2.16. Antigen presenting assay

The evaluation of antigen presentation on MHC class I molecules in the splenic dendritic cells was performed by in-vitro antigen presentation assay using CD8-OVA1.3 cells, which are T cell hybridomas with specificity for OVA. The $CD11c^+$ cells isolated from immunized mice were plated in a 96-well plate at various cells numbers and co-cultured with CD8-OVA1.3 cells (1×10^5) for 20 h. The antigen presentation on MHC class I molecules was evaluated by IL-2 secreted from activated CD8-OVA1.3 cells measured by a commercial IL-2 ELISA Kit (Bay bioscience Co., Ltd., Hyogo, Japan).

2.17. Evaluation of OVA-specific cytokine secretion from the splenic cells

At 2 weeks after the last immunization, the splenic cells collected from immunized mice were plated in 96-well plates and incubated for predetermined times at 37 °C in the presence or absence of OVA (100 μg). IFN-γ and IL-4 in the culture medium were measured by the commercial ELISA Kit, respectively (Bay bioscience Co., Ltd., Hyogo, Japan).

2.18. OVA-specific CTL assay

At 2 weeks after the last immunization, the splenic cells harvested from immunized mice were plated in 6-well plates and co-incubated with mitomycin C-treated E.G7-OVA cells or EL4 cells for 4 days. After co-incubation, non-adherent cells were collected, washed and plated in 96-well plates with target cells (E.G7-OVA cells or EL4 cells) at various effector/target (E/T) ratios. The target 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 resultant supernatant of 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). The percentage of OVA-specific ^{51}Cr release was calculated as (% of ^{51}Cr release from E.G7-OVA cells) − (% of ^{51}Cr release from EL4 cells).

2.19. Therapeutic effects

C57BL/6 mice were immunized three times biweekly. At 2 weeks after last immunization, E.G7-OVA cells and EL4 cells were transplanted subcutaneously into the back of mice. The tumor growth and survival of mice were monitored up to 80 days after transplantation of E.G7-OVA cells and EL4 cells.

2.20. Statistics

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.

3. Results

3.1. In-vitro gene transfection properties by Man-PEG_{2000} lipoplexes

Polyethylene-glycol (PEG) modification of particles is necessary to enclose US imaging gas stably and to prepare the

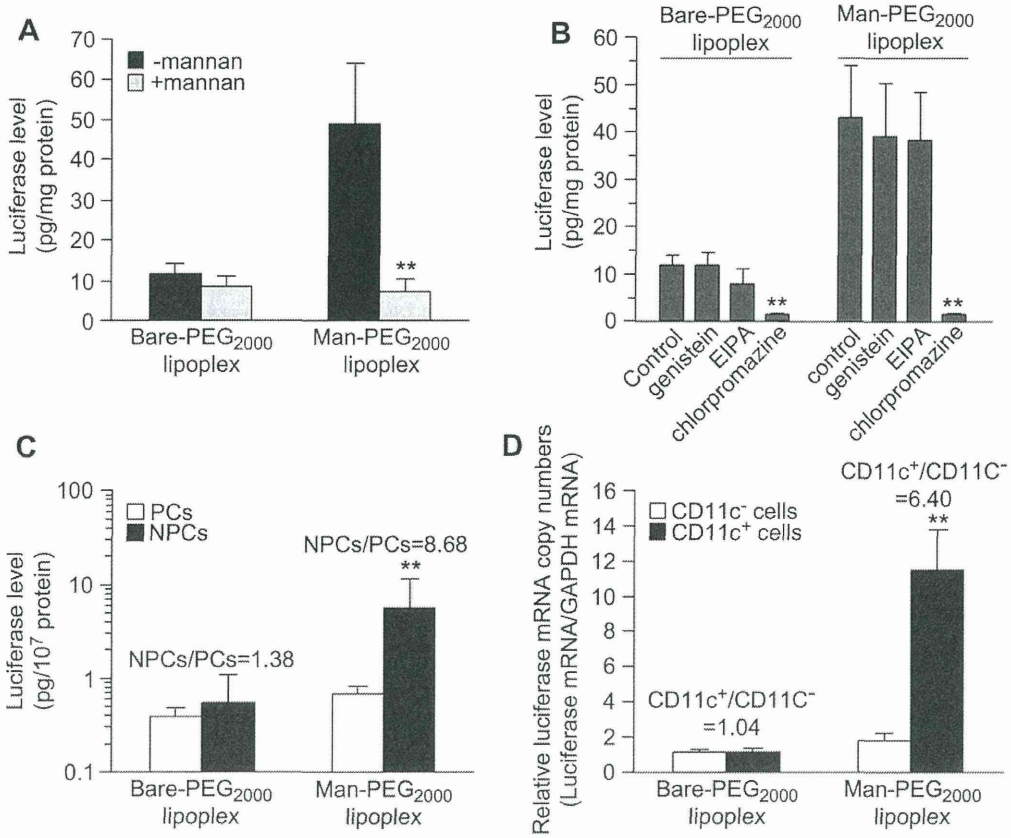

Fig. 2. The mannose receptor-expressing cell-selective gene expression by Man-PEG_{2000} lipoplexes containing Man-PEG_{2000} lipids in vitro and vivo. (A) The level of luciferase expression obtained by Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes (5 μg pDNA) in the absence or presence of 1 mg/mL mannan in mouse cultured macrophages at 24 h after transfection. $^{**}p < 0.01$, compared with the corresponding group of mannan. (B) Inhibition of luciferase expression obtained by Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes (5 μg pDNA) in addition of various endocytosis inhibitors in mouse cultured macrophages at 24 h after transfection. $^{**}p < 0.01$, compared with the corresponding group of control. (C) The level of luciferase expression in mouse hepatic PCs and NPCs after intravenous administration of Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes (50 μg pDNA) in mice at 6 h after transfection. $^{**}p < 0.01$, compared with the corresponding group of PCs. (D) The level of luciferase mRNA expression in mouse splenic $CD11c^+$ cells and $CD11c^-$ cells after intravenous administration of Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes (50 μg pDNA) in mice at 6 h after transfection. $^{**}p < 0.01$, compared with the corresponding group of $CD11c^-$ cells. Each value represents the mean + SD ($n = 3–4$).

small-sized microbubbles for in-vivo administration [12]. Firstly, we developed mannose-conjugated PEG_{2000}-modified lipids (Man-PEG_{2000}-DSPE (Fig. 1)) to prepare the APC-targeted small-sized microbubbles and determined the in-vitro and in-vivo transfection characteristics of mannose-conjugated PEG_{2000}-modified lipoplexes (Man-PEG_{2000} lipoplexes) containing Man-PEG_{2000} lipids. The particle sizes and zeta potentials of Man-PEG_{2000} lipoplexes and non-modified PEG_{2000}-lipoplexes (Bare-PEG_{2000} lipoplexes) were approximately 150 nm and +40 mV, respectively (Supplementary Table 1). In mouse cultured macrophages expressing mannose receptors abundantly, the level of gene expression obtained by Man-PEG_{2000} lipoplexes were significantly higher than those by Bare-PEG_{2000} lipoplexes (Fig. 2A and B). Then, the level of gene expression obtained by Man-PEG_{2000} lipoplexes was suppressed to same extent as that by Bare-PEG_{2000} lipoplexes in the presence of an excess of mannan (Fig. 2A). Moreover, this level of gene expression obtained by Man-PEG_{2000} lipoplexes was also suppressed to same extent as that by Bare-PEG_{2000} lipoplexes in the presence of chlorpromazine (Fig. 2B), which is the inhibitor of clathrin-mediated endocytosis [22]. These results agreed with the results of cellular association of pDNA (Supplementary Fig. 1), and suggest that Man-PEG_{2000} lipoplexes are taken up into the cells via clathrin-mediated endocytosis following the interaction with mannose receptors.

