA are double-stranded with phosphodiester bonds with an abundance of nucleotides likely to generate multiple CpG motifs. Therefore, it is important to elucidate the cellular uptake characteristics of pDNA and the subsequent activation in human macrophages.

Naked pDNA was taken up by human macrophage-like cells, both PMA-treated U937 cells and THP-1 cells. The amounts of naked pDNA associated with the cells were comparable with those of the pDNA complexed with LA. However, the internalization of naked pDNA showed a more pronounced temperature-dependence than complexed pDNA and the recognition of pDNA by the cells involved specific mechanisms for polyanion, which have similar characteristics to those previously observed in mouse macrophages and DCs. Some other groups have also reported that human leucocytes are able to bind and internalize lambda phage DNA mediated by a specific protein on the cellular membrane (Bennett et al., 1985). This shows that naked pDNA is recognized by a specific membrane protein or receptor for polyanions.

In human immune cells, it is well known that only pDCs and B cells, but not monocytes or macrophages, express TLR9 (Hornung et al., 2002) and mainly recognize CpG motifs in DNA. In the present study, the immune response induced by naked pDNA or CpG ODN was investigated in monocytic cell-lines, U937 cells. PMA-treated U937 cells did not release a significant amount of TNF-α by stimulation of either DNA containing CpG motifs or CpG non-containing DNA probably due to lack of TLR9 expression. We

have also observed same phenomena in human primary macrophages differentiated from PBMCs (Fukuhara et al., unpublished results). Murine macrophages, which express TLR9 were activated by the same DNA in a CpG motif-dependent manner. Since human macrophage-like cells release TNF-α following stimulation with LPS, this showed that the cells were fully able to produce inflammatory cytokines. Moreover, LPS is recognized by TLR4, which shares many signaling molecules downstream of TLR9, thus, irresponsiveness to CpG DNA in human macrophages might be due to TLR9 itself. It has been reported that in DCs from TLR9 knock out mice, the responsiveness to pDNA disappeared (Spies et al., 2003). Therefore, these findings suggest that in human immune cells, pDNA also recognizes its own CpG motif by TLR9.

DNA/cationic liposome complex is also used as a gene vector or carrier in human clinical trials of non-viral gene therapy. However, pDNA/cationic liposome complex is well known to induce high levels of inflammatory cytokines in in vivo studies in mice (Alton et al., 1998; Freimark et al., 1998; Paillard, 1999; Yew et al., 2000; Sakurai et al., 2002). When pDNA was complexed with cationic liposomes, a significant amount of TNF-α was released from human macrophage-like cells, although TNF-α was not induced by naked pDNA. Since liposomes alone are unable to stimulate cells sufficiently to release TNF-α, these results show that pDNA is indispensable for TNF-α release by these liposome formulations. However, methylated pDNA or CTDNA, which contain few unmethylated CpG motifs are also able to induce. We have previously observed that in murine macrophages, cellular activation in complexed form was caused in a CpG-indepemdent manner. Similar results were obtained in human primary macrophages differentiated from PBMCs (Fukuhara et al., unpublished results). These results suggest that in human macrophage-like cells, cytokine induction by DNA/LA complex is independent of the CpG motif and TLR9. It is probably due to a change in the mechanism of cellular uptake or intracellular trafficking after uptake. pDNA/cationic liposome complex is able to induce inflammatory cytokines in a CpG motif-independent manner. It has been reported that in TLR9 knock out mice, some markers of toxicity are not affected or reduced but are not eliminated completely (Zhao et al., 2004). Since under in vivo conditions, other immune cells probably influence the responsiveness of macrophages to pDNA/cationic liposomes, more information is required about human immune cells purified from PBMCs.

In conclusion, it has been shown that pDNA/cationic liposome complex is recognized as a danger signal by human macrophage-like cells. These findings will be an important basis for future DNA-based therapies, including gene therapy and DNA vaccination.

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# Gene silencing in primary and metastatic tumors by small interfering RNA delivery in mice: Quantitative analysis using melanoma cells expressing firefly and sea pansy luciferases

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**Abstract**

Silencing of oncogenes or other genes contributing to tumor malignancy or progression by RNA interference (RNAi) offers a promising approach to treating tumor patients. To achieve RNAi-based tumor therapy, a small interfering RNA (siRNA) or siRNA-expressing vector needs to be delivered to tumor cells, but little information about its in vivo delivery has been reported. In this study, we examined whether the expression of the target gene in tumor cells can be suppressed by the delivery of RNAi effectors to primary and metastatic tumor cells. To quantitatively evaluate the RNAi effects in tumor cells, mouse melanoma B16-BL6 cells were stably transfected with both firefly (a model target gene) and sea pansy (an internal standard gene) luciferase genes to obtain B16-BL6/dual Luc cells. The target gene expression in subcutaneous primary tumors of B16-BL6/dual Luc cells was significantly suppressed by direct injection of the RNAi effectors followed by electroporation. The expression in metastatic hepatic tumors was also significantly reduced by an intravenous injection of either RNAi effector by the hydrodynamics-based procedure. These results indicate that the both RNAi effectors have a potential to silence target gene in tumor cells in vivo when successfully delivered to tumor cells.


