prevention of metastasis and relapse but also the suppression of tumor growth.

To achieve potent therapeutic effects by DNA vaccination against cancer, it is essential to transfer the antigen-coding gene selectively and efficiently into APCs such as macrophages and dendritic cells, which play a pivotal role in the initiation, programming and regulation of cancer-specific immune responses.[14,15] Our group has also developed mannose-modified liposome/plasmid DNA (pDNA) complexes (mannose-modified lipoplexes) for APC-selective gene transfer via mannose receptors expressing on APCs, and obtained APC-selective gene expression in the liver and spleen by mannose-modified lipoplexes.[16,17] Moreover, our group also succeeded in obtaining DNA vaccine effects against cancer by intraperitoneal administration of mannose-modified lipoplexes constructed with tumor-specific antigen coding pDNA, such as ovalbumin (OVA) and melanoma-related antigens.[18,19] However, the gene transfection efficiency into APCs was lower than that in other cells;[20] therefore, it could be difficult to induce a potent cancer vaccine effect for the prevention of metastasis and relapse by DNA vaccination using conventional lipofection methods.

It has been reported that cancer vaccine effects can be enhanced by physical stimulation-mediated gene transfer such as electroporation,[21,22] hydrodynamic injection[23,24] and sonoporation methods.[25] These transfection methods enable the delivery of a large amount of antigen-coding gene and antigen peptides into APCs, since exogenous materials are directly introduced into the cytoplasm without endocytosis in these methods.[26−29] Recently, we have applied "sonoporation methods[25,29−31]" using US exposure and microbubbles enclosing US imaging gas to enhance gene expression in APCs[32] and developed a gene transfection method for DNA vaccination using US-responsive and mannose-modified gene carriers, Man-PEG_{2000} bubble lipoplexes.[33] This method enables APC-selective and -efficient gene expression, and moreover, effective vaccine effects against OVA-expressing cancer cells were obtained by applying this method to DNA vaccination using OVA-encoding pDNA.[33] However, the antigenicity of OVA is extremely high compared with other antigens,[34] and it is difficult to extrapolate the result obtained by DNA vaccination against OVA-expressing cells to actual cancer therapy, since OVA-expressing cells are transfectant constructed by gene transfer. Therefore, it is unclear if DNA vaccination by gene transfer using Man-PEG_{2000} bubble lipoplexes and US exposure is effective against cancer, i.e. melanoma, with metastatic properties.

In this study, we examined DNA vaccine effects against melanoma by transfection of pUb-M, coexpressing ubiquitylated gp100 and TRP-2, using Man-PEG_{2000} bubble lipoplexes and US exposure. First, we examined the level of gene expression in the splenic dendritic cells by gene transfer using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure. Second, we studied the characteristics of cytokine secretion and the induction of CTL activities against B16BL6 cell-derived melanoma by DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure. Then, we investigated the cancer vaccine effects against solid and metastatic tumors derived from B16BL6 cells by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Finally, we evaluated the duration of cancer vaccine effects against solid and metastatic melanoma after pUb-M transfection using Man-PEG_{2000} bubble lipoplexes and US exposure.

EXPERIMENTAL SECTION

Materials. 1,2-Stearoyl-3-trimethylammoniumpropane (DSTAP), 1,2-distearoyl-*sn*-glycero- 3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino-(polyethylene glycol)-2000] (NH_2-PEG_{2000}-DSPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), Sigma Chemicals Inc. (St. Louis, MO, USA) and NOF Co. (Tokyo, Japan), respectively. Anti-CD11c monoclonal antibody (N418)-labeled magnetic beads were obtained from Miltenyi Biotec Inc. (Auburn, CA, USA). Fetal bovine serum (FBS) was purchased from Equitech-bio Inc. (Kerrville, TX, USA). RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity available.

pDNA, Cell Lines and Mice. pUb-M containing murine melanoma glycoprotein-100_{25-33} (gp100) and tyrosinase-related protein-$2_{181-188}$ (TRP-2) peptide epitopes was kindly provided by Prof. R. A. Reisfeld.[35] The B16BL6 melanoma cells, colon-26 adenocarcinoma cells and EL4 lymphoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The B16BL6/Luc cells and colon-26/Luc cells, which are cell lines expressing firefly luciferase stably, were established as previously reported.[36,37] The B16BL6 cells and EL4 cells were cultured in DMEM, and the colon-26 cells were cultured in RPMI-1640 at 37 °C in 5% CO_2. Both media were supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Female C57BL/6 mice (6 weeks old) and female Balb/c mice (6 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the U.S. National Institutes of Health and the Guidelines for Animal Experiments of Kyoto University.

Construction of Man-PEG_{2000} Bubble Lipoplexes. Man-PEG_{2000} bubble lipoplexes were constructed according to our previous report.[33] Briefly, DSTAP, DSPC and NH_2-PEG_{2000}-DSPE or mannose-modified PEG_{2000}-DSPE were mixed in chloroform at a molar ratio of 7:2:1 to produce the liposomes for bubble lipoplexes. The mixture for construction of liposomes was dried by evaporation and vacuum desiccated, and the resultant lipid film was resuspended in sterile 5% dextrose. After hydration for 30 min at 65 °C, the dispersion was sonicated for 10 min in a bath sonicator and for 3 min in a tip sonicator to produce liposomes. Then, the liposomes were sterilized by passage through a 0.45 μm filter (Nihon-Millipore, Tokyo, Japan). The lipoplexes were prepared by gently mixing equal volumes of pDNA and liposome solution at a charge ratio of 1.0:2.3 (−:+). To enclose US imaging gas in lipoplexes, the prepared lipoplexes were pressured with perfluoropropane gas (Takachiho Chemical Industries Co., Ltd., Tokyo, Japan) and sonicated using a bath-type sonicator (AS ONE Co., Osaka, Japan) for 5 min. The particle sizes and zeta potentials of the liposomes/lipoplexes were determined by a Zetasizer Nano ZS instrument (Malvern Instrument, Ltd., Worcestershire, U.K.).

In Vivo Gene Transfection Method. Six week old C57BL/6 female mice were intravenously injected with 400 μL of bubble lipoplexes via the tail vein using a 26 gauge syringe needle at a dose of 50 μg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 1.045 MHz; duty, 50%; burst rate,

Table 1. Particle Sizes and Zeta Potentials of Lipoplexes and Bubble Lipoplexes Constructed with pUb-M[a]

	particle size (nm)	zeta-potential (mV)
Bare-PEG_{2000} lipoplex (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE = 7:2:1 (mol))	144 ± 13	45.7 ± 4.5
Man-PEG_{2000} lipoplex (DSTAP:DSPC:Man-PEG_{2000}-DSPE = 7:2:1 (mol))	143 ± 10	44.5 ± 5.8
Bare-PEG_{2000} bubble lipoplex (DSTAP:DSPC:NH_2-PEG_{2000}-DSPE = 7:2:1 (mol))	557 ± 20	46.7 ± 4.2
Man-PEG_{2000} bubble lipoplex (DSTAP:DSPC:Man-PEG_{2000}-DSPE = 7:2:1 (mol))	555 ± 19	45.1 ± 2.2

[a] Each value represents the mean ± SD (n = 3).

10 Hz; intensity 1.0 W/cm^2; time, 2 min) was exposed transdermally to the abdominal area using a Sonopore-4000 sonicator with a probe of diameter 20 mm. At predetermined times after injection, mice were sacrificed and spleens were collected for each experiment. In the intradermal transfection study, mice were intradermally injected with 200 μL of bubble lipoplexes at a dose of 50 μg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2; time, 2 min) was directly exposed to the injected site using a probe of diameter 6 mm. In the intrasplenic transfection, mice were directly injected into the spleen with 200 μL of bubble lipoplexes at a dose of 50 μg of pDNA. At 5 min after the injection of the bubble lipoplexes, US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2; time, 2 min) was directly exposed to the spleen using a probe of diameter 6 mm.

Measurement of the Level of mRNA Expression. Total RNA was isolated from the spleen using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of mRNA was carried out using a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). The detection of the Ub-M cDNA was carried out by real-time PCR using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN, USA) with primers. The primers for Ub-M, gp100, TRP-2 and GAPDH cDNA were constructed as follows: primer for Ub-M cDNA, 5′-GAG CCC AGT GAC ACC ATA GA-3′ (forward) and 5′-GTG CAG GGT GGA CTC TTT CT-3′ (reverse); primer for gp100, 5′-GCA CCC AAC TTG TTG TTC CT-3′ (forward) and 5′-GTG CTA CCA TGT GGC ATT TG-3′ (reverse); primer for TRP-2, 5′-CTT CCT AAC CGC AGA GCA AC-3′ (forward) and 5′-CAG GTA GGA GCA TGC TAG GC-3′ (reverse); primer for GAPDH, 5′-TCT CCT GCG ACT TCA ACA-3′ (forward) and 5′-GCT GTA GCC GTA TTC ATT GT-3′ (reverse) (Sigma-Aldrich, St. Louis, MO, USA). The mRNA copy numbers were calculated for each sample from the standard curve using the instrument software ("Arithmetic Fit Point analysis" for the Lightcycler). Results were expressed as relative copy numbers calculated relative to GAPDH mRNA (copy numbers of Ub-M, gp100 and TRP-2 mRNA/copy numbers of GAPDH mRNA).

Isolation of Splenic $CD11c^+$ Cells (Dendritic Cells) in Mice. At 6 h after transfection, spleens were harvested and spleen cells were suspended in ice-cold RPMI-1640 medium on ice. Then, red blood cells were removed by incubation with hemolytic reagent (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 3 min at room temperature. $CD11c^+$ and $CD11c^-$ cells were separated by magnetic cell sorting with an auto MACS (Miltenyi Biotec Inc., Auburn, CA, USA) following the manufacturer's instructions.

Evaluation of Antigen-Specific Cytokine Secretion. To prepare the tumor cell lysates (B16BL6 cells, EL4 cells and colon-26 cells), the cells were scraped from the plates and suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). After three cycles of freezing and thawing, the lysates were centrifuged at 10000g, 4 °C for 10 min and the resultant supernatants were collected. The protein concentration of cell lysates was determined with a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 96-well plates and incubated for 72 h at 37 °C in the presence or absence of tumor cell lysates (100 μg of proteins). IFN-γ, TNF-α, IL-4 and IL-6 in the culture medium were measured using a suitable commercial ELISA Kit (Bay Bioscience Co., Ltd., Hyogo, Japan).

CTL Assay. At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 6-well plates and coincubated with mitomycin C-treated tumor cells (B16BL6 cells, EL4 cells and colon-26 cells) for 4 days. After 4 days of coincubation, nonadherent cells were harvested, washed and plated in 96-well plates with target cells (B16BL6 cells, EL4 cells and colon-26 cells) at various effector cell/target cell (E/T) ratios. The target tumor cells were labeled with ^{51}Cr by incubating with $Na_2{}^{51}CrO_4$ (PerkinElmer, Inc., MA, USA) in culture medium for 1 h at 37 °C. At 4 h after incubation, the plates were centrifuged, and the supernatant in each well was collected and the radioactivity of released ^{51}Cr was measured in a gamma counter. The percentage of ^{51}Cr release was calculated as follows: specific lysis (%) = [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100).

Therapeutic Experiments in Solid Tumor Models. At 2 weeks after the last immunization or on the immunization day, B16BL6 cells, EL4 cells and colon-26 cells were transplanted subcutaneously into the back of the mice (1×10^6 cells). The tumor size was measured with calipers in two dimensions, and the tumor volume was calculated using the following equation: volume (mm^3) = $\pi/6 \times$ longer diameter × (shorter diameter)^2. The survival of the mice was monitored up to 100 days after the transplantation of tumor cells.

Therapeutic Experiments in Lung Metastatic Tumor Models. At 2 weeks after the last immunization or on the immunization day, B16BL6 cells or colon-26 cells were intravenously administered via the tail vein (1×10^5 cells) and the survival of the mice was monitored up to 100 days after administration of the tumor cells. To evaluate metastasis, B16BL6/Luc cells or colon-26/Luc cells were intravenously administered via the tail vein (1×10^5 cells). At 14 days after the administration of the tumor cells, the number of B16BL6/Luc cells and colon-26/Luc cells in the lung was quantitatively evaluated by measuring luciferase activity as previously reported.[36,37]

Statistical Analysis. Results were presented as the mean ± SD of more than three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Two-group comparisons were performed by the Student's t test. Multiple comparisons between control groups and other groups were performed by the Dunnett's test, and multiple

comparisons between all groups were performed by the Tukey–Kramer test.

RESULTS

Physicochemical Properties of Bubble Lipoplexes Constructed with pUb-M. The physicochemical properties of lipoplexes and bubble lipoplexes constructed with pUb-M used in all experiments were evaluated by measuring the particle sizes and zeta potentials. The mean particle sizes and zeta potentials of nonmodified PEG_{2000}-lipoplexes (Bare-PEG_{2000} lipoplexes) and mannose-conjugated PEG_{2000}-lipoplexes (Man-PEG_{2000} lipoplexes) were 144 ± 13 nm, 45.7 ± 4.5 mV and 143 ± 10 nm, 44.5 ± 5.8 mV, respectively (Table 1). Moreover, the mean particle sizes and zeta potentials of nonmodified bubble lipoplexes (Bare-PEG_{2000} bubble lipoplexes) and Man-PEG_{2000} bubble lipoplexes were 557 ± 20 nm, 46.7 ± 4.2 mV and 555 ± 19 nm, 45.1 ± 2.2 mV, respectively (Table 1). These results corresponded to our previous reports using other pDNA,[33] suggesting that pDNA had no effect on the physicochemical properties of Man-PEG_{2000} bubble lipoplexes.