3.2. In-vivo gene transfection properties by Man-PEG_{2000} lipoplexes

Since the degradation of pDNA by nuclease in the blood is one of the critical factors in the in-vivo gene transfection by intravenously administration of lipoplexes, we investigated the stability of Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes against nucleases. Following electrophoresis of naked pDNA and lipoplexes after incubation with DNase I, although naked pDNA underwent the degradation by DNase I, lipoplexes did not undergo the degradation and retained the complex forms (Supplementary Fig. 2). Then, we investigated the gene expression characteristics of Man-PEG_{2000} lipoplexes in the liver and spleen, which are the targeted organs of mannose-modified carriers [27]. In this study, liver was separated in the parenchymal cells (PCs) and non-parenchymal cells (NPCs), and spleen was separated in the dendritic cells ($CD11c^+$ cells) and other cells ($CD11c^-$ cells). As shown in Fig. 2C and D, following intravenous administration of Man-PEG_{2000} lipoplexes, selective gene expression was observed in the hepatic NPCs and the splenic $CD11c^+$ cells, which are the APCs expressing mannose receptors abundantly [28–30].

3.3. In-vitro gene transfection efficiency by Man-PEG_{2000} bubble lipoplexes and US exposure

Although Man-lipoplexes showed the APC-selective gene transfection properties in vivo, this level of gene expression was

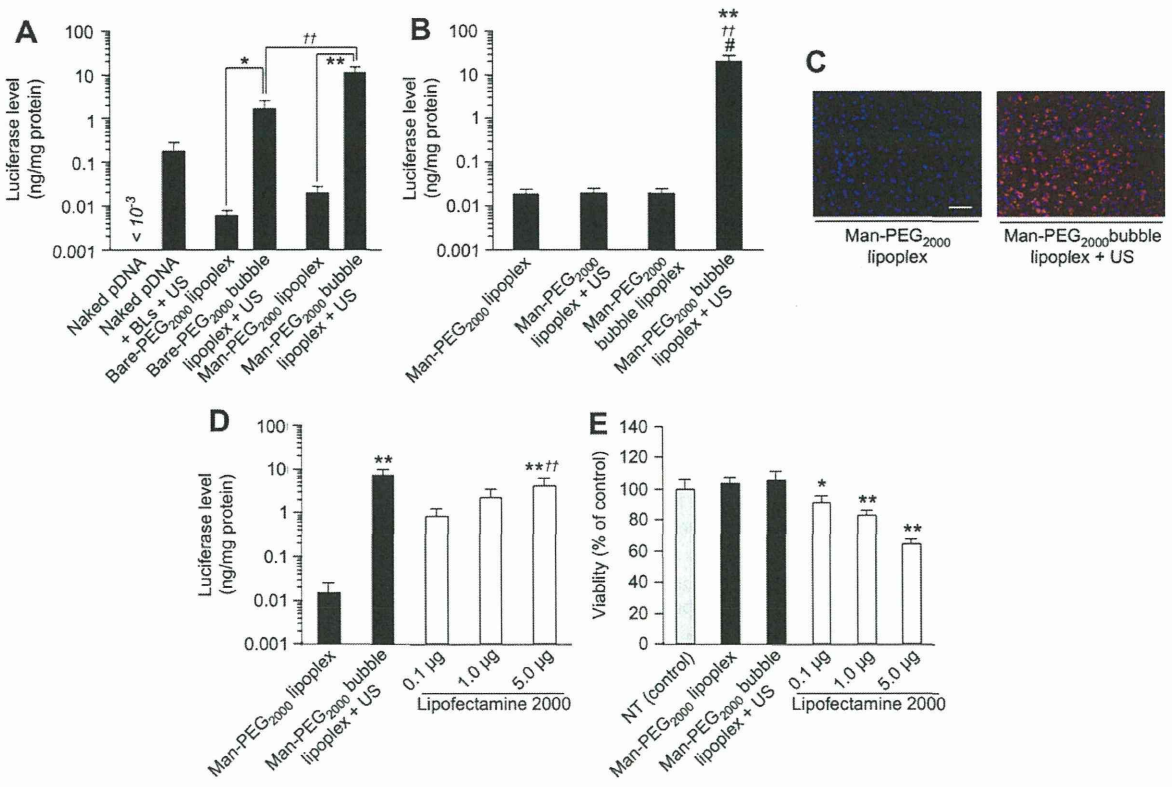

Fig. 3. Enhancement of gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure in vitro. (A) The level of luciferase expression obtained by naked pDNA, naked pDNA + BLs with US exposure, Bare-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure, Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure (5 μg pDNA) at 24 h after transfection. Significant difference; $^*p < 0.05$; **, $^{\dagger\dagger}p < 0.01$. (B) The level of luciferase expression obtained by Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with or without US exposure (5 μg pDNA) at 24 h after transfection. $^{**}p < 0.01$, compared with Man-PEG_{2000} lipoplex, $^{\dagger\dagger}p < 0.01$, compared with Man-$PEG_{2000}$ lipoplex + US, $^{\#\#}p < 0.01$, compared with Man-PEG_{2000} bubble lipoplex. (C) Representative fluorescent images of cellular association of pDNA obtained by Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure (5 μg pDNA) at 2 h after treatment. Lipoplexes were constructed with TM-rhodamine-labeled pDNA. TM-rhodamine-labeled pDNA (red), nuclei counterstained by DAPI (blue). Scale bars, 100 μm. (D) Comparison of the level of luciferase expression obtained by Man-PEG_{2000} bubble lipoplexes (5 μg pDNA) and US exposure with that by Lipofectamine 2000. $^{**}p < 0.01$, compared with Man-PEG_{2000} lipoplexes, $^{\dagger\dagger}p < 0.01$, compared with Lipofectamine 2000 (0.1 μg). (E) Comparison of cell viability by transfection using Man-PEG_{2000} bubble lipoplexes (5 μg pDNA) and US exposure with that by Lipofectamine 2000. N.T., non-treatment. $^*p < 0.05$; $^{**}p < 0.01$, compared with N.T. Each value represents the mean + SD ($n = 4$).

low compared with our previous reports [1,19,25]. To enhance the level of gene expression by sonoporation method, we developed Man-PEG_{2000} bubble lipoplexes (Fig. 1) by enclosing US imaging gas (perfluoropropane gas) into Man-PEG_{2000} lipoplexes. The lipid composition of lipoplexes is important for the stable enclosure of US imaging gas. Following optimization of lipid composition, lipoplexes constructed with the saturated lipids only, which have a high melting temperature (T_m), were enclosed US imaging gas stably (Supplementary Table 2). Following enclosure of US imaging gas in lipoplexes, lipoplexes became cloudy and their particle sizes were increased (from 150 nm to 550 nm, approximately) (Supplementary Fig. 3A and Table 3). Then, since the zeta potentials of bubble lipoplexes were lower than that of bubble liposomes and same as that of lipoplexes (Supplementary Tables 1 and 3), it is considered that pDNA is attached on the surface of bubble liposomes. Moreover, the stability against nucleases observed in Man-PEG_{2000} lipoplexes (Supplementary Fig. 2) was maintained after enclosure of US imaging gas into lipoplexes (Supplementary Fig. 3B).

The level of gene expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was 500-fold higher than that by Man-PEG_{2000} lipoplexes in mouse cultured macrophages expressing mannose receptors abundantly, and also higher than that by non-modified bubble lipoplexes (Bare-PEG_{2000} bubble lipoplexes, Fig. 1) and US exposure or conventional sonoporation method using naked pDNA and BLs (Fig. 3A). This enhanced gene expression was observed when bubble lipoplexes and US exposure were used for in-vitro gene transfer (Fig. 3B). The cellular association of pDNA obtained by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was also 10-fold higher than that by Man-PEG_{2000} lipoplexes, and also higher than that by Bare-PEG_{2000} bubble lipoplexes and US exposure or conventional sonoporation method using naked pDNA and BLs (Fig. 3C and Supplementary Fig. 4A). Moreover, this level of gene expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was comparable to that by Lipofectamine® 2000, which is widely used as a gene transfection reagent (Fig. 3D). On the other hand, the cytotoxicity by Man-PEG_{2000} bubble lipoplexes and US exposure was lower than that by Lipofectamine® 2000 (Fig. 3E).

