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Title: Development of an ultrasound-responsive and mannose-modified gene carrier for DNA vaccine therapy

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Corresponding Author: Dr. Mitsuru Hashida, Ph.D.

Corresponding Author's Institution: Graduate School of Pharmaceutical Sciences, Kyoto University

First Author: Keita Un

Order of Authors: Keita Un; Shigeru Kawakami, Ph.D.; Ryo Suzuki, Ph.D.; Kazuo Maruyama, Ph.D.; Fumiyoshi Yamashita, Ph.D.; Mitsuru Hashida, Ph.D.

**Abstract:** Development of a gene delivery system to transfer the gene of interest selectively and efficiently into targeted cells is essential for achievement of sufficient therapeutic effects by gene therapy. Here, we succeeded in developing the gene transfection method using ultrasound (US)-responsive and mannose-modified gene carriers, named Man-PEG2000 bubble lipoplexes. Compared with the conventional lipofection method using mannose-modified carriers, this transfection method using Man-PEG2000 bubble lipoplexes and US exposure enabled approximately 500~800-fold higher gene expressions in the antigen presenting cells (APCs) selectively in vivo. This enhanced gene expression was contributed by the improvement of delivering efficiency of nucleic acids to the targeted organs, and by the increase of introducing efficiency of nucleic acids into the cytoplasm followed by US exposure. Moreover, high anti-tumor effects were demonstrated by applying this method to DNA vaccine therapy using ovalbumin (OVA)-expressing plasmid DNA (pDNA). This US-responsive and cell-specific gene delivery system can be widely applied to medical treatments such as vaccine therapy and anti-inflammation therapy, which its targeted cells are APCs, and our findings may help in establishing innovative methods for in-vivo gene delivery to overcome the poor introducing efficiency of carriers into cytoplasm which the major obstacle associated with gene delivery by non-viral carriers.

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5 **Development of an ultrasound-responsive and mannose-modified**  
6 **gene carrier for DNA vaccine therapy**  
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16 Keita Un<sup>a,b</sup>, Shigeru Kawakami<sup>a</sup>, Ryo Suzuki<sup>c</sup>, Kazuo Maruyama<sup>c</sup>,  
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18 Fumiyoshi Yamashita<sup>a</sup> and Mitsuru Hashida<sup>a,d\*</sup>  
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22

23 <sup>a</sup>Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto  
24 University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan  
25  
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27  
28 <sup>b</sup>The Japan Society for the Promotion of Science (JSPS), Chiyoda-ku, Tokyo 102-8471, Japan  
29  
30

31 <sup>c</sup>Department of Biopharmaceutics, School of Pharmaceutical Sciences, Teikyo University,  
32 1091-1 Suwarashi, Sagamiko, Sagamihara, Kanagawa 229-0195, Japan  
33  
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35  
36 <sup>d</sup>Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University,  
37 Yoshida-ushinomiya-cho, Sakyo-ku, Kyoto 606-8302, Japan  
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47 **\*Corresponding author:**  
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49 Mitsuru Hashida, Ph.D.,  
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51 Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences,  
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53 Kyoto University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan.  
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57 Phone: +81-75-753-4545. Fax: +81-75-753-4575.  
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59 E-mail: hashidam@pharm.kyoto-u.ac.jp  
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## 1. Introduction

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3 In the post-genome era, the analysis of disease-related genes has rapidly advanced, and  
4 the medical application of the information obtained from gene analysis is being put into  
5 practice. In particular, the development of effective method to transfer the gene of interest  
6 selectively and efficiently into the targeted cells is essential for the gene therapy of refractory  
7 diseases, in-vivo functional analysis of genes and establishment of animal models for diseases.  
8 However, a suitable carrier for selective and efficient gene transfer to the targeted cells is still  
9 being developed. Although various types of viral and non-viral carriers have been  
10 developed for gene transfer, they are limited to use by viral-associated pathogenesis and low  
11 transfection efficiency, respectively. For the cell-selective gene transfer, many investigators  
12 have focused on ligand-modified non-viral carriers such as liposomes [1-4], emulsions [5],  
13 micelles [6] and polymers [7], because of their high productivity and low toxicity. On the  
14 other hand, since the gene transfection efficiency by non-viral carriers is poor, it is difficult to  
15 obtain the effective therapeutic effects by gene therapy using non-viral carriers. Moreover,  
16 in the gene transfection using conventional ligand-modified non-viral carriers, since the  
17 carriers need to be taken up into cells via endocytosis following by interaction with targeted  
18 molecules on the cell membrane, the number of candidates which are suitable as ligands for  
19 targeted gene delivery is limited.