Splenic Dendritic Cell-Selective and -Efficient Gene Expression by Gene Transfer Using Man-PEG_{2000} Bubble Lipoplexes and US Exposure. First, to investigate the level of gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure in the spleen, we measured the relative mRNA copy numbers of Ub-M after transfection. As shown in Figures 1A and 1B, the level of Ub-M mRNA expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure reached a peak at 6 h after transfection. Moreover, that level of Ub-M mRNA expression was markedly higher than that obtained by Bare- and Man-PEG_{2000} lipoplexes, and significantly higher than that obtained by Bare-PEG_{2000} bubble lipoplexes and US exposure. Then, we investigated the mannose receptor-expressing cell selectivity of Ub-M mRNA expression obtained by gene transfer using Man-PEG_{2000} bubble lipoplexes and US exposure. In the spleen, the relative mRNA copy numbers of Ub-M in $CD11c^+$ cells was significantly higher than that in $CD11c^-$ cells following transfection using Man-PEG_{2000} bubble lipoplexes and US exposure (Figure 1C). On the other hand, no selective gene expression in $CD11c^+$ cells was observed by gene transfer using Bare-PEG_{2000} bubble lipoplexes and US exposure (Figure 1C).

Antigen-Stimulatory Th1 Cytokine Secretion from the Splenic Cells Immunized by Man-PEG_{2000} Bubble Lipoplexes and US Exposure. To evaluate the melanoma-specific cytokine secretion from immunized splenic cells, splenic cells immunized by pUb-M were incubated with each tumor cell-lysate in vitro, and then, Th1 and Th2 cytokines secreted in the supernatants were measured. Following investigation of the expression level of gp100 and TRP-2, a melanoma-specific antigen, in each cell used in this study, the expression of gp100 and TRP-2 was only detected in B16BL6 cells which are melanoma cell lines (Supplementary Figure 1 in the Supporting Information). As results of the immunization according to the protocol shown in Figure 2A, the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes and US exposure secreted the highest amount of IFN-γ and TNF-α, which are Th1 cytokines, in the presence of B16BL6 cell lysates (Figures 2B and 2C). On the other hand, the secretion of Th1 cytokines (IFN-γ and TNF-α) was lower in all the groups in the presence of EL4 and colon-26 cell lysates. Moreover, the secretion of IL-4 and IL-6, which are Th2 cytokines, was also lower in all the groups in the presence of each cell lysate (Figures 2D and 2E). These observations suggest that pUb-M transfer by Man-PEG_{2000} bubble lipoplexes and US exposure significantly enhances the differentiation of helper T cells into Th1.

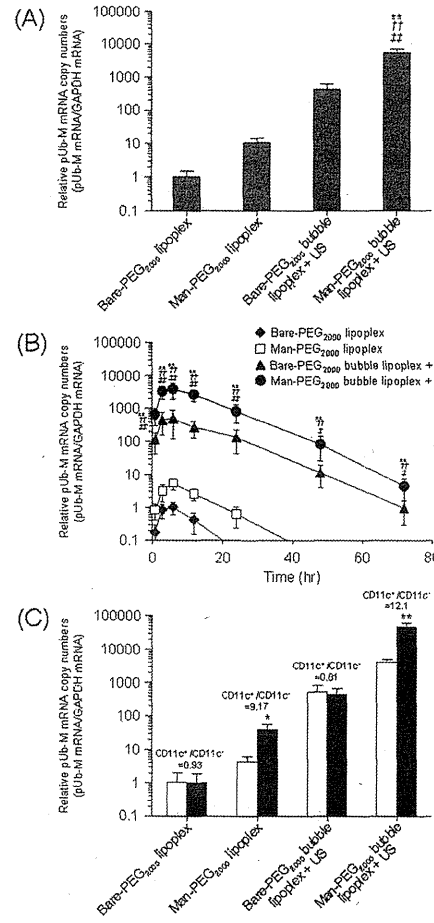

Figure 1. Enhanced Ub-M mRNA expression in the spleen and the splenic dendritic cells ($CD11c^+$ cells) by Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure in vivo. (A) The level of Ub-M mRNA expression obtained by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA) in the spleen at 6 h after transfection. Each value represents the mean + SD (n = 4). $^{**}p < 0.01$, compared with Bare-PEG_{2000} lipoplex; $^{\dagger\dagger}p < 0.01$, compared with Man-$PEG_{2000}$ lipoplex; $^{\ddagger\ddagger}p < 0.01$, compared with Bare-$PEG_{2000}$ bubble lipoplex + US. (B) Time-course of Ub-M mRNA expression in the spleen after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). Each value represents the mean ± SD (n = 4). $^{**}p < 0.01$, compared with the corresponding group of Bare-PEG_{2000} lipoplex; $^{\dagger\dagger}p < 0.01$, compared with the corresponding group of Man-PEG_{2000} lipoplex; $^{\ddagger}p < 0.05$; $^{\ddagger\ddagger}p < 0.01$, compared with the corresponding group of Bare-PEG_{2000} bubble lipoplex + US. (C) Splenic cellular localization of Ub-M mRNA expression at 6 h after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). Each value represents the mean + SD (n = 4). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of $CD11c^-$ cells.

Induction of Melanoma-Specific CTLs by pUb-M Transfer Using Man-PEG_{2000} Bubble Lipoplexes and US Exposure. We investigated the melanoma-specific CTL activities in the

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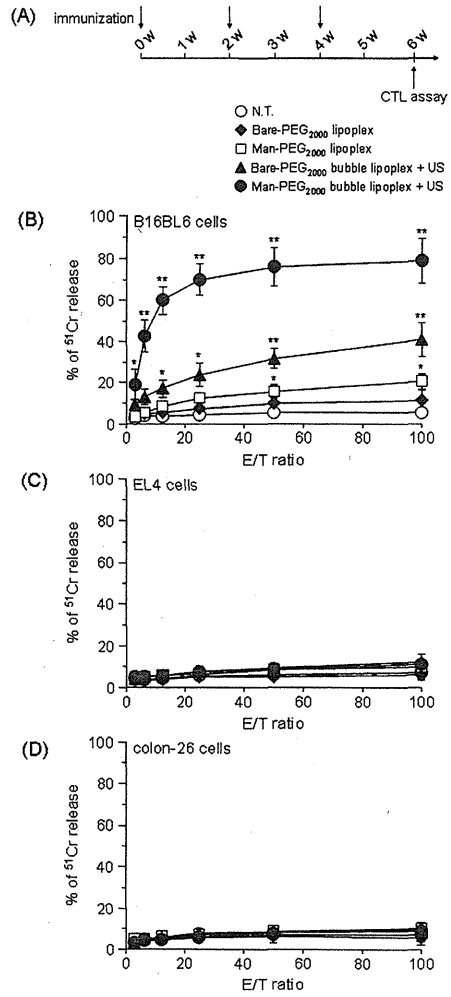

Figure 3. Evaluation of melanoma-specific CTL activities by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of immunization for the assay of melanoma-specific CTL activities. (B–D) Each cancer cell lysate-specific CTL activities after immunization three times with Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). The splenic cells were collected at 2 weeks after the last immunization, and then, the splenic cells were coincubated with ^{51}Cr-labeled cancer cells. CTL activities against B16BL6 cells (B), EL4 cells (C) and colon-26 cells (D) in the immunized splenic cells were determined by ^{51}Cr release assay. Each value represents the mean ± SD (n = 4). *p < 0.05; **p < 0.01, compared with the corresponding "N.T." (no treatment) group.

TRP-2 expression in each tumor used in this study, and confirmed that the expression of gp100 and TRP-2 was only detected in B16BL6 tumor (Supplementary Figure 2 in the Supporting Information). Following investigation of cancer vaccine effects against solid tumors according to the protocol shown in Figure 4A, B16BL6-transplanted tumor growth was significantly suppressed in mice immunized with Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure (Figures 4B and 4D). Moreover, the survival of B16BL6-transplanted mice was significantly prolonged by DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure, and complete tumor-rejection was observed in 7/10 of B16BL6-transplanted mice (Figure 4C). These vaccine effects were obtained against B16F1-transplanted mice (Supplementary Figure 4B in the Supporting Information); on the other hand, no cancer vaccine effects against EL4 and colon-26 cell-derived tumors, which do not express gp100 and TRP-2, were observed in all groups (Figures 4B–D). In addition, these DNA vaccine effects against B16BL6-derived tumors were not observed in mice immunized by Man-PEG_{2000} bubble lipoplexes constructed with pcDNA3.1 (control vector) and US exposure (Supplementary Figure 3 in the Supporting Information), suggesting that DNA vaccine effects against melanoma are attributed to not pDNA transfer itself but melanoma-related antigens expressed by pUb-M.

Then, we investigated the cancer vaccine effects against a pulmonary metastatic tumor obtained by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Figure 5A, the level of luciferase expression derived from B16BL6/Luc cells in the lung, which express gp100 and TRP-2 (Supplementary Figures 1 and 2 in the Supporting Information), was significantly suppressed in mice immunized by Man-PEG_{2000} bubble lipoplexes and US exposure (Figures 5B and 5D). Moreover, the survival of the pulmonary metastatic tumor model mice constructed with B16BL6 cells was significantly prolonged by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure (Figure 5C). These vaccine effects were obtained against pulmonary metastatic B16F1-derived tumor model mice (Supplementary Figure 4C in the Supporting Information); on the other hand, no therapeutic effects against colon-26 cells by DNA vaccination using this method were observed in any of the groups (Figures 5B–D).

Effect of Administration Routes of Man-PEG_{2000} Bubble Lipoplexes on Cancer Vaccine Effects. Next, we evaluated the effects of the administration routes of Man-PEG_{2000} bubble lipoplexes to obtain effective DNA vaccine effects. In this experiment, in addition to pUb-M transfer using intravenous administration of Man-PEG_{2000} bubble lipoplexes and external US exposure, we investigated the DNA vaccine effects by pUb-M transfer using intradermal and intrasplenic administration of Man-PEG_{2000} bubble lipoplexes and direct US exposure to the administration sites. In the preliminary experiments about US intensity for obtaining the highest gene expression in the spleen and skin, the optimized intensities of US exposure to the abdominal area by a probe of diameter 20 mm and to the injected sites directly by a probe of diameter 6 mm are 1.0 W/cm^2 and 4.0 W/cm^2, respectively (data not shown). Based on these investigations, we used the different US intensity depending on the probe size and US-exposed sites in this study. Following immunization against melanoma according to the protocol shown in Figure 6A, B16BL6-transplanted tumor growth was suppressed the best in mice transfected with pUb-M using intravenous injection of Man-PEG_{2000} bubble lipoplexes and external US exposure (Figure 6B). Moreover, the survival of B16BL6-transplanted mice was also prolonged the best by DNA vaccination using intravenous injection of Man-PEG_{2000} bubble lipoplexes and external US exposure (Figure 6C).

Duration of DNA Vaccine Effects by Man-PEG_{2000} Bubble Lipoplexes and US Exposure. Finally, to investigate the duration of DNA vaccine effects following pUb-M transfer using Man-PEG_{2000} bubble lipoplexes and US exposure, B16BL6 cells were retransplanted in mice in which first-transplanted tumors derived from B16BL6 cells were completely rejected by DNA