3.4. Intracellular uptake properties of pDNA by Man-PEG_{2000} bubble lipoplexes and US exposure

The gene expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was significantly suppressed in the presence of an excess of mannan (Fig. 4A). Therefore, the interaction with mannose receptors on the cell membrane is involved in the gene transfection by Man-PEG_{2000} bubble lipoplexes and US exposure, similar to the gene transfection by Man-PEG_{2000} lipoplexes. On the other hand, unlike Man-PEG_{2000} lipoplexes (Fig. 2B), the gene expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was not suppressed in the presence of chlorpromazine (Fig. 4B), which is a clathrin-mediated endocytosis inhibitor [22]. These results agreed with the results of cellular association of pDNA (Supplementary Fig. 4B), and indicated that pDNA delivered by Man-PEG_{2000} bubble lipoplexes was directly introduced into the cytoplasm without mediating endocytosis by the gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure.

3.5. In-vivo gene transfection efficiency by Man-PEG_{2000} bubble lipoplexes and US exposure

As shown in Fig. 5A and B, the level of gene expression obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was

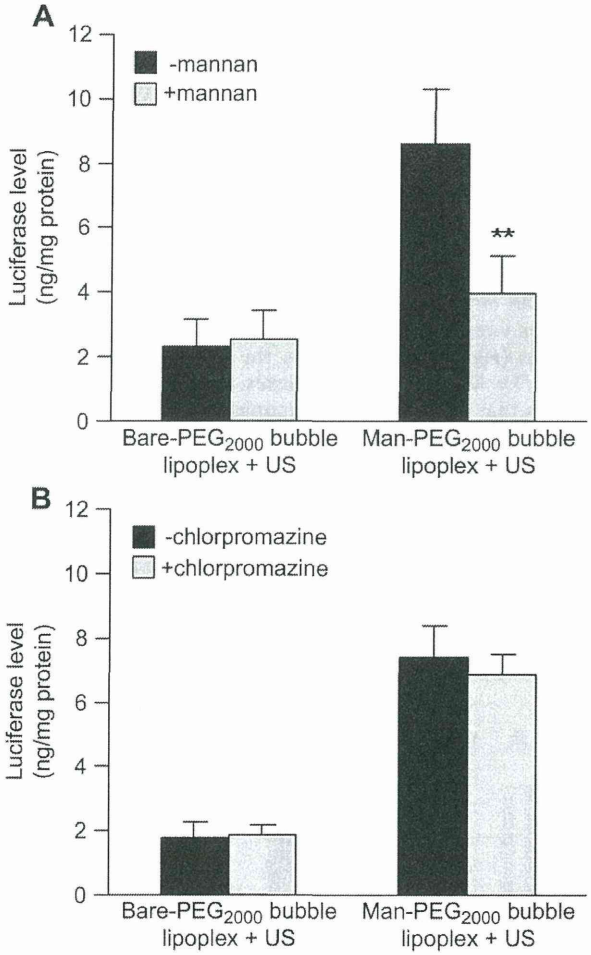

Fig. 4. Effects of mannan and chlorpromazine on gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure in vitro. (A) The level of luciferase expression obtained by Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (5 μg pDNA) in the absence or presence of 1 mg/mL mannan at 24 h after transfection. $^{**}p < 0.01$, compared with the corresponding group of mannan. (B) The level of luciferase expression by Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (5 μg pDNA) in the absence or presence of 50 μM chlorpromazine at 24 h after transfection. Each value represents the mean + SD ($n = 4$).

500 ~ 800-fold higher than that by Man-PEG_{2000} lipoplexes, and also higher than that by Bare-PEG_{2000} bubble lipoplexes and US exposure or the conventional sonoporation method using naked pDNA and BLs in the liver and spleen, which are the targeted organs of mannose-modified carriers [27]. This enhanced gene expression in the liver and spleen was observed when bubble lipoplexes and US exposure were used for in-vivo gene transfer (Fig. 5C and D). Moreover, this gene expression obtained by Bare-PEG_{2000} bubble lipoplexes with US exposure or Man-PEG_{2000} bubble lipoplexes with US exposure in the liver and spleen remained higher than that by Bare-PEG_{2000} lipoplexes or Man-PEG_{2000} lipoplexes for at least 48 h, respectively (Fig. 5E and F). In addition, the gene expression was also enhanced in the US-exposed organ specifically following gene transfection by direct US exposure to the targeted organ after intravenous administration of Man-PEG_{2000} bubble lipoplexes (Supplementary Fig. 5). On the other hand, the increase of gene expression by bubble lipoplexes and US exposure was not observed in other organ such as lung, kidney and heart (Fig. 5G and H).