*Keywords:* RNAi; Gene delivery; Hydrodynamics-based procedure; Hepatic tumor; Luciferase

## 1. Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing event in which short double-stranded RNA (siRNA) degrades target mRNA in a sequence-specific manner [1–3]. After the discovery that the use of short double-stranded RNA can induce RNAi in

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mammalian cells without a sequence-nonspecific response [4,5], RNAi has been widely used as an experimental tool to suppress specific gene expression for research involving gene function. This is because RNAi is attractive for its speed, usefulness, and lower cost, compared with conventional strategies to suppress gene function, such as gene knock-out by homologous recombination, and is generally more powerful than antisense strategies [6,7]. Moreover, RNAi is expected to be used as a therapeutic tool in the treatment of various diseases, such as cancer, viral infections, and neurodegenerative disorders [8,9].

Tumor cells have at least two major abnormalities: dysregulation of the cell cycle leading to uncontrolled growth, and resistance to death resulting from abnormalities in one or more genes that mediate apoptosis. Therefore, silencing these genes by RNAi offers a therapeutic treatment for cancer patients. In the last 5 years, there have been a number of reports in the literature describing how induction of RNAi suppresses these genes and decreases the malignancy of tumor cells in vitro. However, a few reports have described the successful treatment of tumors by the induction of RNAi in vivo [10–13]. Poor delivery of RNAi effectors may be the major reason for the limited success in tumor therapy by RNAi in vivo in contrast to the many successes in vitro. One reason for this is that RNAi effectors are water soluble macromolecules and so they have difficulty in crossing cellular membranes and their gene silencing effect is limited in the cells that have received RNAi effectors [14,15]. Regarding the delivery of RNAi effectors, the authors reporting successful in vivo tumor therapy administrated naked siRNA, siRNA or siRNA-expressing vector complexed with a cationic carrier. Duxbury et al. [13] and Verma et al. [10] did not evaluate the delivery of siRNA to tumor cells in detail but they examined the therapeutic effects produced by silencing the target genes. To inhibit tumor growth by siRNA targeting to vascular endothelial growth factor, Filleur et al. [11] investigated the effect of the routes of administration of siRNA on the gene silencing efficiency in subcutaneous tumors. Zhang et al. [12] administered siRNA-expressing plasmid DNA (pDNA) encapsulated in liposomes coated with antibody targeting the brain tumor and succeeded in suppressing gene expression in the tumor. However, the delivery of RNAi effectors to tumor cells and intratumoral gene silencing in vivo has received little attention in any of these previous studies.

RNAi can be induced by the delivery of siRNA or siRNA-expressing vector, which works as a platform to produce siRNA within the target cell for a relatively long period. Some groups have developed vector-based siRNA expression systems [16–19], which showed effective RNAi induction. The molecular weight of siRNA-expressing vector is several hundred-fold greater than that of siRNA and must be delivered to the nucleus of target cells, unlike siRNA which can work if it is present in the cytoplasm [14]. Therefore, siRNA-expressing vector is more problematic than siRNA as far as delivery to the target cells is concerned. However, siRNA-expressing vector has the advantages of a sustained effect and ease in regulating its function compared with siRNA. Moreover, siRNA-expressing vectors that have an on-off switch for siRNA have also been reported [20]. Both RNAi effectors have advantages and disadvantages, and so should be used as the situation demands.

In either case of siRNA or siRNA-expressing vectors, the delivery of RNAi effectors to the tumor cells is the key factor for treating tumor patients with RNAi, because the gene silencing effect is limited in the cells that receive RNAi effectors. To improve RNAi-based tumor therapy, efficient RNAi effector delivery systems and delivery methods need to be developed. We considered that a model system in which the gene silencing effect can be sensitively evaluated may be a powerful tool for achieving successful in vivo RNAi-based tumor therapy. As used in previous studies involving the simultaneous administration of target gene and RNAi effectors [21], we think that clones of tumor cells stably expressing reporter genes, such as luciferase and green fluorescent protein, will be useful for examining whether siRNA or siRNA-expressing vector is effective in reducing target gene expression in tumor cells in vivo. Some research groups have introduced marker genes to detect tumor cells in vivo [22,23]. We selected firefly (model target gene of RNAi) and sea pansy luciferases (indicator of tumor cell number) for detecting in vivo RNAi, because (i) these luciferases can be detected with a high degree of sensitivity and (ii) they can be simultaneously and quantitatively measured using a simple luminometric assay. Therefore, mouse melanoma B16-BL6 cells were stably