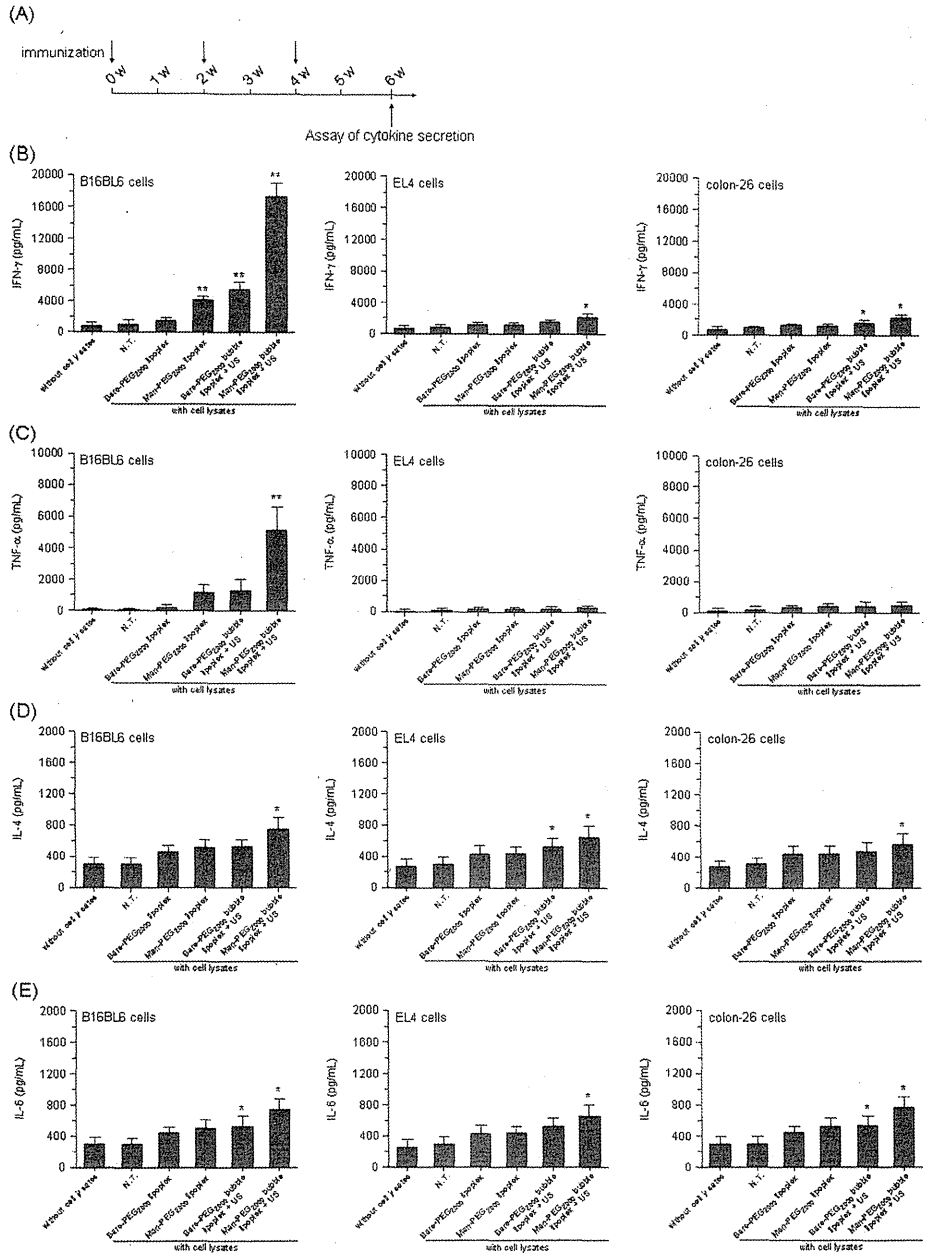

Figure 2. Melanoma-stimulatory cytokine secretion characteristics by DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure. (A) Schedule of immunization for the evaluation of melanoma-stimulatory cytokine secretion characteristics. (B−E) Each cancer cell lysate-specific IFN-γ (B), TNF-α (C), IL-4 (D) and IL-6 (E) secretion from the splenic cells immunized three times biweekly with Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). The splenic cells were collected at 2 weeks after the last immunization. After the immunized splenic cells were cultured for 72 h in the presence of each cancer cell lysate (100 μg protein), IFN-γ, TNF-α, IL-4 and IL-6 secreted in the medium were measured by ELISA. Each value represents the mean + SD ($n = 4$). $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding "without cell lysate" group.

splenic cells immunized by pUb-M. This experiment was performed according to the protocol shown in Figure 3A. As shown in Figure 3B, the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes and US exposure showed the highest CTL activities of all groups stimulated by B16BL6 cells. In contrast, no CTL activity was obtained in all groups stimulated by EL4 and colon-26 cells (Figures 3C and 3D). These results suggest that melanoma-specific CTLs are induced effectively in the splenic cells transfected pUb-M by Man-PEG_{2000} bubble lipoplexes and US exposure.

Cancer Vaccine Effects against Melanoma-Derived Solid and Metastatic Tumors by DNA Vaccination Using Man-PEG_{2000} Bubble Lipoplexes and US Exposure. Cancer vaccine effects against solid and metastatic tumors obtained by DNA vaccination using Man-PEG_{2000} lipoplexes and US exposure were examined. First, we evaluated the level of gp100 and

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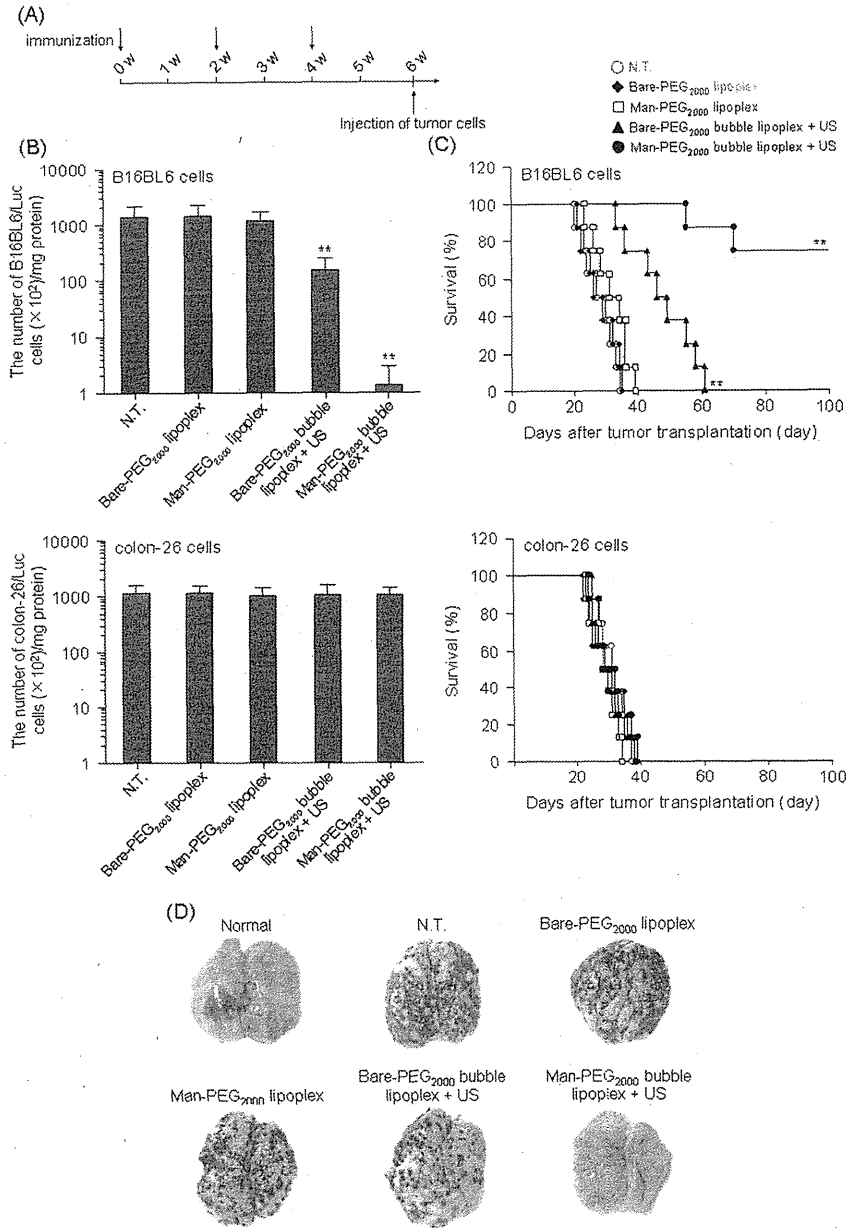

Figure 5. Cancer vaccine effects against pulmonary metastatic tumors by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments involving pulmonary metastatic tumors. (B, C) The suppressing effects of pulmonary metastatic tumors (B) and the prolonging of survival (C) by DNA vaccination using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg of pDNA). Two weeks after the last immunization, B16BL6/Luc and colon-26/Luc cells (for the evaluation of tumor metastasis) and B16BL6 and colon-26 cells (for the evaluation of survival) were injected intravenously (1×10^5 cells) into mice. The pulmonary metastatic tumors at 14 days after the tumor injection were evaluated by the luciferase activity (n = 5, each value represents the mean ± SD), and the survival was monitored up to 100 days after the tumor injection (n = 8). **$p < 0.01$, compared with the corresponding "N.T." (no treatment) group. (D) Photograph of a B16BL6-derived pulmonary metastatic tumor at 14 days after the tumor injection in mice immunized by each transfection method.

were sustained for at least 100 days against both solid and metastatic tumors.

DISCUSSION

The prognosis is poor for patients with melanoma, who exhibit a high rate of metastasis and relapse; therefore, the development of therapy for suppressing this melanoma metastasis and relapse is required.[2,3] It has been reported that DNA vaccination is effective for the prevention of metastasis and relapse,[5,7,8] and especially the application of DNA vaccination against melanoma has been focused since the identification of cancer antigens such as gp100, MART-1 and TRP is proceeding in melanoma.[10−13]

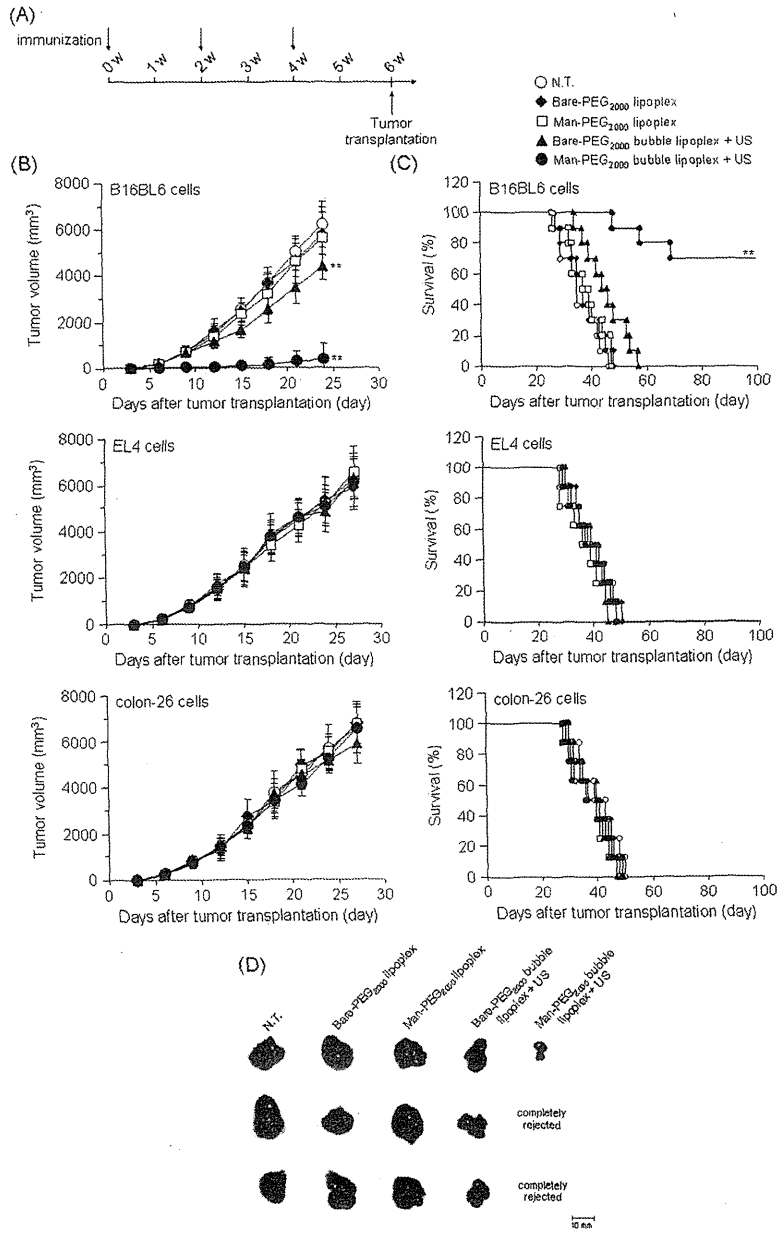

Figure 4. Cancer vaccine effects against solid tumors by DNA vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments on solid tumors. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging effects of survival in tumor-transplanted mice (C) by DNA vaccination using Bare-PEG$_{2000}$ lipoplexes, Man-PEG$_{2000}$ lipoplexes, Bare-PEG$_{2000}$ bubble lipoplexes with US exposure and Man-PEG$_{2000}$ bubble lipoplexes with US exposure (50 μg of pDNA). Two weeks after the last immunization, B16BL6 cells, EL4 cells and colon-26 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8-10$). The tumor volume was evaluated (each value represents the mean $\pm$ SD), and the survival was monitored up to 100 days after the tumor transplantation. $^{**}p < 0.01$, compared with the corresponding "N.T." (no treatment) group. (D) Photograph of a B16BL6 cell-derived solid tumor at 15 days after the tumor transplantation in mice immunized by each transfection method ($n = 3$).

vaccination using Man-PEG$_{2000}$ bubble lipoplexes and US exposure at 100 days after the first transplantation (Figure 7A). As shown in Figure 7B, compared with N.T. mice, the second-transplanted tumor growth derived from B16BL6 cells was markedly suppressed and the survival of B16BL6-transplanted mice was significantly prolonged. In addition, we also evaluated the duration of DNA vaccine effects against a pulmonary metastatic tumor. Following intravenous injection of B16BL6/Luc cells into mice at 100 days after the last immunization (Figure 7C), the level of luciferase expression derived from B16BL6/Luc cells in the lung was significantly suppressed and the survival of pulmonary metastatic tumor model mice constructed with B16BL6 cells was significantly prolonged in mice transfected with pUb-M using Man-PEG$_{2000}$ bubble lipoplexes and US exposure (Figure 7D). These results suggest that DNA vaccine effects by pUb-M transfer using Man-PEG$_{2000}$ bubble lipoplexes and US exposure

the spleen was obtained by gene transfer using intravenous injection of Man-PEG_{2000} bubble lipoplexes and external US exposure (Figures 1A and 1B). Moreover, this gene expression was obtained selectively in the splenic $CD11c^+$ cells, known as dendritic cells[46] (Figure 1C), and these findings corresponded to those in our previous reports of using firefly luciferase-encoding pDNA.[33] In our previous report using pCMV-Luc and pCMV-OVA, we showed that the enhanced gene expression in the spleen was obtained by gene transfer using Man-PEG_{2000} bubble lipoplexes and US exposure, and not observed in gene transfer using Man-PEG_{2000} lipoplexes or Man-PEG_{2000} bubble lipoplexes only and Man-PEG_{2000} lipoplexes with US exposure.[33] These observations suggest that splenic dendritic cell-selective and -efficient expression of melanoma-related antigens can be specifically obtained by the gene transfer using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure.