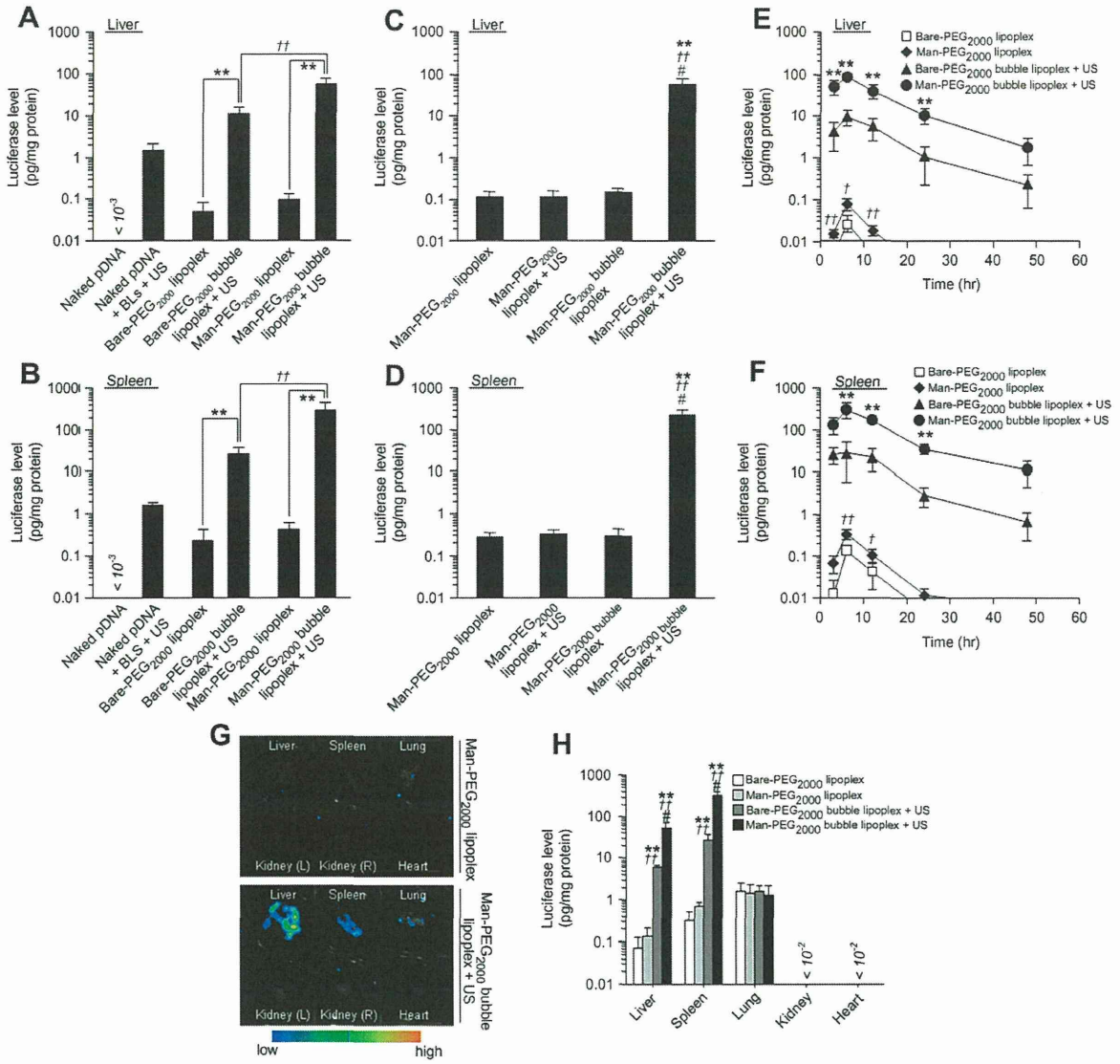

Fig. 5. Enhancement of mannose receptor-expressing cells-selective gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure in vivo. (A, B) The level of luciferase expression obtained by naked pDNA, naked pDNA + BLs with US exposure, Bare-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure, Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA) in the liver (A) and spleen (B) at 6 h after transfection. Significant difference; **, $^{\dagger\dagger}p < 0.01$. (C, D) The level of luciferase expression obtained by Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with or without US exposure (50 μg pDNA) in the liver (C) and spleen (D) at 6 h after transfection. $^{**}p < 0.01$, compared with Man-PEG_{2000} lipoplex, $^{\dagger\dagger}p < 0.01$, compared with Man-$PEG_{2000}$ lipoplex + US, $^{\ddagger\ddagger}p < 0.01$, compared with Man-$PEG_{2000}$ bubble lipoplex. (E, F) Time-course of luciferase expression in the liver (E) and spleen (F) 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 pDNA). Each value represents the mean ± SD ($n = 4$). $^{**}p < 0.01$, compared with Bare-PEG_{2000} bubble lipoplex + US, $^{\dagger}p < 0.05$; $^{\dagger\dagger}p < 0.01$, compared with Bare-$PEG_{2000}$ lipoplex. (G) In-vivo imaging photographs of luciferase expression in the isolated organs at 6 h after transfection by Man-PEG_{2000} lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). (H) The level of luciferase expression in each organ 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 pDNA). $^{**}p < 0.01$, 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, $^{\ddagger\ddagger}p < 0.01$, compared with the corresponding group of Bare-PEG_{2000} bubble lipoplex + US. Each value represents the mean + SD ($n = 4$).

3.6. *Targeted cell-selective gene transfection properties by Man-PEG_{2000} bubble lipoplexes and US exposure in vivo*

We investigated the mannose receptor-expressing cell selectivity of gene expression by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure. In the liver, the level of gene expression in the hepatic NPCs expressing mannose receptors was significantly higher than that in the hepatic PCs following gene transfection by Man-PEG_{2000} bubble lipoplexes and US exposure (Fig. 6A). This difference in gene expression between the NPCs and PCs obtained by Man-PEG_{2000} bubble lipoplexes and US exposure was similar to that by Man-PEG_{2000} lipoplexes, although the level of gene expression in the NPCs and PCs was markedly higher. On the other hand, selective gene expression in the NPCs was not observed by Bare-PEG_{2000} bubble lipoplexes and US exposure.

In the spleen, the level of mRNA expression in the $CD11c^+$ cells, which are the splenic dendritic cells expressing mannose receptors, was significantly higher than that in the $CD11c^-$ cells following transfection by Man-PEG_{2000} bubble lipoplexes and US exposure (Fig. 6B). On the other hand, selective gene expression in the $CD11c^+$ cells was not observed by Bare-PEG_{2000} bubble lipoplexes and US exposure.

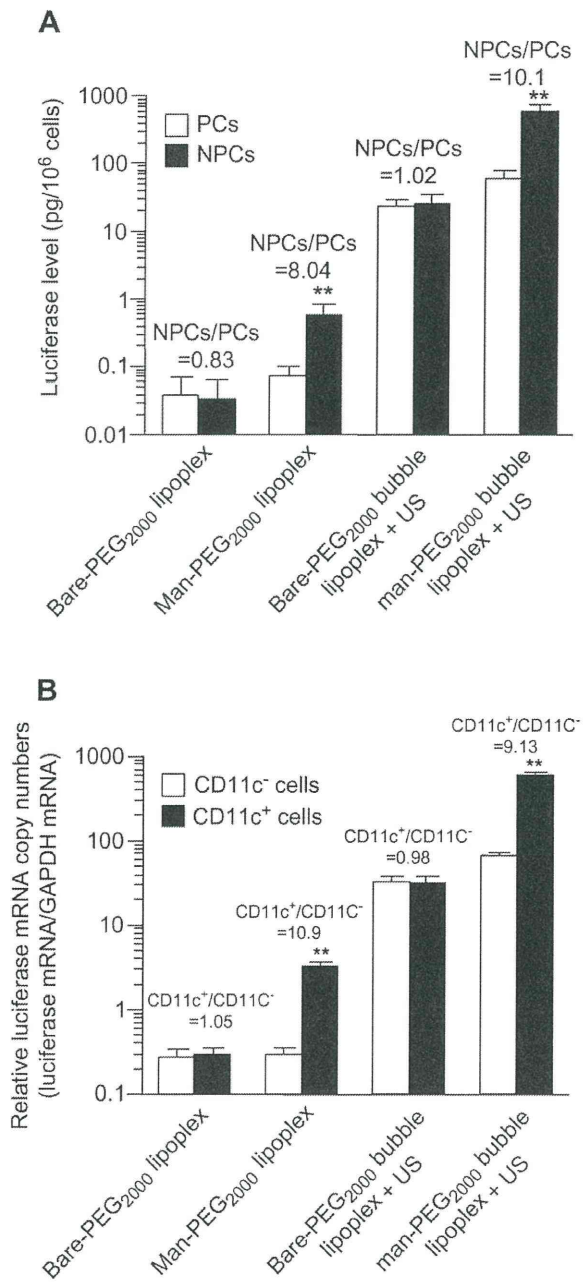

Fig. 6. Hepatic and splenic cellular localization of luciferase expression by Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Hepatic cellular localization of luciferase expression at 6 h after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). $^{**}p < 0.01$, compared with the corresponding group of PCs. (B) Splenic cellular localization of luciferase 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 pDNA). $^{**}p < 0.01$, compared with the corresponding group of $CD11c^-$ cells. Each value represents the mean + SD ($n = 4$).

3.7. *In-vivo distribution properties of pDNA by Man-PEG_{2000} bubble lipoplexes and US exposure*

Next, to elucidate the mechanism of enhanced in-vivo gene expression using Man-PEG_{2000} bubble lipoplexes and US exposure, we investigated the effect on the tissue distribution of pDNA followed by gene transfection. In this study, Bare-PEG_{2000} bubble lipoplexes and Man-PEG_{2000} bubble lipoplexes constructed with radio-labeled pDNA were intravenously administrated, and then mice were subjected to external US exposure. As shown in Fig. 7, in the case of both bubble lipoplexes, the retention time of pDNA in the blood was slightly reduced and the distribution of pDNA delivered by bubble lipoplexes was significantly increased by US exposure in the liver and spleen (Fig. 7). Moreover, the amount of pDNA distributed in the liver and spleen by Man-PEG_{2000} bubble lipoplexes and US exposure (Fig. 7A) was higher than that by Bare-PEG_{2000} bubble lipoplexes and US exposure (Fig. 7B). On the other hand, no increase of pDNA distribution followed by US exposure was observed in the lung.

3.8. *The liver toxicity by Man-PEG_{2000} bubble lipoplexes and US exposure*

We examined ALT and AST activities in the serum to investigate the liver toxicity by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure. ALT and AST activities in the serum were increased by gene transfection using Bare-PEG_{2000} lipoplexes and Man-PEG_{2000} lipoplexes. On the other hands, the increase of ALT and AST activities was not observed by gene transfection using Bare-PEG_{2000} bubble lipoplexes and Man-PEG_{2000} bubble lipoplexes with US exposure (Fig. 8).