transfected with firefly and sea pansy luciferases to obtain B16-BL6/dual Luc cells. This cell clone has advantages in evaluating in vivo intratumoral gene silencing effect in that we can investigate the effect easily, sensitively, and quantitatively. A subcutaneous primary tumor model and a hepatic metastasis model were selected as the targets of the delivery. There are no literature reports about gene silencing in metastatic tumors in the liver. As delivery methods, we used naked siRNA or pDNA without any viral or cationic carriers. We investigated whether the gene transfer methods reported to result in high transgene expression in the liver or muscle can deliver RNAi effectors into tumor cells inoculated in the liver or under the skin. Our data suggest one solution to the question of how the in vivo intratumoral delivery of siRNA can be achieved.

## 2. Materials and methods

### 2.1. Plasmid DNA

pCMV-Luc encoding firefly luciferase$^+$ and neomycin-resistant gene was used to construct the cell line stably expressing firefly luciferase. The pDNA was constructed by subcloning the Hind III /Xba I firefly luciferase$^+$ cDNA fragment from pGL3-control (Promega, Madison, WI, USA) vector into the polylinker of the pcDNA3 vector [24]. pCMV-RL/Hygro encoding sea pansy *Renilla reniformis* luciferase and hygromycin-resistant gene was transfected to obtain the cell line stably expressing sea pansy luciferase. The pDNA was constructed by subcloning the Nhe I/BamH I sea pansy luciferase cDNA fragment from pRL-CMV vector (Promega) into the multicloning site of the pcDNA3.1/Hygro vector (Invitrogen, Carlsbad, CA, USA). pEGFP-F encoding farnesylated EGFP (enhanced green fluorescent protein), a modified form to bind to the plasma membrane, and pDsRed2-N1 encoding red fluorescent protein Dsred2 were purchased from BD Biosciences Clontech (Palo Alto, CA, USA). We used pEGFP-F for the genetic labeling of tumor cells to avoid effusion of the gene product following the hydrodynamics-based injection, since the unmodified EGFP might diffuse into the circulation following a large volume injection [25]. Each pDNA was amplified in the DH5α strain of *Escherichia coli* and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

### 2.2. Construction of stably expressing cell lines

Murine melanoma cell line B16-BL6 [26] and genetically labeled B16 cell lines were cultured in Dulbecco's modified Eagle's minimum essential medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/L-glutamine (PSG) at 37 °C and 5% $CO_2$. B16-BL6 plated on 6-well plates was transfected with pCMV-Luc using Lipofectamine 2000 (Invitrogen) to obtain B16-BL6 expressing the firefly luciferase, B16-BL6/Luc as reported previously [27]. B16-BL6/Luc plated on 6-well plates was then transfected with pCMV-RL/Hygro using Lipofectamine 2000, and the cells were selected using 1 mg/ml of hygromycin for 14–20 days to obtain a clone expressing the highest sea pansy luciferase activity, B16-BL6/dual Luc. The selected clone continuously expressed the firefly and sea pansy luciferases of activities to about 3 and 10 RLU/cell, respectively. In addition, B16-BL6/EGFP-F that stably expresses EGFP-F was also prepared by the same procedure as B16-BL6/Luc using pEGFP-F. The EGFP-F-expressing cell clones were screened and selected by confocal microscopic observations (MRC-1024; Bio-Rad, Hercules, CA, USA).

### 2.3. siRNA and siRNA-expressing pDNA

siRNA-expressing pDNAs driven by human U6 promoter were constructed from piGENE hU6 vector (iGENE Therapeutics, Tsukuba, Japan) according to the manufacturer's instructions. pU6-siGL3 transcribes a single-stranded RNA 5′-GUG CGU UGU UGG UGU UAA UCC UUC AAG AGA GGG UUG GCA CCA GCA GCG CAC UUU U-3′, which forms stem-loop-structured siRNA, targeted to firefly luciferase$^+$ mRNA (targeted sequence: GTG CGC TGC TGG TGC CAA CCC), with loop sequences of UUCAAGAGA [17]. pU6-siGL2 produces siRNA whose target sequence is not found in this study. piGENE hU6 vector, which transcribes the non related sequence of RNA 5′-GUG AGC AGG UGU AAA GCC ACC AUG GAA GAC ACC UGC CAA CUU UU-3′ with partial duplex formation, was used as a