To achieve potent therapeutic effects by DNA vaccination against cancer, the activation of Th1 immunity and the effective induction of CTLs with high antitumor activities are important.[47] The antigen presentation on MHC class I molecules is essential for efficient CTL induction.[6,8,9] Antigens function as endogenous antigens since the cancer antigens are expressed intracellularly in DNA vaccination; consequently, the antigens are presented on MHC class I molecules.[8] As shown in Figure 2, the enhanced secretion of Th1 cytokines (IFN-γ and TNF-α) was observed in the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure by addition of B16BL6 cell lysates, compared with that of Th2 cytokines (IL-4 and IL-6). Moreover, the effective induction of CTLs against B16BL6 cells was also observed by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure (Figure 3). Recently, we have reported that the antigen presentation on MHC class I molecules was also observed in DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with OVA-encoding pDNA.[33] These results suggest that antigen presentation of melanoma antigens on MHC class I molecules is responsible for the enhanced secretion of Th1 cytokines stimulated by B16BL6 cell and the induction of CTLs against B16BL6 cells in this study. As shown in Figure 4, the growth of B16BL6 cell-derived tumors was suppressed and the survival of B16BL6 cell-transplanted mice was prolonged by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Since the prognosis of patients with melanoma is poor, because of the high metastatic properties of melanoma as mentioned above,[2,3] we also investigated the vaccine effects against metastatic melanoma by DNA vaccination using this method. As shown in Figure 5, B16BL6 cell-derived pulmonary metastasis constructed by intravenous injection of tumor cells was suppressed by DNA vaccination using this gene transfection method. These DNA vaccine effects followed by Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure were obtained against not only B16BL6 cells but also B16F1-derived tumors (Supplementary Figure 4 in the Supporting Information), suggesting that this DNA vaccination might be potent against various types of melanoma. On the other hand, the potent therapeutic effects against B16BL6-derived solid and metastatic tumor transplanted mice were not observed in DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M and US exposure (data not shown). These findings suggest that the optimized duration for immunization is essential for obtaining potent antitumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure, and DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure may be suitable for the prevention of cancer metastasis and relapse. In addition, these vaccine effects against solid and metastatic tumors were sustained for at least 100 days (Figure 7). These observations lead us to believe that the enhanced secretion of Th1 cytokines and the induction of B16BL6 cell-specific CTLs contribute to the effective and long-term DNA vaccine effects against solid and metastatic tumors, following pUb-M transfer into splenic dendritic cells using Man-PEG_{2000} bubble lipoplexes and US exposure.

Intradermal and intrasplenic routes are widely used to transfer pDNA into the Langerhans cells known as dendritic cells in the skin or splenic dendritic cells.[48,49] On the other hand, we obtained potent therapeutic effects by DNA vaccination using intravenous administration of Man-PEG_{2000} bubble lipoplexes and external US exposure in this study. As shown in Figure 6, the DNA vaccine effects obtained by immunization using intravenous administration of Man-PEG_{2000} bubble lipoplexes were higher, compared with that using intradermal and intrasplenic administration. In the gene transfer using intradermal/intrasplenic administration of Man-PEG_{2000} bubble lipoplexes, it is assumed that the diffusibility of Man-PEG_{2000} bubble lipoplexes is not good and the delivering efficiency to the dendritic cells may be low, because of the large particle size of Man-PEG_{2000} bubble lipoplexes (approximately 500 nm (Table 1)). Therefore, it may be difficult to deliver the antigen-encoding pDNA into a large number of dendritic cells in the gene transfection process using intradermal/intrasplenic administration of Man-PEG_{2000} bubble lipoplexes. On the other hand, when Man-PEG_{2000} bubble lipoplexes were administered intravenously, the antigen-encoding pDNA may be delivered into a large number of dendritic cells widely distributed in the spleen through the blood vessels. Therefore, it is assumed that potent vaccine effects are obtained by gene transfer in the dendritic cells widely distributed in the spleen. These results suggest that the intravenous administration of Man-PEG_{2000} bubble lipoplexes is suitable to obtain high therapeutic effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure.

In this study, melanoma-specific vaccine effects were induced by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure, and moreover, intravenous administration of ManPEG_{2000} bubble lipoplexes was found to be suitable for DNA vaccination using this method in mice (Figure 6). For clinical application to achieve efficient DNA vaccination, Man-PEG_{2000} bubble lipoplexes need to be delivered to the spleen efficiently at a low dose. Recently, a medical catheter, which possesses a device to inject the microbubbles and to expose US, has been developed for the treatment of thrombolysis in clinical situation.[44,45] During treatment, this catheter is positioned within the lesion sites via the vessels, and various types of drugs, such as the lytic agents and microbubbles, are infused simultaneously with US exposure. Since this system may enable the local injection of Man-PEG_{2000} bubble lipoplexes and direct US exposure to the spleen by catheter delivery via the blood vessels, more potent DNA vaccine effects against melanoma are expected to be obtained at a low dose of Man-PEG_{2000} bubble lipoplexes by applying this catheter-based US system in the future.

CONCLUSION

In the present study, we developed DNA vaccination using Man-PEG_{2000} bubble lipoplexes constructed with pUb-M encoding ubiquitylated melanoma-specific antigens (gp100 and TRP-2) and US exposure, and succeeded in obtaining potent DNA vaccine effects against solid and metastatic cancers derived from B16BL6

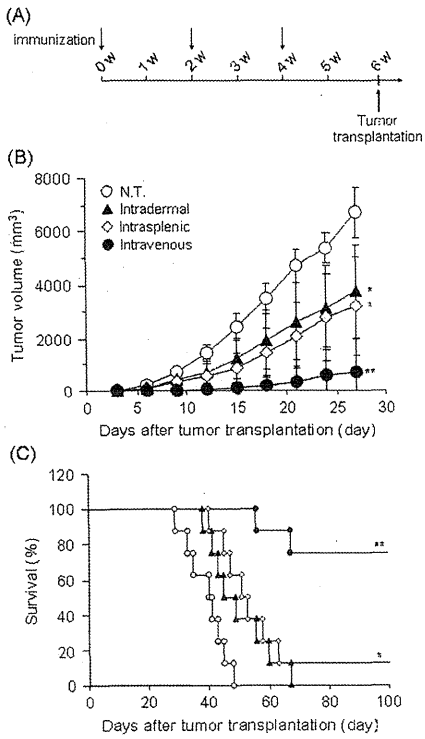

Figure 6. Effects of administration routes of Man-PEG_{2000} bubble lipoplexes on DNA vaccine effects. (A) Schedule of therapeutic experiments. (B, C) The suppressing effects of tumor growth against solid tumors (B) and the prolonging of survival in tumor-transplanted mice (C) by DNA vaccination using various administration routes of Man-PEG_{2000} bubble lipoplexes (50 μg of pDNA) and US exposure. Man-PEG_{2000} bubble lipoplexes were given by intradermal, intrasplenic and intravenous administration into mice, and US was exposed to the injected site directly or to the abdominal area externally. Two weeks after the last immunization, B16BL6 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8$). The tumor volume was evaluated (each value represents the mean $\pm$ SD), and the survival was monitored up to 100 days after the tumor transplantation. $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

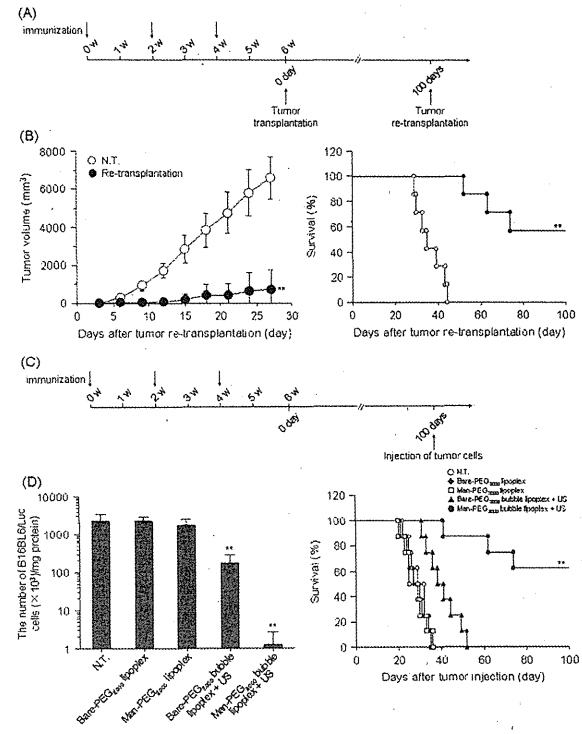

Figure 7. Duration of DNA vaccine effects by Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of therapeutic experiments against solid tumors. At 100 days after first transplantation of B16BL6 cells into mice immunized three times by Man-PEG_{2000} bubble lipoplexes and US exposure, B16BL6 cells (1×10^6 cells) were retransplanted subcutaneously into the back of mice who completely rejected the first transplanted tumors ($n = 7$). (B) The suppressing effects of tumor growth against solid tumors and the prolonging of survival in tumor-transplanted mice by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure (50 μg of pDNA). The tumor volume was evaluated (each value represents the mean $\pm$ SD), and the survival was monitored up to 100 days after the tumor retransplantation. (C) Schedule of therapeutic experiments against metastatic tumors. At 100 days after the last immunization using Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA), B16BL6 cells (for the evaluation of tumor metastasis) and B16BL6 cells (for the evaluation of survival days) were injected intravenously (1×10^5 cells) into mice. (D) The suppressing effects of B16BL6 cell-derived pulmonary metastatic tumors and the prolonging of survival by DNA vaccination using Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg of pDNA). The pulmonary metastatic tumors at 14 days after the injection of B16BL6 cells were evaluated by the luciferase activity ($n = 4$), and the survival was monitored up to 100 days ($n = 8$). $^{**}p < 0.01$, compared with the corresponding "N.T." (no treatment) group.

On the other hand, it is essential to transfer effectively into APCs such as dendritic cells to obtain potent therapeutic effects by DNA vaccination.[14,15] In the present study, we applied an APC-selective and -efficient gene transfection method using Man-PEG_{2000} bubble lipoplexes constructed with gp100 and TRP-2-encoding pDNA and US exposure to DNA vaccination against melanoma with metastatic and relapsed properties.

The delivery of antigen-encoding gene into the dendritic cells, known as a major target cells for cancer immunotherapy, is necessary to achieve potent therapeutic effects with DNA vaccination.[14,15] However, it seems that the number of dendritic cells distributed in organs, such as spleen and skin, is low for DNA vaccination.[38] Moreover, gene transfection efficiency in dendritic cells is low,[20] because dendritic cells are poorly dividing cells[39,40] and immune effector cells are highly sensitive to cationic lipids.[41] To overcome these obstacles, gene transfection methods using external physical stimulation, such as electropolation, hydrodynamic injection and sonoporation, have been investigated for cancer vaccination.[21−25] In particular, sonoporation methods using microbubbles and US exposure are expected to be suitable as a gene transfection method for DNA vaccination in clinical situation, because microbubbles and US exposure systems have been used for diagnostic imaging[42,43] and calculus fragmentation[44,45] in clinical situation. We have developed a gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure, and succeeded in obtaining APC-selective and -efficient gene expression following experiments using luciferase-encoding pDNA.[33] In this study using pUb-M which expresses melanoma-related antigens (gp100 and TRP-2), a high level of expression in

dx.doi.org/10.1021/mp100369n |*Mol. Pharmaceutics* 2011, 8, 543–554

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melanoma specifically. Moreover, its vaccine effects against melanoma were sustained for 100 days at least. The findings obtained in this study suggest that the gene transfection method using Man-PEG$_{2000}$ bubble lipoplexes and US exposure could be suitable for DNA vaccination aimed at the prevention of metastatic and relapsed cancer.

■ ASSOCIATED CONTENT

Supporting Information. Additional figures as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

The authors are very grateful to Prof. R. A. Reisfeld (Department of Immunology, The Scripps Research Institute, USA) for providing pUb-M for this research. This work was supported in part by a Grant-in-Aid for Young Scientists (A) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health and Labour Sciences Research Grants for Research on Noninvasive and Minimally Invasive Medical Devices from the Ministry of Health, Labour and Welfare of Japan, and by the Programs for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and by the Japan Society for the Promotion of Sciences (JSPS) through a JSPS Research Fellowship for Young Scientists.

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Biomaterials 32 (2011) 4659–4669

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The elucidation of gene transferring mechanism by ultrasound-responsive unmodified and mannose-modified lipoplexes

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ARTICLE INFO

Article history:
Received 1 March 2011
Accepted 7 March 2011
Available online 9 April 2011

Keywords:
Bubble lipoplex
US exposure
Gene transfer
Endocytosis
Pro-inflammatory cytokine production

ABSTRACT

The development of gene transfection methods enhancing the level of gene expression under simple and low-toxic condition is required for gene therapy in clinical. Our group has developed the ultrasound (US)-mediated gene transfection method using Man-PEG_{2000} bubble lipoplexes, which are US-responsive and mannose-modified gene carriers, and succeeded in obtaining the enhanced gene expression in mannose receptor-expressing cells selectively by the gene transfer using Man-PEG_{2000} bubble lipoplexes with US exposure *in vitro* and *in vivo*. Here, we investigated pDNA transferring mechanism followed by US exposure to unmodified and Man-PEG_{2000} bubble lipoplexes, in particular, focused on US exposure timing. Following investigation of intracellular transferring characteristics, a large amount of pDNA was transferred into the cytoplasm followed by US-mediated destruction of bubble lipoplexes in the gene transfer using both bubble lipoplexes with US exposure. Moreover, the effective gene expression was obtained without TNF-α production when US was exposed until 5 min after the addition of bubble lipoplexes. These findings suggest that the gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure enables to transfer pDNA into the cytoplasm, and optimized US exposure timing is important to achieve the high level of gene expression and the low level of pro-inflammatory cytokine production.