3.9. *Antigen presentation on MHC class I molecules in immunized splenic dendritic cells*

To investigate the DNA vaccine effects by Man-PEG_{2000} bubble lipoplexes and US exposure, we prepared Man-PEG_{2000} bubble lipoplexes constructed with pDNA expressing OVA as a model antigen. Firstly, to investigate the antigen (OVA) presentation on MHC class I molecules in the splenic dendritic cells ($CD11c^+$ cells) by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure, the splenic $CD11c^+$ cells isolated from once-immunized mice were co-incubated with CD8-OVA1.3 cells, which are T cell hybridomas with specificity for OVA. Following measurement of IL-2 to evaluate the activation of T cells, the IL-2 secretion from activated CD8-OVA1.3 cells co-incubated with the $CD11c^+$ cells isolated from mice immunized by Man-PEG_{2000} bubble lipoplexes and US exposure was the highest of all (Fig. 9A). This result indicates that DNA vaccination by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure can induce significantly high $CD8^+$-T lymphocyte activation.

3.10. *Antigen-specific cytokine secretion from immunized splenic cells*

We evaluated the OVA-specific cytokine secretion from the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure. Following optimization of immunization schedule, it was shown that a 2 week interval was necessary to achieve the same level of gene expression as former transfection in the spleen (Supplementary Fig. 6) and at least three times immunization was necessary to effective anti-tumor effects by DNA vaccination using this method (Supplementary Fig. 7). Therefore, the immunization to mice was performed according to the protocol shown in Fig. 9B. As shown in Fig. 9C, in the presence of OVA, the highest amount of IFN-γ (Th1 cytokine) was secreted from splenic cells harvested from mice immunized with Man-PEG_{2000} bubble lipoplexes and US exposure. On the other hand, no secretion of IFN-γ was observed in any of the groups in the absence of OVA. Moreover, the secretion of IL-4 (Th2 cytokine) was not increased in any of the groups both in the presence or absence of OVA (Fig. 9C). These results suggest that immunization by Man-PEG_{2000} bubble lipoplexes constructed with

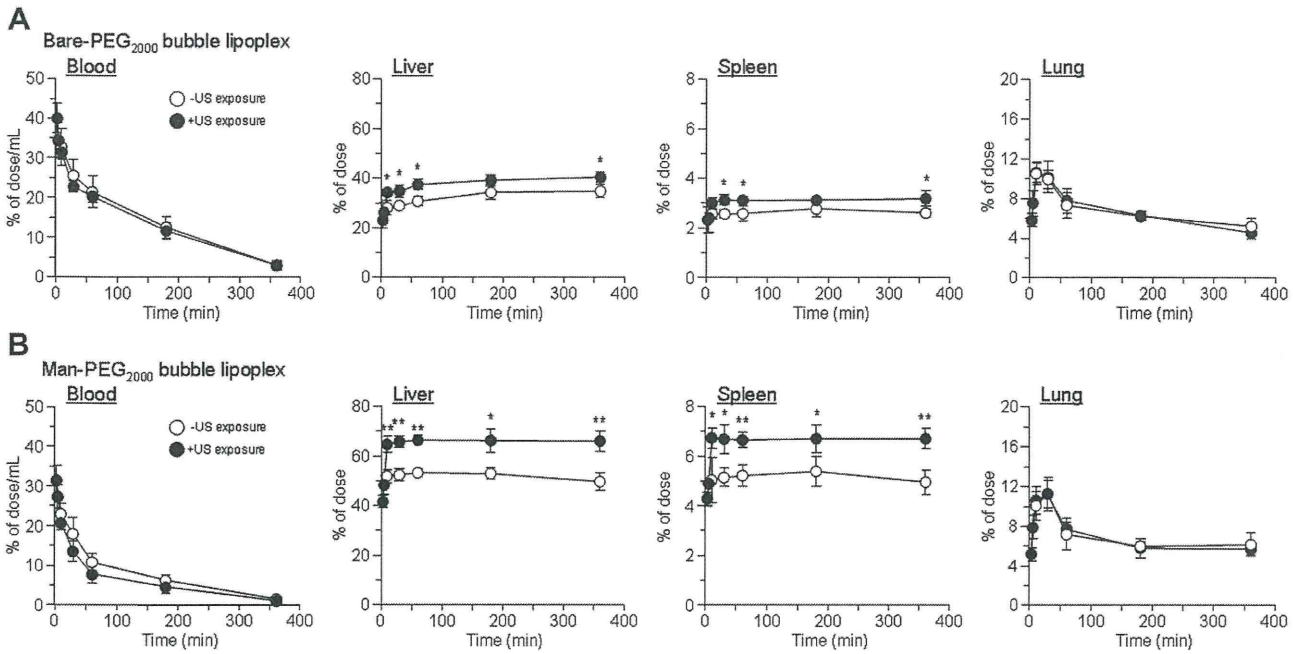

Fig. 7. Tissue distribution of pDNA by Man-PEG_{2000} bubble lipoplexes and US exposure. Tissue distribution after intravenous administration of (A) Bare-PEG_{2000} bubble lipoplexes and (B) Man-PEG_{2000} bubble lipoplexes (50 μg pDNA) with or without US exposure in mice. US was exposed at 5 min after intravenous administration of bubble lipoplexes. Each value represents the mean ± SD ($n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of US exposure.

pCMV-OVA and US exposure significantly enhances the differentiation of helper T cells to Th1 cells, which are pivotal cells for the activation of cytotoxic T lymphocytes (CTL) with high anti-tumor activity, by OVA stimulation.

3.11. Antigen-expressing cell-specific CTL activity in immunized splenic cells

Next, we assessed the CTL activity in the splenic cells harvested from mice immunized by Man-PEG_{2000} bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Fig. 9B, the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure showed the highest CTL activity in all groups against E.G7-OVA cells which are the lymphoma cells expressing OVA (Fig. 9D). In contrast, the CTL activity was not observed in EL4 cells which are the lymphoma cells not expressing OVA in all groups (Fig. 9D). These results indicate that the splenic cells immunized by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure induce the OVA-expressing cell-specific CTL activity.

3.12. Therapeutic effects against antigen-expressing tumor by DNA vaccination

Finally, we investigated the anti-tumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Following experiments according to the protocol shown in Fig. 10A, significantly high anti-tumor effects against E.G7-OVA cells were observed in mice immunized by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure (Fig. 10B). However, in mice transplanted EL4 cells, no anti-tumor effects were observed in any of the groups (Fig. 10C). Moreover, we investigated the maintenance of DNA vaccine effects following administration of Man-PEG_{2000} bubble lipoplexes and US exposure. According to the protocol shown in Fig. 11A, E.G7-OVA cells were re-transplanted into mice which first-transplanted tumors were completely rejected by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. As results, high anti-tumor effects were observed in mice following re-transplantation of E.G7-OVA cells (Fig. 11B); therefore it was demonstrated that DNA vaccine effects obtained by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure were maintained for at least 80 days.

4. Discussion

To obtain high therapeutic effects by DNA vaccination using tumor-specific antigen-coding gene, it is essential to transfer the gene selectively and efficiently into the APCs, such as macrophages and dendritic cells [31,32]. However, it is difficult to transfer the gene into the APCs selectively because of the number of APCs is limited in the organ [33]. Since the APCs are expressed a large number of mannose receptors [28,29], we and other groups have developed mannose-modified non-viral carriers for gene delivery to the APCs [7,25,34]. On the other hand, our group also reported that the gene transfection efficiency in the APCs was lower than that in other cells [35]; therefore it is difficult to achieve high gene transfection efficiency to induce high therapeutic effects by DNA vaccination in vivo. In the present study, to establish an APC-selective and efficient gene delivery system, we developed US-responsive and mannose-modified carriers, named Man-PEG_{2000} bubble lipoplexes, which had selectivity to the APCs and responded to US exposure. The gene delivery system using Man-PEG_{2000} bubble lipoplexes and US exposure enabled to achieve markedly high gene expression in macrophages and dendritic cells selectively in vivo, in spite of the handy system used intravenous administration and external US exposure. Moreover, we succeeded in obtaining high anti-tumor effects by applying this method to DNA vaccine therapy using OVA-expressing pDNA.