1. Introduction

The development of gene carriers for delivering nucleic acids into the cells is essential to obtain high therapeutic effects by gene therapy. Non-viral carriers, such as liposomes, emulsions and polymers, continue to be valuable as gene carriers, because of their safety, versatility, and simplicity of preparation [1–6]. However, since these non-viral carriers are taken up into the cells *via* endocytosis, the nucleic acids are degraded in the endosomes/lysosomes [7,8]. Therefore, the improvement of endosomal escapes of nucleic acids is important to obtain the sufficient gene expression for achieving appreciable clinical therapeutic effects in the gene transfer using non-viral carriers.

Many studies have investigated gene transfection methods using physical stimulations, such as electroporation [9,10], hydrodynamic injection [11,12], tissue pressure-mediated method [13,14] and sonoporation [15–19], to achieve a high level of tissue/cell-selective gene expression *in vitro* and *in vivo*. In particular, sonoporation methods using microbubbles and ultrasound (US) exposure are promising methods for gene therapy, since these methods have been applied to diagnosis and calculus fragmentation in clinical settings [20–23]. However, naked plasmid DNA (pDNA) is rapidly eliminated from the blood following systemic administration, *via* enzymatic degradation and uptake by Kupffer cells in mice [24]; therefore, stability of pDNA in the blood and control of the *in vivo* distribution of pDNA is required for effective *in vivo* gene transfection.

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doi:10.1016/j.biomaterials.2011.03.013

Recently, we developed unmodified and/or mannose-modified bubble liposome/pDNA complexes (unmodified-PEG_{2000} bubble lipoplexes and Man-PEG_{2000} bubble lipoplexes) for US-responsive and/or cell-selective efficient gene transfer in mice [25], and showed that this complex formation protects pDNA from enzymatic degradation by nucleases [25]. As far as the cell-selective transfection is concerned, high level of gene expression was observed in mannose receptor-expressing cells, such as macrophages [26] and dendritic cells [27], selectively following intravenous administration of Man-PEG_{2000} bubble lipoplexes with US exposure in mice [25]. Moreover, potential therapeutic effects for the suppression of tumor growth and metastasis were obtained by applying the gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure to DNA vaccination, *via* antigen-specific cytotoxic T-lymphocyte (CTL) activity in mice [25,28].

Our group previously reported that the escape of pDNA from the endosomes/lysosomes to the cytoplasm in the target cells is critical for a high level of gene expression after intravenous administration in mice [29]. In the sonoporation method using microbubbles and naked pDNA separately, the transient pores are created on the cell membrane by the cavitation energy followed by the rapid destruction of microbubbles by US exposure [30]; consequently, a large amount of extracellular nucleic acids including pDNA is directly transferred to the cytoplasm in the cultured cells, and a high level of gene expression can be obtained [17–19]. As far as the gene transfection methods using unmodified and Man-PEG_{2000} bubble lipoplexes by US exposure, pDNA are expected to transfer into the cytoplasm by the same mechanism as previously described. However, the detailed mechanism of this transfection method using unmodified and Man-PEG_{2000} bubble lipoplexes remains unclear.

In the present study, we attempted to investigate the mechanism of pDNA transfer by US exposure to unmodified and Man-PEG_{2000} bubble lipoplexes. We examined intracellular uptake using unmodified and Man-PEG_{2000} bubble lipoplexes complexed with ^{32}P or TM-rhodamine labeled pDNA, with regard to US exposure timing in primary cultured mouse peritoneal macrophages that express a high level of the mannose receptor [26]. Moreover, it has been reported that the pro-inflammatory cytokines produced, such as TNF-α and IL-12, followed by the interaction of double-strand DNA with endosomal TLR-9, affect gene expression using conventional lipoplexes [31–36]. Since gene transfection method using both bubble lipoplexes with US exposure is expected to transfer pDNA into the cytoplasm, the production of pro-inflammatory cytokines is likely to be low, because of escaped recognition of pDNA with endosomal TLR-9. Therefore, gene expression and TNF-α production as a result of gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure were investigated with particular regard to US exposure timing in primary cultured mouse peritoneal macrophages and mice.

2. Materials and methods

2.1. Materials

1,2-Distearoyl-*sn*-glycero-3-trimethylammoniumpropane (DSTAP), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (NH_2-PEG_{2000}-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and NOF (Tokyo, Japan), respectively. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and chlorpromazine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). [α-^{32}P]-dCTP and Soluene-350 were obtained from PerkinElmer (Waltham, MA, USA), and DNA polymerase I, DNase I, E. coli DNA polymerase I buffer and dNTP mixture were purchased from Takara Bio (Shiga, Japan). RPMI-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan) and fetal bovine serum (FBS) was purchased from Japan Bioserum (Hiroshima, Japan). AlexaFluor®-488 transferrin conjugate and Opti-MEM I was obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals purchased were of the highest purity available.

2.2. pDNA and mice

The pCMV-Luc was constructed in our previous reports [37]. Briefly, pCMV-Luc was constructed by subcloning the HindIII/Xba I firefly luciferase cDNA fragment obtained from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen). The pDNA was amplified in the *E. coli* strain DH5α, isolated and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN, Hilden, Germany). Female C57BL/6 mice (6-week-old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were performed in accordance with the Principles of Laboratory Animal Care as adopted and propagated by the U.S. National Institutes of Health, as well as the Guidelines for Animal Experiments of Kyoto University.

2.3. Preparation of TM-rhodamine and ^{32}P-labeled pDNA

For gene transfer studies, ^{32}P-labeled pDNA was prepared by nick translation [38]. Briefly, template pDNA, DNA polymerase I, DNase I, *E. coli* DNA polymerase I buffer, dNTP mixture and [α-^{32}P]-dCTP were mixed on ice. The mixture was incubated at 15 °C for 2 h, and heated to 70 °C for 10 min, and unincorporated ^{32}P-labeled dCTP were removed from the resultant mixture by gel filtration. The TM-rhodamine-labeled pDNA was prepared using a Label IT TM-rhodamine nucleic acid labeling kit (Mirus, Madison, WI, USA) according to the manufacturer's instructions. Briefly, pDNA was incubated with labeling reagents at 37 °C for 2 h, and pDNA was then precipitated by the addition of 100% ethanol and washed with 70% ethanol. The concentration of TM-rhodamine-labeled pDNA was measured by UV absorbance at 260 nm.

2.4. Harvesting of mouse peritoneal macrophages

Mouse peritoneal macrophages were harvested and cultured according to our previous report [37]. Briefly, the macrophages were harvested from the peritoneal cavity of female C57BL/6 mice, and then, the collected macrophages were washed and suspended in RPMI-1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine, and plated on 24-well culture plates at a density of 2×10^5 cells/1.88 cm^2. After incubation for 2 h at 37 °C in 5% CO_2, adherent cells were washed with culture medium, and the macrophages were incubated for an additional 72 h.

2.5. Construction of Man-PEG_{2000} bubble lipoplexes

Man-PEG_{2000} bubble lipoplexes were constructed according to our previous report [25]. Briefly, DSTAP, DSPC and NH_2-PEG_{2000}-DSPE or mannose-modified PEG_{2000}-DSPE were mixed in chloroform at a molar ratio of 7:2:1 to produce the liposomes for bubble lipoplexes. The mixture used for the construction of liposomes was dried by evaporation, vacuum desiccated and the resultant lipid film was resuspended in sterile 5% dextrose. After hydration for 30 min at 65 °C, the dispersion was sonicated for 10 min in a bath-type sonicator and for 3 min in a tip-type sonicator to produce liposomes. Liposomes were sterilized by passage through a 0.45 μm membrane filter (Nihon-Millipore, Tokyo, Japan). The lipoplexes were prepared by gently mixing with equal volumes of pDNA and liposome solution at a charge ratio of 1.0:2.3 (-:+). To enclose US imaging gas into lipoplexes, perfluoropropane gas (Takachiho Chemical Industries, Tokyo, Japan) was applied to prepare lipoplexes under pressured and sonicated using a bath-type sonicator (AS ONE, Osaka, Japan) for 5 min. The particle sizes and ζ-potentials of liposomes/lipoplexes were determined by a Zetasizer Nano ZS instrument (Malvern Instrument, Worcestershire, UK).

2.6. Cellular association and internalization study of pDNA

After the macrophages were collected and incubated for 72 h, cells were washed twice with Hank's balanced salt solution (HBSS, pH 7.4), and the culture medium was replaced with 500 μL Opti-MEM I containing bubble lipoplexes (5 μg pDNA) constructed with ^{32}P-labeled pDNA. At a pre-determined time after the addition of bubble lipoplexes, macrophages were exposed to US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2) for 20 s using a 6 mm diameter probe placed in the well. US was generated using a Sonopore-4000 sonicator (NEPA GENE, Chiba, Japan). Macrophages were washed five times with ice-cold HBSS and for the internalization study, the cells were washed five times with ice-cold acetate buffer (pH 4.0) in PBS buffer to remove lipoplexes bound to the cell surface at pre-determined times after the addition of bubble lipoplexes. The macrophages were solubilized with 0.3 M NaOH solution containing 10% Triton X-100, and the resultant cell lysates were dissolved in Soluene-350 (PerkinElmer). Lysates were decolorized with isopropanol and 30% H_2O_2, and neutralized with 5 N HCl. Radioactivity of ^{32}P-labeled pDNA was measured in scintillation counter (LSA-500; Beckman Coulter, CA, USA) after the addition of Clear-Sol I solution. The protein concentration was determined using a Protein Quantification Kit (Dojindo Molecular Technologies, Tokyo, Japan).

2.7. Confocal microscopy study

After the macrophages were collected and incubated for 72 h, bubble lipoplexes constructed with TM-rhodamine-labeled pDNA were added at a dose of

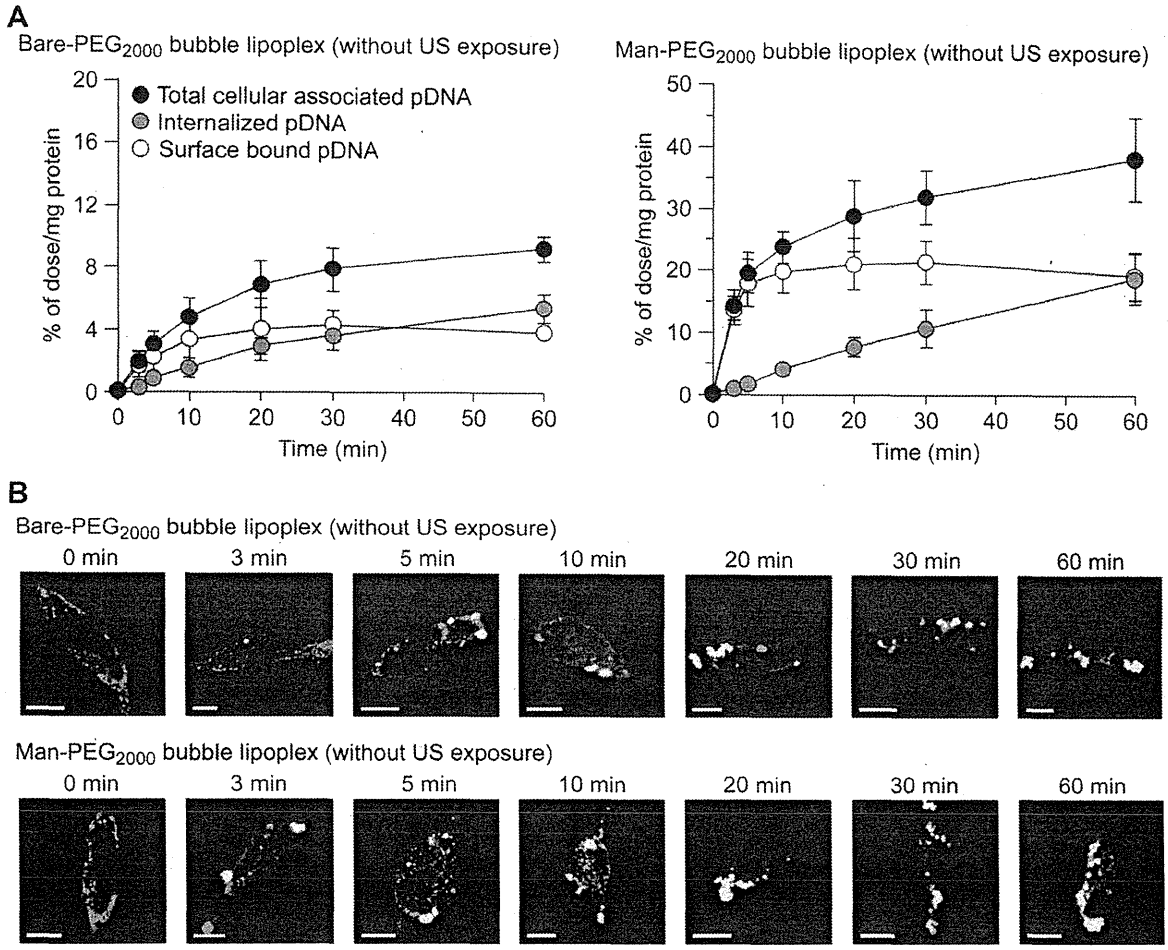

Fig. 1. Cellular association and internalization of pDNA obtained by unmodified and Man-PEG_{2000} bubble lipoplexes in cultured mouse macrophages. (A) Time-course of the cellular association and internalization of pDNA obtained by unmodified and Man-PEG_{2000} bubble lipoplexes constructed with ^{32}P-labeled pDNA (5 μg pDNA) in mouse cultured macrophages. To evaluate the amount of internalized pDNA, macrophages were washed with ice-cold acetate buffer (pH 4.0) in PBS to remove lipoplexes bound to the cell surface at pre-determined times after the addition of bubble lipoplexes. The amount of surface bound pDNA was determined by subtracting the amount of internalized pDNA from the amount of associated pDNA. Each value represents the mean ± SD ($n = 3$). (B) *In vitro* confocal images of cellular associated pDNA obtained by unmodified and Man-PEG_{2000} bubble lipoplexes (5 μg pDNA) at pre-determined times after the addition of bubble lipoplexes. The pDNA complexed with each bubble liposome and the endosomes of macrophages were labeled with TM-rhodamine (red) and AlexaFluor®-488 transferrin conjugates (green), respectively. Nuclei were counterstained by DAPI (blue). Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5 μg pDNA. At pre-determined time after the addition of bubble lipoplexes, macrophages were exposed to US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2) for 20 s using a 6 mm diameter probe placed in the well. Endosomes were labeled with 100 μg/mL AlexaFluor®-488 transferrin conjugates before addition of lipoplexes or after US exposure. Macrophages were then washed five times with ice-cold PBS, and fixed with 4% paraformaldehyde. After washing twice with ice-cold PBS, cover glasses were mounted on slide glasses with VECTASHIELD® mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were observed using a Fluoview® FV10i confocal laser microscope (Olympus, Tokyo, Japan).

2.8. *In vitro gene transfer*

After the macrophages were collected and incubated for 72 h, the culture medium was replaced with Opti-MEM I containing bubble lipoplexes (5 μg pDNA). Macrophages were exposed to US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm^2) for 20 s using a 6 mm diameter probe placed in the well at pre-determined times after the addition of bubble lipoplexes. Then, at 1 h after the addition of bubble lipoplexes, the incubation medium was replaced with RPMI-1640 and incubated for an additional 23 h. After incubation for 24 h, the cells were scraped from the plates and suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris; pH 7.8). Cell suspensions were mixed and then centrifuged at 10,000 × g, 4 °C for 10 min. The supernatant was mixed with luciferase assay buffer (Picagene; Toyo Ink, Tokyo, Japan) and the luciferase activity was measured by a luminometer (Lumat LB 9507; EG&G Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to the protein content of cells. Protein concentration was determined using a Protein Quantification Kit (Dojindo Molecular Technologies).

2.9. *In vivo gene transfer*

Six-week-old C57BL/6 female mice were intravenously injected with 400 μL bubble lipoplexes *via* the tail vein using a 26-gauge syringe needle at a dose of 50 μg pDNA. At pre-determined times after intravenous administration of bubble lipoplexes, US (frequency, 1.045 MHz; duty, 50%; burst rate, 10 Hz; intensity 1.0 W/cm^2; time, 2 min) was exposed transdermally to the abdominal area using a Sonopore-4000 sonicator with a probe of diameter 20 mm. At 6 h after administration, mice were sacrificed and tissues were collected. Tissues were washed twice with cold saline and homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris; pH 7.8). The lysis buffer was added at 5 mL/g for the liver or 4 mL/g for other tissues. After three cycles of freeze-and-thaw cycles, homogenates were centrifuged at 10,000 × g, 4 °C for 10 min. Luciferase activity of supernatants was determined by luciferase assay, as previously described.

2.10. *MTT assay*

The cytotoxicity was evaluated by MTT assay [39]. Briefly, at 24 h after *in vitro* gene transfection, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyltetrazolium bromide (MTT; Nacalai Tesque, Inc., Kyoto, Japan) solution was added to each well and incubated for 4 h. The resultant formazan crystals were dissolved in 0.04 M HCl-

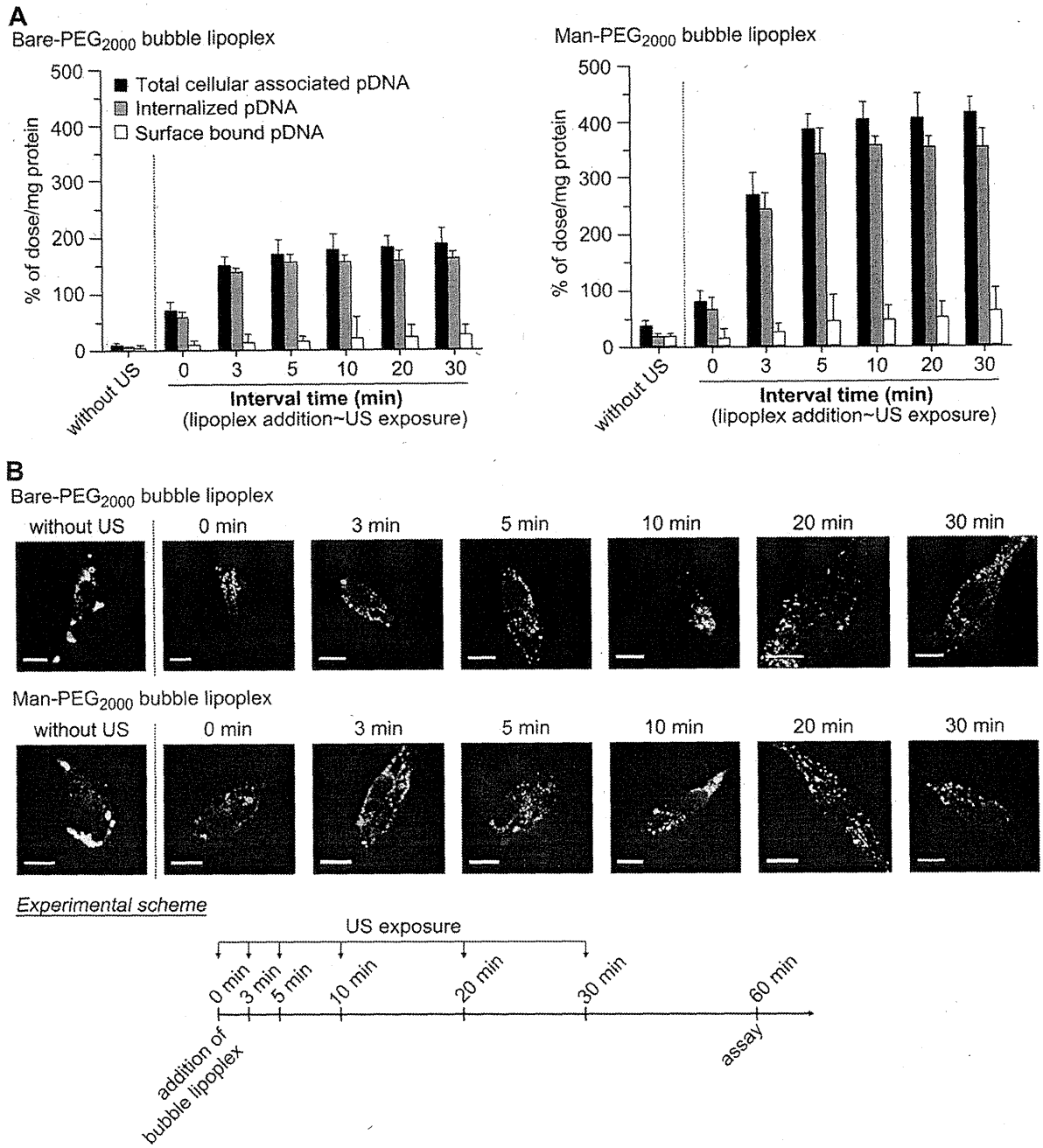

Fig. 2. The effects of US exposure timing on cellular association and internalization of pDNA obtained by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure in cultured mouse macrophages. (A) The amounts of cellular associated and internalized pDNA followed by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes (5 μg pDNA) with US exposure in the macrophages, when US was exposed at 0, 3, 5, 10, 20, and 30 min after the addition of bubble lipoplexes. Unmodified and Man-PEG_{2000} bubble lipoplexes were constructed with ^{32}P-labeled pDNA. Each value represents the mean ± SD ($n = 3$). (B) *In-vitro* confocal images of cellular associated pDNA obtained by unmodified and Man-PEG_{2000} lipoplexes (5 μg pDNA) with US exposure at 60 min after the addition of bubble lipoplexes. US was exposed at 0, 3, 5, 10, 20, and 30 min after the addition of bubble lipoplexes. The pDNA complexed with each bubble liposome and the endosomes of macrophages were labeled with TM-rhodamine (red) and AlexaFluor®-488 transferrin conjugates (green), respectively. Nuclei were counterstained by DAPI (blue). Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isopropanol and sonicated for 10 min in a bath-type sonicator. Absorbance values at 550 nm (test wavelength) and 655 nm (reference wavelength) were measured and the results were expressed as viability (%).

2.11. Measurement of secreted TNF-α

At 3 h after in vitro gene transfection, supernatants were collected, and TNF-α levels in the supernatants were determined by the commercial ELISA Kit (Bay bioscience, Hyogo, Japan) according to recommended procedures. At 3 h after *in-vivo* gene transfer, tissues were collected, washed twice with cold saline, and stored at −80 °C until ELISA was performed. Tissues were thawed on ice and were then homogenized in PBS containing a cocktail of protease inhibitors (aprotinin; 2.0 μg/mL, leupeptin; 1.0 μg/mL, Pepstatin A; 1.0 μg/mL and PMSF; 100 μg/mL). The cytoplasmic fractions were isolated as the supernatant after centrifugation at 10,000 × g, 4 °C for 10 min. TNF-α levels containing these fractions were immediately analyzed for cytokines by ELISA.

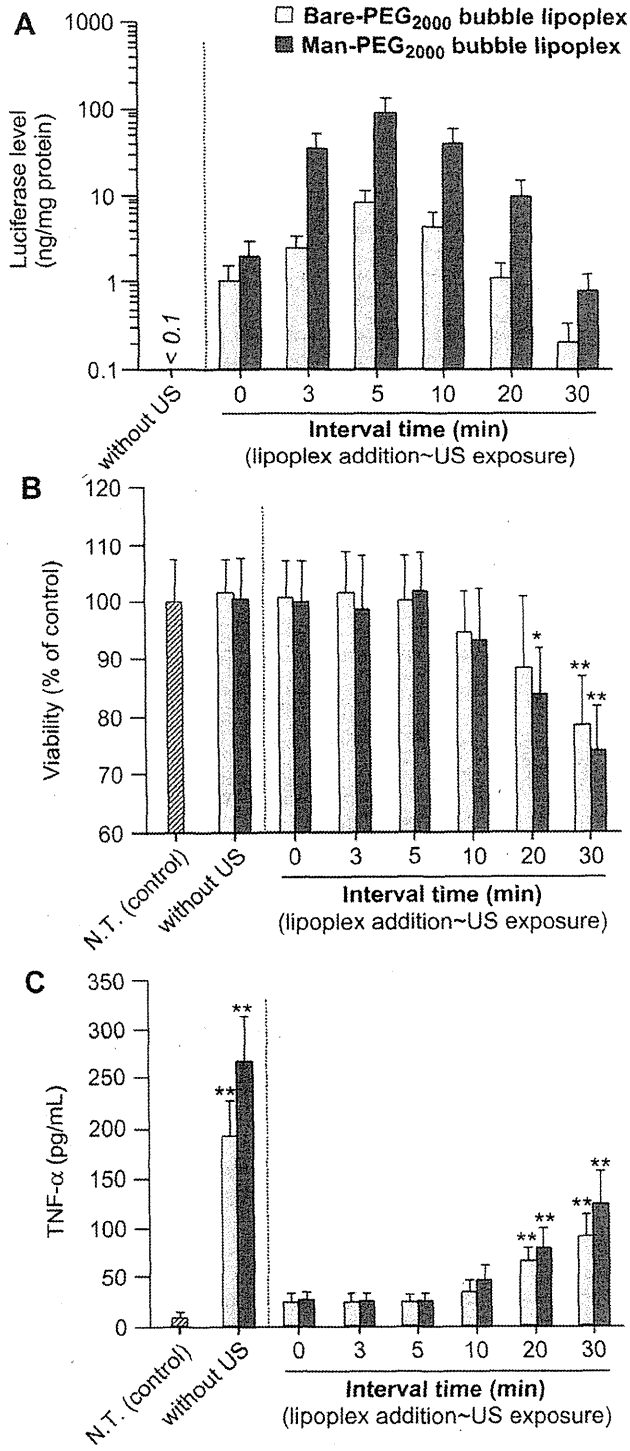

Fig. 3. The effects of US exposure timing on gene expression, cytotoxicity and TNF-α production followed by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure in cultured mouse macrophages. (A,B) The level of luciferase expression (A) and cell viability (B) followed by unmodified and Man-PEG_{2000} bubble lipoplexes (5 μg pDNA) with US exposure at 24 h after transfection in mouse cultured macrophages, when US was exposed at 0, 3, 5, 10, 20, and 30 min after the addition of bubble lipoplexes. (C) The level of TNF-α production in the supernatants followed by unmodified and Man-PEG_{2000} bubble lipoplexes (5 μg pDNA) with US exposure at 3 h after transfection, when US was exposed at 0, 3, 5, 10, 20, and 30 min after the addition of bubble lipoplexes. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T.(control). N.T., no-treatment. Each value represents the mean + SD ($n = 4$).