Firstly, since PEG_{2000}-modification is necessary to enclose US imaging gas stably [12], we prepared Man-PEG_{2000} lipoplexes

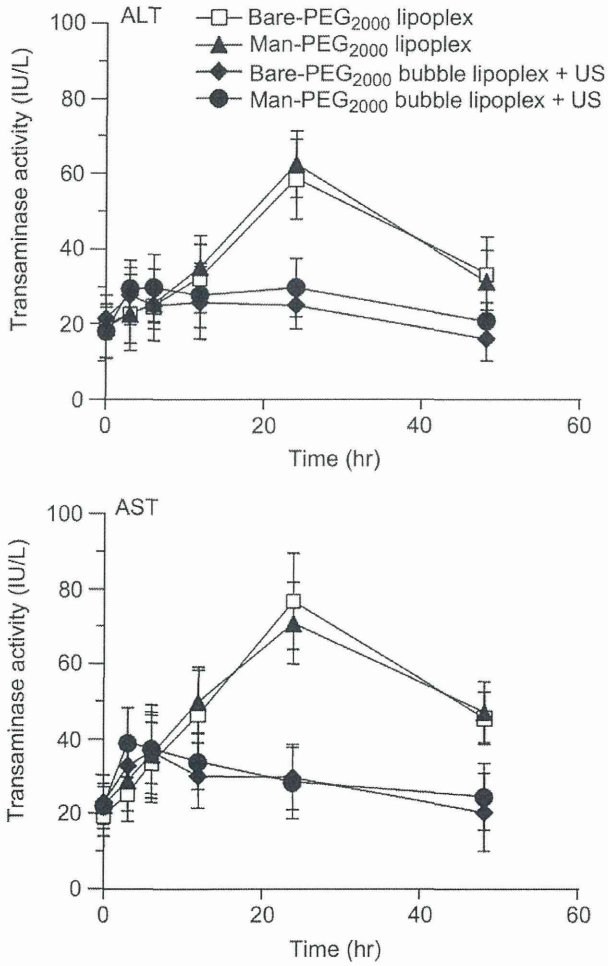

Fig. 8. Liver toxicity by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure. Time-course of serum transaminase activities after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were measured at predetermined times after transfection. Each value represents the mean ± SD ($n = 4$).

containing Man-PEG_{2000} lipids. This Man-PEG_{2000} lipoplexes exhibited mannose receptor-expressing cell-selective gene expression both in vitro and vivo (Fig. 2). On the other hand, the level of gene expression by Man-PEG_{2000} lipoplexes was lower than that by mannosylated lipoplexes without PEG-modification, as reported previously by our group [1,25]. However, this result was considered to be contributed by the reduced interaction with the cell membrane and the reduction of endosomal escape efficiency by PEG_{2000}-modification [36,37]. In the sonoporation method, Tachibana et al. demonstrated that a transient pore is created on the cell membrane followed by the degradation of microbubbles [38]. Then, nucleic acids, such as pDNA, siRNA and oligonucleotides, are introduced into the cell through the generated pore [13,15,16]. Consequently, since the nucleic acids are directly introduced into cytoplasm in the sonoporation method [13,14], it is considered that the low level of transfection efficiency obtained by Man-PEG_{2000} lipoplexes can be overcome by applying sonoporation method. As shown in Figs. 3 and 4, a large amount of pDNA is directly introduced into the cytoplasm and high level of gene expression is observed by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure. Therefore, by delivering pDNA to the APCs using Man-PEG_{2000} bubble lipoplexes, it is suggested that high level of gene expression in the APCs can easily achieve by following US exposure in this gene transfection method.

In this study, the level of gene expression obtained by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was higher than that obtained by Man-PEG_{2000} lipoplexes or Bare-PEG_{2000} bubble lipoplexes with US exposure in the liver and spleen (Fig. 5). Moreover, gene expression by Man-PEG_{2000} bubble lipoplexes and US exposure was observed selectively in the hepatic NPCs and the splenic dendritic cells (Fig. 6), known as mannose receptor-expressing cells [28–30]. Although this selectivity of gene expression was the same as that obtained by mannosylated lipoplexes reported previously by our group [1,25], this level of gene expression was markedly higher. It is considered that this enhanced and cell-selective gene expression is contributed by the increase of interaction with mannose receptor-expressing cells by mannose modification (Supplementary Fig. 1), by the improvement of delivering efficiency of nucleic acids to the targeted organs (Fig. 7) and by the direct introduction of nucleic acids into the cytoplasm of targeted cells followed by US exposure to Man-PEG_{2000} bubble lipoplexes (Figs. 3C and 4B and Supplementary Fig. 4). Moreover, the enhanced gene expression was not observed in the lung, kidney and spleen (Fig. 5G and H). It is guessed that the reason why the enhanced gene expression was not observed in the lung is because US is not spread to the thoracic cavity by the diaphragm, and the reason why the enhanced gene expression was not observed in the kidney and heart was because the distributed amounts of bubble lipoplexes were markedly small. In addition, since the particle size of bubble lipoplexes (approximately 500 nm) is suitable for delivery to the liver and spleen, compared with stabilized liposomes (approximately 100 nm) [39], the gene transfection system using Man-PEG_{2000} bubble lipoplexes and US exposure is a suitable method for the selective delivery of nucleic acids into the mannose receptor-expressing cells in the liver and spleen.

On the other hand, the liver toxicity followed by gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was lower than that by Man-PEG_{2000} lipoplexes (Fig. 8). It was reported that the CpG motifs in the pDNA sequence are recognized to Toll-like receptor 9 (TLR9) in the endosomes [40,41]; therefore it has been considered that the production of proinflammatory cytokines, such as TNF-α, IFN-γ and IL-12, could be induced in the lipofection method using liposomes and emulsions, and these cytokines cause liver injury [42]. However, in the gene transfection using Man-PEG_{2000} bubble lipoplexes and US exposure, a large amount of pDNA was directly introduced into the cytoplasm not-mediated endocytosis (Figs. 3C and 4B and Supplementary Fig. 4). Therefore, it is considered that pDNA is not recognized to TLR9 in the endosomes, and consequently liver toxicity followed by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure is low.

In the previous study [16], we developed combination-use method using mannosylated lipoplexes [1] and BLs [12] with US exposure to achieve targeted cell-selective gene transfer. However, this combination-use method is complicated because of the necessity of twice injection of mannosylated lipoplexes and BLs, therefore it is difficult to apply for medical treatments using multiple injection. Moreover, it is considered that the difference of in-vivo distribution characteristics between mannosylated lipoplexes and BLs might be decreased its transfection efficacy. On the other hand, this transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure is handy because of using only once injection of Man-PEG_{2000} bubble lipoplexes and external US exposure. In addition, this method using Man-PEG_{2000} bubble lipoplexes and US exposure overcame the difference of in-vivo distribution of formulations, which might lead to the decrease of