2.12. Inhibitory experiments of endocytosis and signaling via TLR-9

Endocytosis was inhibited by chlorpromazine (50 μM) as a clathrin-mediated endocytosis inhibitor [40]. Chlorpromazine was added to the macrophages at 30 min before the addition of lipoplexes or bubble lipoplexes. TLR-9 signaling was inhibited by iODN-2088 (Enzo Life Sciences, Plymouth Meeting, PA, USA) [41]. The iODN-2088 was added to the macrophages at a final concentration of 2 μM at 30 min before the addition of bubble lipoplexes.

2.13. Statistical analyses

Results were presented as the mean ± S.D. of greater than three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Two-group comparisons were performed by Student's *t*-test. Multiple comparisons between control groups and other groups were performed using Dunnett's test and multiple comparisons between all groups were performed by Tukey–Kramer test.

3. Results

3.1. Physicochemical properties of unmodified and Man-PEG_{2000} bubble lipoplexes

To investigate the physicochemical properties of unmodified and Man-PEG_{2000} bubble lipoplexes used in all experiments, their particle sizes and ζ-potentials were evaluated. The mean particle sizes and ζ-potentials of unmodified and Man-PEG_{2000} lipoplexes were 137 ± 13 nm, 46.1 ± 4.8 mV and 139 ± 15 nm, 47.2 ± 4.0 mV, respectively. Following enclosure of US imaging gas (perfluoropropane gas) into lipoplexes, the mean particle sizes and ζ-potentials of unmodified and Man-PEG_{2000} bubble lipoplexes were 550 ± 18 nm, 47.9 ± 4.8 mV and 551 ± 15 nm, 47.1 ± 6.6 mV, respectively. The physicochemical properties of lipoplexes and bubble lipoplexes used in this study are consistent with our previous reports [25,28].

3.2. Time-dependent cellular association and internalization of pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes in cultured mouse macrophages

To characterize the time-dependent cellular association and internalization of pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes in cultured mouse macrophages, cells were subjected to unmodified and Man-PEG_{2000} bubble lipoplexes constructed with ^{32}P-labeled pDNA. As shown in Fig. 1A, the amounts of cell-associated pDNA obtained by both bubble lipoplexes were increased time-dependently. Moreover, the amount of cell-associated pDNA obtained by Man-PEG_{2000} bubble lipoplexes was significantly higher than that obtained by unmodified-PEG_{2000} bubble lipoplexes in cultured mouse macrophages expressing mannose receptors [26] (Fig. 1A). These values are consistent with our previous studies obtained by unmodified and Man-PEG_{2000} lipoplexes that are not enclosed US imaging gas [25]. Then, to determine the amount of surface bound and internalized pDNA in mouse cultured macrophages, cells were washed with acetate buffer (pH 4.0) in PBS. The amount of internalized pDNA obtained by both bubble lipoplexes were increased in a time-dependent manner (Fig. 1A). However, the amount of surface bound pDNA obtained by both bubble lipoplexes reached the plateau at 10 min after the addition of bubble lipoplexes (Fig. 1A). In addition, confocal microscopy images of the cell-associated pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes at pre-determined times after the addition of bubble lipoplexes is shown in Fig. 1B. Since pDNA was co-localized with endosomes (Fig. 1B), it is suggested that pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes is taken up into the cells *via* endocytosis in a time-dependent manner.

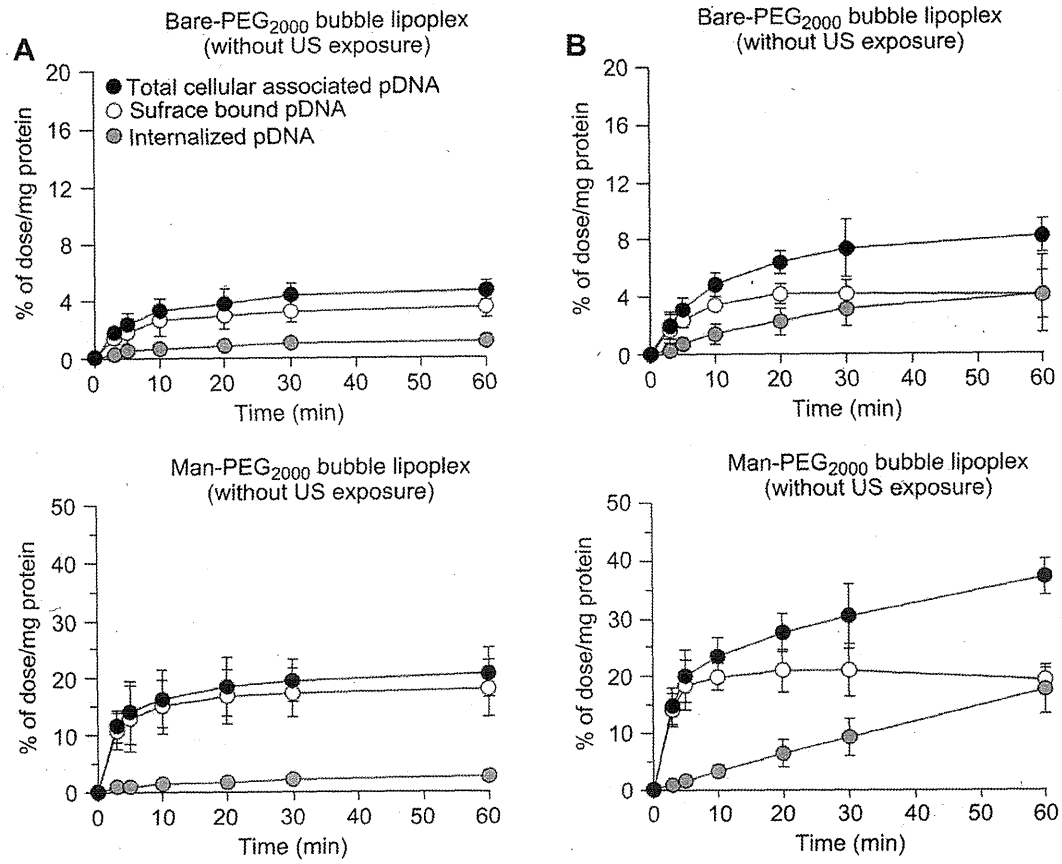

Fig. 4. The inhibitory effects of endocytosis and TLR-9 signaling on the cellular association and internalization cellular association and internalization of pDNA obtained by unmodified and Man-PEG_{2000} bubble lipoplexes. Time-course of the cellular association and internalization of pDNA obtained by unmodified and Man-PEG_{2000} bubble lipoplexes constructed with ^{32}P-labeled pDNA (5 μg pDNA) in the presence of (A) 50 μM chlorpromazine (an inhibitor of clathrin-mediated endocytosis) or (B) 2 μM iODN-2088 (an inhibitor of TLR-9 signaling). Each value represents the mean ± SD ($n = 3$).

3.3. Effects of US exposure timing on cellular association and internalization of pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure in mouse cultured macrophages

The effects of US exposure timing on cellular association and internalization of pDNA were investigated in the gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure in cultured mouse macrophages. As shown in Fig. 2A, the amount of cell-associated pDNA obtained by both bubble lipoplexes and US exposure was significantly higher than that obtained by both bubble lipoplexes only. Moreover, the amount of cell-associated pDNA obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was higher than that obtained by unmodified-PEG_{2000} bubble lipoplexes and US exposure in cultured mouse macrophages expressing mannose receptors [26] (Fig. 2A). In addition, the amount of cell-associated pDNA obtained by both bubble lipoplexes and US exposure was the highest when US was exposed at over 5 min after the addition of bubble lipoplexes (Fig. 2A). Following the evaluation of the amount of surface bound and internalized pDNA, the majority of pDNA delivered by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure was transferred into cells (Fig. 2A). As shown in Fig. 2B, following observation of the confocal microscopy images of cellular association of pDNA, pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure was not co-localized with endosomes. This observation suggests that pDNA is directly introduced into the cells not-mediated by endocytosis, or the endosomal escape of pDNA taken up into the cells *via* endocytosis is enhanced by sonoporation in gene transfer using bubble lipoplexes and US exposure.

3.4. Effects of US exposure timing on gene expression, cytotoxicity, and TNF-α production by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes and US exposure in cultured mouse macrophages

The effects of US exposure timing on the gene expression by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure were investigated in cultured mouse macrophages. As shown in Fig. 3A, the level of gene expression obtained by both bubble lipoplexes and US exposure was markedly higher than that obtained by bubble lipoplexes only, and the level of gene expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was higher than that obtained by unmodified-PEG_{2000} bubble lipoplexes and US exposure in cultured mouse macrophages expressing mannose receptors [26]. Moreover, the level of gene expression obtained by both bubble lipoplexes and US exposure was highest when US was exposed at 5 min after the addition of bubble lipoplexes (Fig. 3A).

We next evaluated the effects of US exposure timing on the cytotoxicity and TNF-α production followed by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure.

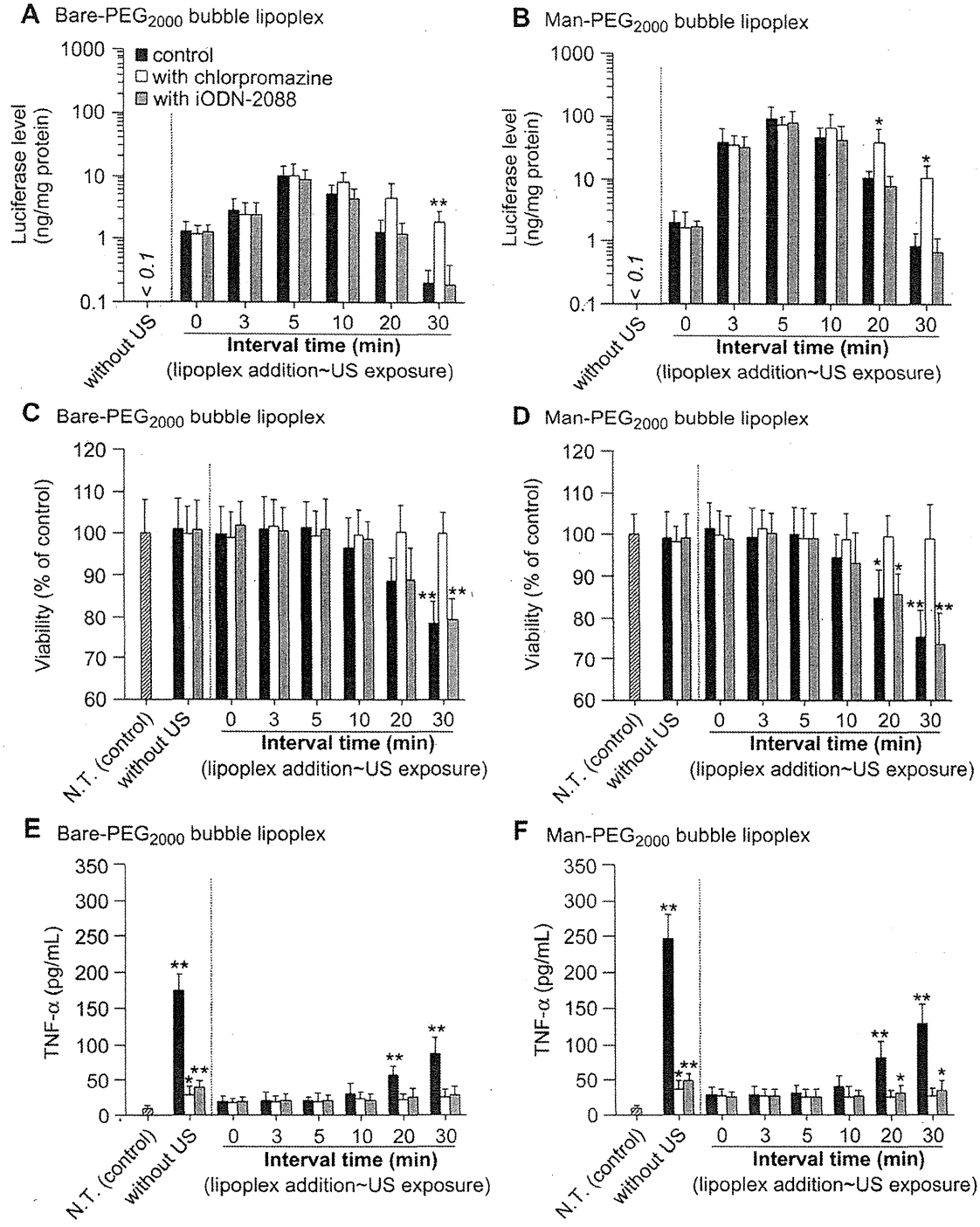

Fig. 5. The inhibitory effects of endocytosis and TLR-9 signaling on gene expression, viability, and TNF-α production followed by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure. (A,B) The level of luciferase expression followed by unmodified (A) and Man-PEG_{2000} bubble lipoplexes (B) (5 μg pDNA) with US exposure at 24 h after transfection in mouse cultured macrophages. (C,D) The viability followed by unmodified (C) and Man-PEG_{2000} bubble lipoplexes (D) (5 μg pDNA) with US exposure at 24 h after transfection in mouse cultured macrophages. (E,F) The level of TNF-α production in the supernatants followed by unmodified (E) and Man-PEG_{2000} bubble lipoplexes (F) (5 μg pDNA) with US exposure at 3 h after transfection. US was exposed at 0, 3, 5, 10, 20, and 30 min after the addition of unmodified and Man-PEG_{2000} bubble lipoplexes. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T.(control). N.T., no-treatment. Each value represents the mean + SD ($n = 4$).