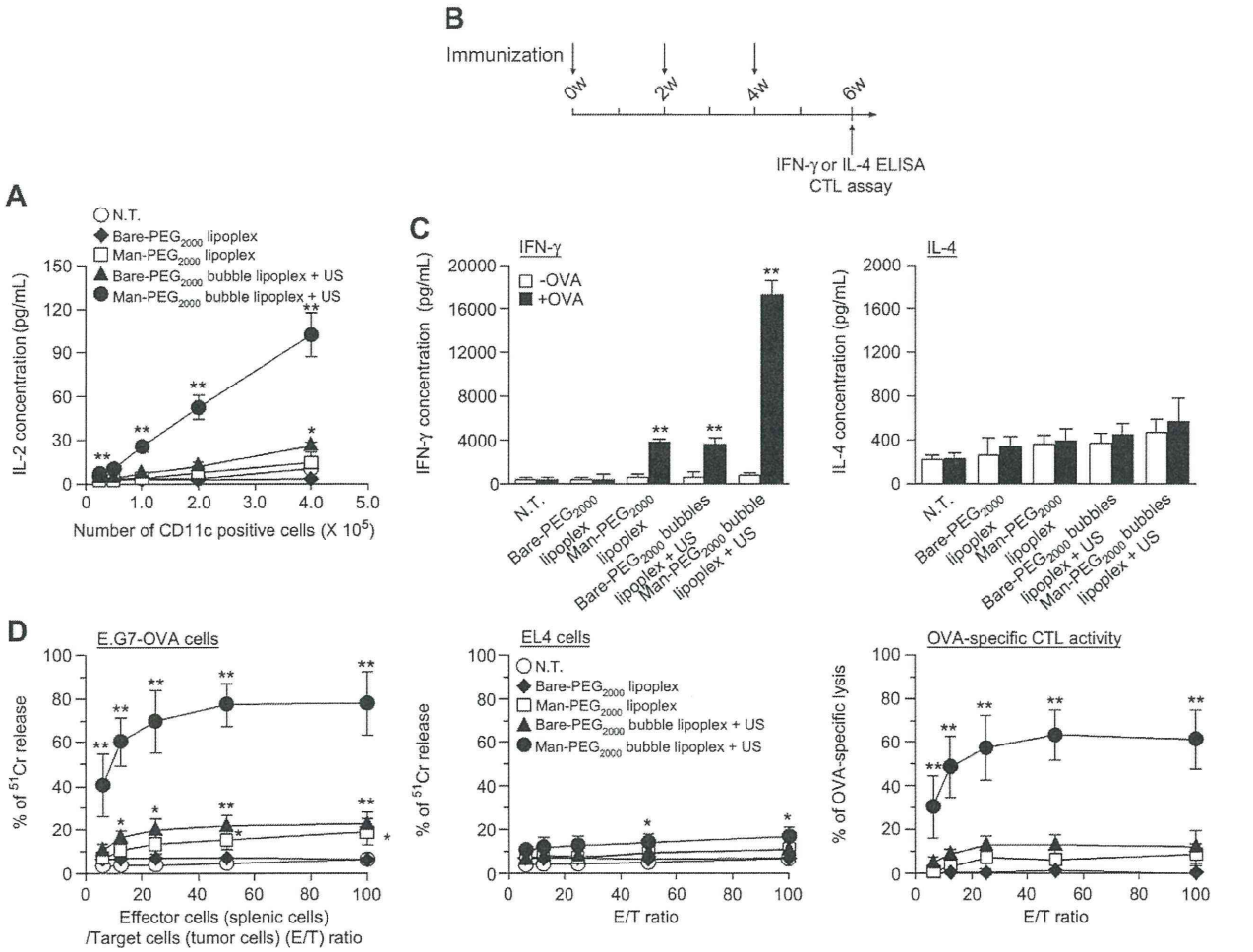

Fig. 9. Cytokine secretion characteristics and CTL activities by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) OVA presentation on MHC class I molecules in the splenic $CD11c^+$ cells at 24 h after transfection by Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). OVA presentation on MHC class I molecules was determined by IL-2 level secreted from CD8-OVA1.3 cells co-incubated with the $CD11c^+$ cells isolated from once-immunized mice. Each value represents the mean ± SD ($n = 4$). $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T. (B) Schedule of immunization for OVA-specific cytokine secretion experiments and CTL assay. (C) OVA-specific IFN-γ and IL-4 secretion from the splenic cells immunized three times biweekly 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 pDNA). The splenic cells were collected at 2 weeks after last immunization. After the immunized splenic cells were cultured for 72 h in the absence or presence of 100 μg OVA, IFN-γ and IL-4 secreted in the medium were measured by ELISA. Each value represents the mean + SD ($n = 4$). $^{**}p < 0.01$, compared with the corresponding group of OVA. (D) OVA-specific CTL activities after immunization three times 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 pDNA). CTL activities to E.G7-OVA and EL4 cells 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 group of N.T. N.T., non-treatment.

transfection efficiency. In fact, the level of gene expression by this method was higher than that by combination-use method reported previously in the targeted organs (liver and spleen) (Fig. 5) and targeted cells (hepatic NPC and splenic dendritic cells) (Fig. 6); therefore this gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure is more suitable for APC-selective gene transfer in vivo.

Since APC-selective and efficient gene expression was observed by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure, effective therapeutic effects are to be expected by applying this transfection method to DNA vaccine therapy, which the targeted cells are the APCs, using tumor-specific antigen-coding pDNA [31,32]. However, since the level of gene expression by transfection using Man-PEG_{2000} bubble lipoplexes and US exposure was reduced sequentially (Supplementary Fig. 6), multiple transfections are essential to obtain more effective vaccine effects against cancer (Supplementary Fig. 7). On the other hand, a 2 week interval was needed to achieve the same level of gene expression by lipoplexes or bubble lipoplexes with US exposure as former transfection in the spleen (Supplementary Fig. 7B and C). Meyer et al. reported that the optimal transfection interval was necessary to achieve high gene expression by the second transfection using lipofection methods because of IFN-γ secretion by intravenous administration of lipoplexes [43]; therefore it is considered that a similar phenomenon is contributed to the sonoporation methods using microbubbles constructed with phospholipids. Based on these findings, we performed the optimization of immunization times (Supplementary Fig. 7) and determined the optimal immunization schedule as shown in Figs. 9B, 10A and 11A.

In DNA vaccine therapy, unlike cancer immunotherapy using tumor-specific antigenic peptides, the peptides expressed as gene products in the cells act as the internal antigen. Since the internal antigens are presented on MHC class I molecules, the strong activation of CTL and high anti-tumor effects are expected in DNA vaccination therapy [44,45]. In this study, by applying this gene transfection method to DNA vaccine therapy using OVA-expressing