As shown in Fig. 3B, when US was exposed at over 10 min after the addition of both bubble lipoplexes, the viability gradually decreased depending on the intervals from the addition of bubble lipoplexes to US exposure. The level of TNF-α production followed by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure was lower than that resulting from gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes only (Fig. 3C). Moreover, the level of TNF-α secretion was gradually increased depending on the intervals from the addition of bubble lipoplexes and US exposure, when US was exposed at over

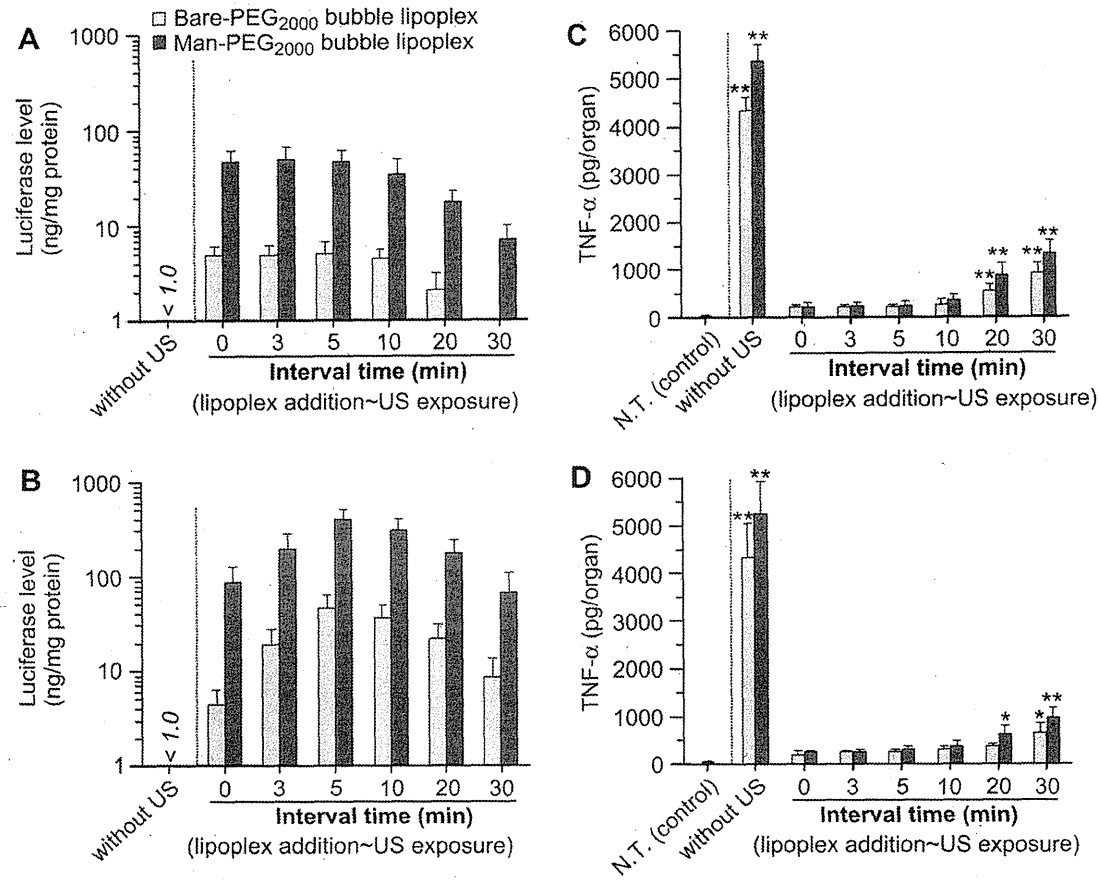

Fig. 6. The effects of US exposure timing on gene expression and TNF-α production followed by unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure in mice. The level of luciferase expression (A,B) and TNF-α production (C,D) in the liver (A,C) and spleen (B,D) followed by unmodified and Man-PEG_{2000} bubble lipoplexes (50 μg pDNA) with US exposure at 6 h after transfection in mice, when US was exposed at 0, 3, 5, 10, 20, and 30 min after intravenous administration of bubble lipoplexes. $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T.(control). N.T., no-treatment. Each value represents the mean + SD ($n = 4$).

10 min after the addition of both bubble lipoplexes (Fig. 3C). The decrease in gene expression and the increase in cytotoxicity and TNF-α production correlated with the amount of internalized pDNA *via* endocytosis (Fig. 1). Therefore, these observations suggest that the sites where the destruction of bubble lipoplexes are induced followed by US exposure, such as on the cell surface or in the endosome, are affected to the level of gene expression, cytotoxicity and TNF-α production followed by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure.

3.5. Time-dependent cellular association and internalization of pDNA transferred by unmodified and Man-PEG_{2000} bubble lipoplexes under inhibitory condition of endocytosis and TLR-9 signaling

The inhibitory effects of endocytosis and TLR-9 signaling on time-dependent cellular association and internalization of pDNA delivered by unmodified and Man-PEG_{2000} bubble lipoplexes were evaluated. First, to investigate the effects of endocytosis inhibition on intracellular uptake of pDNA, macrophages were pre-treated with chlorpromazine, a clathrin-mediated endocytosis inhibitor [40], and then subjected to unmodified and Man-PEG_{2000} bubble lipoplexes constructed with ^{32}P-labeled pDNA. As shown in Fig. 4A, the amount of surface bound pDNA obtained by both bubble lipoplexes was similar to the cellular associated pDNA, and pDNA transferred by both bubble lipoplexes was not internalized in the presence of chlorpromazine (Fig. 4A). Then, to investigate the effects of TLR-9 signaling inhibition on intracellular uptake of pDNA, macrophages were pre-treated with iODN-2088, a TLR-9 signaling inhibitor [41]. Following investigation of intracellular uptake profiles, the cellular associated, surface bound and internalized profiles of pDNA transferred by both bubble lipoplexes in the presence of iODN-2088 (Fig. 4B) were similar to those performed in the absence of iODN-2088 (Fig. 1A).

3.6. Effects of US exposure timing on gene expression, cytotoxicity, and TNF-α production by gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes and US exposure with inhibited endocytosis and TLR-9 signaling

The inhibitory effects of endocytosis and TLR-9 signaling on the gene expression, cytotoxicity, and TNF-α production were evaluated in the gene transfer using unmodified and Man-PEG_{2000} bubble lipoplexes with US exposure. When US was exposed at over 10 min after the addition of both bubble lipoplexes, the decreasing level of gene expression obtained by both bubble lipoplexes and US exposure was low in the presence of chlorpromazine, compared other groups (Fig. 5A and B). Moreover, as shown in Fig. 5C and D, the cytotoxicity followed by gene transfer using both bubble lipoplexes and US exposure was not observed in the presence of

chlorpromazine, when US was exposed at over 10 min after the addition of both bubble lipoplexes. In addition, the level of TNF-α production was not increased in the presence of not only chlorpromazine but also iODN-2088, when US was exposed at over 10 min after the addition of both bubble lipoplexes (Fig. 5E and F). These observations suggest that the decrease in gene expression and the increased in cytotoxicity/TNF-α production are obtained, when the degradation of bubble lipoplexes followed by US exposure is caused into the endosomes. Moreover, it is also suggested the secretion of pro-inflammatory cytokines induced by the interaction of pDNA with endosomal TLR-9 is not involved in the decrease of gene expression and cytotoxicity.

3.7. *Effects of US exposure timing on gene expression and TNF-α production by gene transfer using unmodified and Man-PEG$_{2000}$ bubble lipoplexes and US exposure in mice*

The effects of US exposure timing on the gene expression and TNF-α production in the liver and spleen were examined in the gene transfer using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. The level of gene expression in the liver obtained by both bubble lipoplexes and US exposure was highest when US was exposed until 5 min after administration of bubble lipoplexes (Fig. 6A). On the other hand, the level of gene expression in the spleen obtained by both bubble lipoplexes and US exposure was highest when US was exposed at 5 min after administration of bubble lipoplexes in the spleen (Fig. 6B). We also evaluated the effects of US exposure timing on TNF-α production followed by gene transfer using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. As shown in Fig. 6C and D, the level of TNF-α production in the liver and spleen was gradually increased when US was exposed at over 20 min after the addition of both bubble lipoplexes (Fig. 6C and D). Moreover, the effects of US-exposing timing on TNF-α production were comparable among the organs (Fig. 6C and D).

4. Discussion

The aim of this study was to determine the gene transferring characteristics and the involvement on the gene expression and pro-inflammatory cytokine production in the gene transfer using unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. First, we investigated the intracellular uptake characteristics of pDNA transferred by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with or without US exposure (Figs. 1 and 2). As shown in Fig. 1, time-dependent cellular association and internalization of ^{32}P-labeled pDNA were observed in the gene transfer using unmodified and Man-PEG$_{2000}$ bubble lipoplexes without US exposure (Fig. 1). Moreover, TM-rhodamine-labeled pDNA was co-localized with endosomes (Fig. 1B), and intracellular uptake of pDNA transferred by unmodified and Man-PEG$_{2000}$ bubble lipoplexes was suppressed by the addition of chlorpromazine, a clathrin-mediated endocytosis inhibitor (Fig. 4). These findings suggest that pDNA delivered by unmodified and Man-PEG$_{2000}$ bubble lipoplexes is taken up into the cells *via* endocytosis. Then, we investigated the intracellular uptake characteristics of pDNA transferred by unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure. As shown in Fig. 2, the amount of ^{32}P-labeled pDNA transferred by unmodified and Man-PEG$_{2000}$ bubble lipoplexes was significantly increased by US exposure (Fig. 2A), and TM-rhodamine-labeled pDNA transferred by bubble lipoplexes and US exposure was not co-localized with endosomes (Fig. 2B). Although the intracellular localization of pDNA transferred by both lipoplexes altered from the cell surface to endosomes in time-dependent manner (Fig. 1), the amount of internalized pDNA was relatively consistent when US was exposed at over 5 min after the addition of bubble lipoplexes (Fig. 2A). These results lead us to believe that pDNA is directly delivered into the cytoplasm not-mediated by endocytosis, or the endosomal escape of pDNA taken up into the cells *via* endocytosis is enhanced in the gene transfer using both unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure.

Since the production of excess pro-inflammatory cytokines could affect the hepatic toxicity [32–34,42] as well as suppression of gene expression [43,44] in the gene transfection, the evaluation of pro-inflammatory cytokines is also important from the viewpoint of carrier development. In the gene transfer using lipofection methods, it is known that the production of pro-inflammatory cytokines followed by gene transfer is induced by interaction with pDNA and endosomal TLR-9 [31–36]. On the other hand, the escape from the recognition of pDNA with endosomal TLR-9 by direct gene transfer into the cytoplasm is expected to reduce the production of pro-inflammatory cytokines in the gene transfer using bubble lipoplexes and US exposure. Therefore, to examine whether the gene transfer using both unmodified and Man-PEG$_{2000}$ bubble lipoplexes with US exposure affects the production of pro-inflammatory cytokines, the production of pro-inflammatory cytokines were evaluated. TNF-α production followed by the gene transfer using both bubble lipoplexes with US exposure was significantly lower than without US exposure, not only in primary cultured macrophages (Fig. 3) but also in mice (Fig. 6); suggesting the enhancement of endosomal escape of pDNA followed by US exposure to both bubble lipoplexes. These results support the view that the gene transfer using both unmodified and Man-PEG$_{2000}$ bubble lipoplexes can efficiently distribute pDNA into the cytoplasm followed by US exposure.

In the lipofection methods, it is reported that the cationic carriers are taken up into the cells *via* clathrin-mediated endocytosis followed by the non-specific association with the cell surface [45,46], and then, pDNA interacts with the endosomal TLR-9 [31,35]; consequently, involving in the pro-inflammatory cytokine production [32–34,36]. On the other hand, it is expected that pDNA is directly delivered into the cytoplasm and does not interact with endosomal TLR-9 when US is exposed to cell-associated bubble lipoplexes. Moreover, since it has been reported that gene expression is affected by the production of TNF-α followed by the interaction with endosomal TLR-9 [43,44], US exposure timing is assumed to be one of the major factors for achieving high gene expression and low cytokine production in the gene transfer using bubble lipoplexes and US exposure. Therefore, the effects of US exposure timing on gene expression and cytokine production were evaluated. As far as the intracellular uptake process of pDNA is concerned, we showed that following the addition of both bubble lipoplexes, much of the pDNA were associated with the cell surface at 5 min, and then, pDNA was taken up into the cells *via* endocytosis at over 10 min (Figs. 1 and 4A). Although the amount of internalized pDNA followed by US exposure was similar in all of US exposure timing (Fig. 2A), the highest gene expression was observed when US was exposed at 5 min after the addition of both bubble lipoplexes with US exposure both *in vitro* and *in vivo* experiments (Figs. 3A, 6A and B). Moreover, TNF-α production was not observed when US was exposed within 5 min after the addition of both bubble lipoplexes with US exposure both *in vitro* and *in vivo* experiments (Figs. 3C, 6C and D). On the other hand, TNF-α production was observed when US was exposed at 10, 20, and 30 min (Fig. 5C–F), suggesting that most of the bubble lipoplexes are endocytosed, and then, interacted with TLR-9 in the endosomes at over 10 min after the addition of lipoplexes. Therefore, to confirm the effect of endocytosis, the inhibitory experiment of endocytosis was performed under the same experimental conditions. As