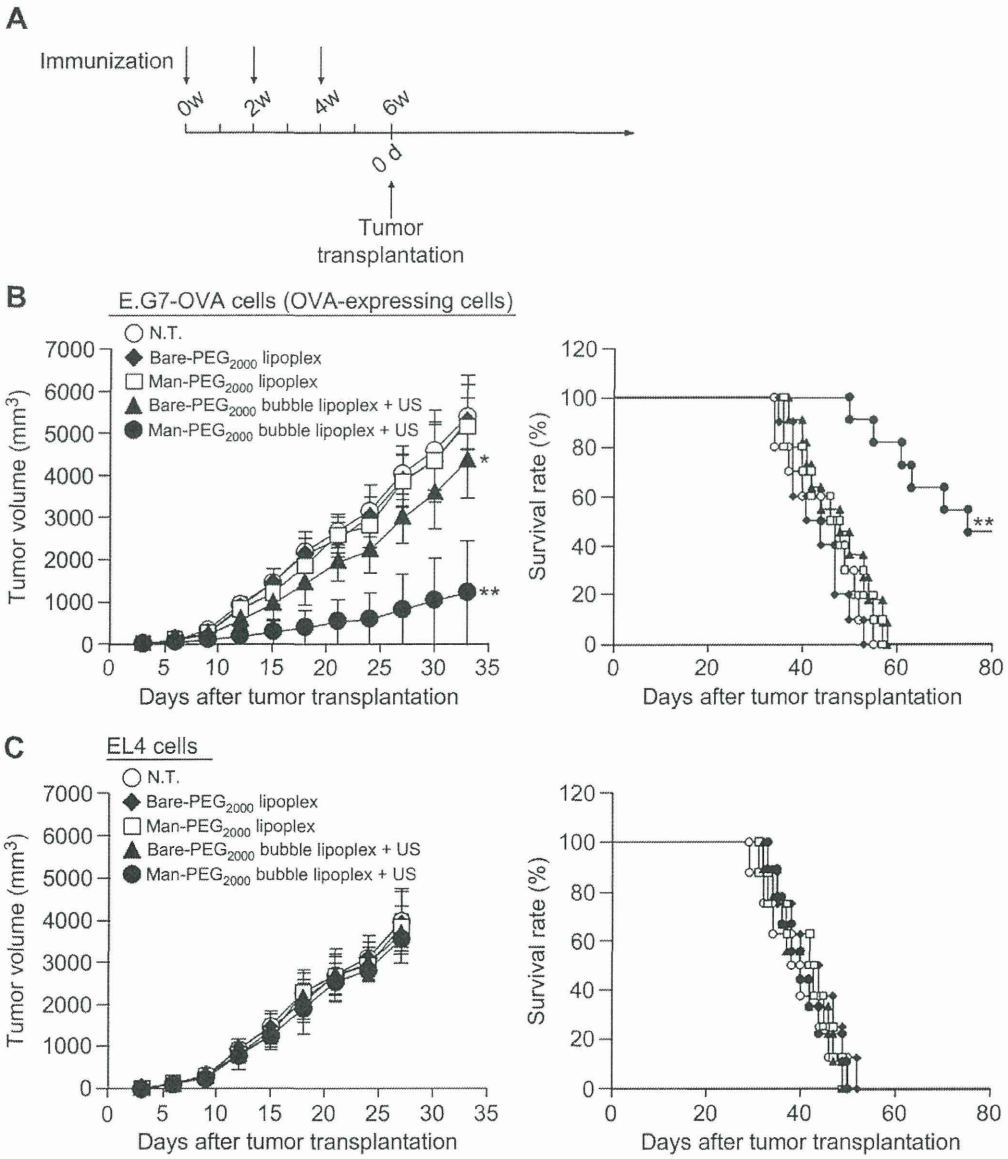

Fig. 10. Anti-tumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. (A) Schedule of immunization for experiments of therapeutic effects. (B, C) Anti-tumor effects by immunization using Bare-PEG_{2000} lipoplexes, Man-PEG_{2000} lipoplexes, Bare-PEG_{2000} bubble lipoplexes with US exposure and Man-PEG_{2000} bubble lipoplexes with US exposure (50 μg pDNA). Two weeks after last immunization, (B) E.G7-OVA cells or (C) EL4 cells (1×10^6 cells) were transplanted subcutaneously into the back of mice ($n = 8–11$). The tumor volume was evaluated (each value represents the mean ± SD) and the survival was monitored up to 80 days after the tumor transplantation. $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T. N.T., non-treatment.

pDNA, i) the presentation of OVA-peptides on MHC class I molecules of splenic dendritic cells, ii) OVA-specific Th1 cytokine secretion from splenic cells by OVA stimulation and iii) marked activation of CTL against OVA-expressing tumor were observed by gene transfection using Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure (Fig. 9). Moreover, high and long-term anti-tumor effects against OVA-expressing tumor were observed in mice transfected by Man-PEG_{2000} bubble lipoplexes constructed with pCMV-OVA and US exposure (Figs. 10 and 11). It is considered that these results are contributed by APS-selective and efficient gene transfection efficiency using Man-PEG_{2000} bubble lipoplexes and US exposure. Although more detailed examination by pDNA encoding other tumor-specific antigens, such as gp100 in melanoma or PSA in prostate cancer [45], is necessary, this transfection method by Man-PEG_{2000} bubble lipoplexes and US exposure might be available for DNA vaccine therapy.

The gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure was enabled selective and efficient gene transfer to the mannose receptor-expressing cells in the liver such as Kupffer cells and hepatic endothelial cells, which are components of the APCs (Fig. 6A). Therefore, this method is also suitable for anti-inflammatory therapy targeted to Kupffer cells and hepatic endothelial cells, known to play a key role in inflammation [46,47]. In spite of low liver toxicity, since this gene transfection system showed NPC-selective and efficient gene expression in the liver (Fig. 8), better therapeutic effects could be expected by Man-PEG_{2000} bubble lipoplexes constructed with

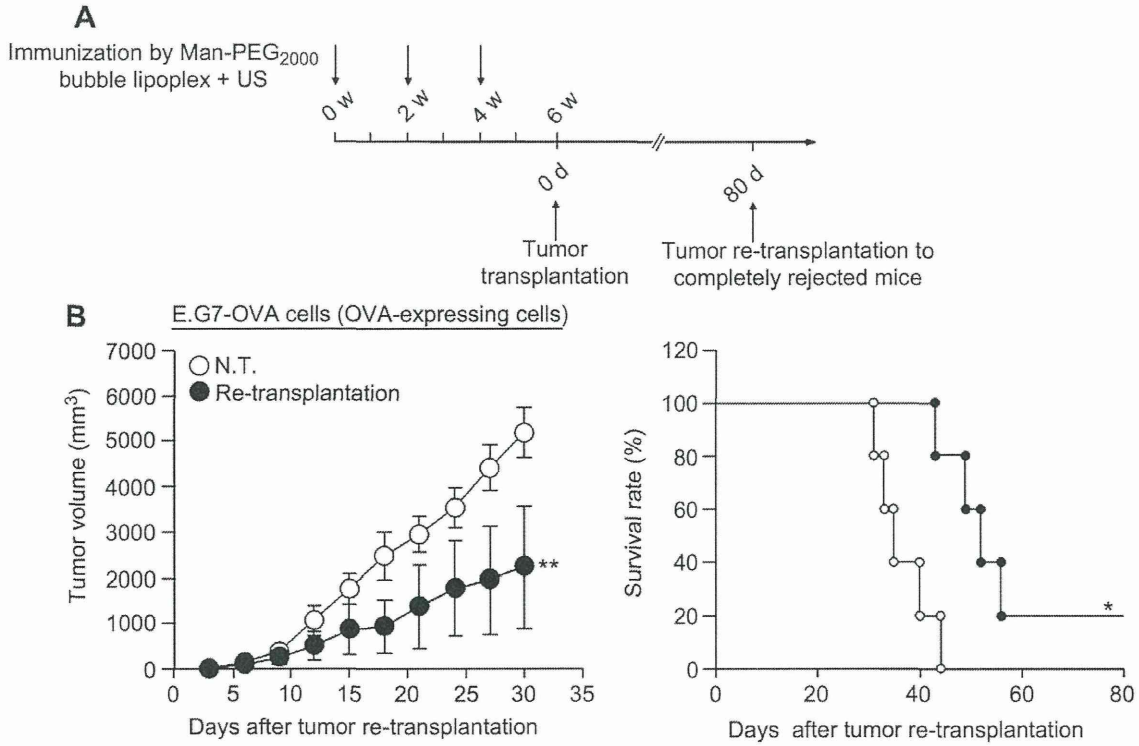

Fig. 11. Maintaining of Anti-tumor effects by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. At 80 days after first transplantation of E.G7-OVA cells to immunized mice three times by Man-PEG_{2000} bubble lipoplexes and US exposure, E.G7-OVA cells (1×10^6 cells) were re-transplanted subcutaneously into the back of mice which the first-transplanted tumors were completely rejected ($n = 5$). The tumor volume was evaluated (each value represents the mean ± SD) and the survival was monitored up to 80 days after the tumor re-transplantation. $^*p < 0.05$; $^{**}p < 0.01$, compared with the corresponding group of N.T. N.T., non-treatment.

various types of nucleic acids, such as NF-κB decoy [48], ICAM-1 antisense oligonucleotides [49], with low doses of nucleic acids. Moreover, organ-specific gene expression was observed in US-exposed organ by exposing US to the organ directly after intravenous administration of Man-PEG_{2000} bubble lipoplexes (Supplementary Fig. 5); therefore the beforehand knockdown of inflammatory factors such as NF-κB or ICAM-1 by Man-PEG_{2000} bubble lipoplexes and US exposure might be available for the prevention of ischemia reperfusion injury, a major problem in living donor liver transplantation.

5. Conclusion

In this study, we developed the gene transfection method using Man-PEG_{2000} bubble lipoplexes and US exposure. This transfection method enabled APC-selective and efficient gene expression, and moreover, effective anti-tumor effects was obtained by applying this method to DNA vaccine therapy against cancer. This method could be widely used in a variety of targeted cell-selective and efficient gene transfection methods by substituting mannose with various ligands reported previously [2–6]. In addition, in this gene transfection method, pDNA can directly introduce the nucleic acids into the cells through the transient pores created by US-responsive degradation of bubble lipoplexes, therefore this method could apply to many ligands which are not taken up via endocytosis. These findings make a valuable contribution to overcome the poor introducing efficiency into cytoplasm which is a major obstacle for gene delivery by non-viral vectors, and show that this method is an effective method for in-vivo gene delivery.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2010.06.058.

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