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47 Some researchers have attempted to develop the transfection method using external  
48 stimulation, such as electrical energy [8], physical pressure [9] and water pressure [10], to  
49 enhance the gene transfection efficiency. Among these, gene transfection method using US  
50 exposure and microbubbles enclosing US imaging gas, called “sonoporation method”, have  
51 been focused as effective drug/gene delivery systems [11-14]. In the sonoporation method,  
52 microbubbles are degraded by US exposure with optimized intensity, then cavitation energy is  
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1 generated by the destruction of microbubbles. Consequently, the transient pores are created  
2 on the cell membrane, and large amount of nucleic acids are directly introduced into the  
3 cytoplasm through the created pores [13,15,16]. However, the in-vivo gene transfection  
4 efficiency by conventional sonoporation method administering the nucleic acids and  
5 microbubbles separately is low because of the rapid degradation of nucleic acids in the body  
6 [17], the large particle size of conventional microbubbles [15] and the different  
7 pharmacokinetic profiles of the nucleic acids and microbubbles. Moreover, to transfer the  
8 gene into the targeted cells selectively by sonoporation method in vivo, the control of in-vivo  
9 distribution of nucleic acids and microbubbles, which are separately administered, is  
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26 In our previous report [16], we have demonstrated the effective transfection by  
27 combination-use method using our mannose-modified lipoplexes composed of Man-C4-  
28 chol: DOPE [1], and conventional Bubble liposomes (BLs) [12] with US exposure. However, this  
29 combination-use method is complicated because of the necessity for multiple injections of  
30 mannose-modified lipoplexes and BLs; therefore, it is difficult to apply for medical treatments  
31 using multiple transfections. In addition, the difference of in-vivo distribution  
32 characteristics between mannose-modified lipoplexes and BLs might be decreased its transfection  
33 efficacy. Therefore, it is essential to develop the US-responsive and cell-selective gene  
34 carriers constructed with ligand-modified gene carriers and microbubbles.  
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49 Taking these into considerations, we examined the gene transfection system for  
50 effective DNA vaccine therapy using physical stimulation and ligand-modification. First,  
51 we developed US-responsive and mannose-modified gene carriers, Man-PEG<sub>2000</sub> bubble  
52 lipoplexes (Fig. 1), by enclosing perfluoropropane gas into mannose-conjugated  
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65 lipoplexes (Fig. 1), by enclosing perfluoropropane gas into mannose-conjugated

1))pDNA complexes. Then, we evaluated the enhanced and cell-selective gene expression in the APCs by intravenous administration of Man-PEG<sub>2000</sub> bubble lipoplexes and external US exposure in mice. Finally, we examined high anti-tumor effects by applying this method to DNA vaccine therapy using OVA-expressing pDNA.

## 2. Materials and Methods

### 2.1. Mice and cell lines

Female ICR mice (4-5 weeks old) and C57BL/6 mice (6-8 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 promulgated by the US National Institutes of Health and the guideline for animal experiments of Kyoto University. CD8-OVA1.3 cells, T-cell hybridomas with specificity for OVA (257-264)-kb, were kindly provided by Dr. C.V. Harding (Case Western Reserve University, Cleveland, OH, USA) [18]. EL4 cells (C57BL/6 T lymphomas) and E.G7-OVA cells (the OVA-transfected clones of EL4) were purchased from American Type Culture Collection (Manassas, VA). CD8-OVA1.3 cells and EL4 cells were maintained in Dulbecco's modified Eagle's medium and E.G7-OVA cells were maintained in RPMI 1640. Both mediums were supplemented with 10% fetal bovine serum (FBS), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37 °C in 5% CO<sub>2</sub>.

### 2.2. pDNA

pCMV-Luc and pCMV-OVA were constructed in our previous reports [19,20]. Briefly, pCMV-Luc was constructed by subcloning the HindIII/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). pCMV-OVA was constructed by subcloning the EcoRI chicken egg albumin (ovalbumin) cDNA fragment from pAc-neo-OVA, which was kindly provided by Dr. M.J. Bevan (University of Washington, Seattle, WA, USA) into the polylinker of pVAX I. pDNA were amplified in the E. coli strain DH5α, isolated

1 and purified using a QIAGEN Endofree Plasmid Giga Kit (QIAGEN GmbH, Hilden,  
2  
3 Germany).

### 4 5 6 7 8 *2.3. Synthesis of Man-PEG<sub>2000</sub>-DSPE and preparation of Man-PEG<sub>2000</sub> bubble lipoplexes*

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10 Man-PEG<sub>2000</sub>-DSPE was synthesized in a one-step reaction by covalent binding with  
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12 NH<sub>2</sub>-PEG<sub>2000</sub>-DSPE (NOF Co., Tokyo, Japan) and 2-imino-2-methoxyethyl-1-thiomannoside  
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14 (IME-thiomannoside). IME-thiomannoside was prepared according to the method of Lee et  
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16 al. [21]. Next, NH<sub>2</sub>-PEG<sub>2000</sub>-DSPE and IME-thiomannoside were reacted, vacuum dried and  
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18 dialyzed to produce Man-PEG<sub>2000</sub>-DSPE, and then, the resultant dialysates were lyophilized.  
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20 To produce the liposomes for bubble lipoplexes, DSTAP (Avanti Polar Lipids Inc., Alabaster,  
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22 AL, USA), DSPC (Sigma Chemicals Inc., St. Louis, MO, USA) and Man-PEG<sub>2000</sub>-DSPE or  
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24 NH<sub>2</sub>-PEG<sub>2000</sub>-DSPE were mixed in chloroform at a molar ratio of 7:2:1. For construction of  
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26 BLs, DSPC and methoxy-PEG<sub>2000</sub>-DSPE (NOF Co., Tokyo, Japan) were mixed in chloroform  
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28 at a molar ratio of 94:6. The mixture for the construction of liposomes was dried by  
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30 evaporation, vacuum desiccated and the resultant lipid film was resuspended in sterile 5%  
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32 dextrose. After hydration for 30 min at 65 °C, the dispersion was sonicated for 10 min in a  
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34 bath sonicator and for 3 min in a tip sonicator to produce liposomes. Then, liposomes were  
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36 sterilized by passage through a 0.45 μm filter (Nihon-Millipore, Tokyo, Japan). The  
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38 lipoplexes were prepared by gently mixing with equal volumes of pDNA and liposome  
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40 solution at a charge ratio of 1.0:2.3 (-:~). For preparation of BLs and bubble lipoplexes, the  
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42 enclosure of US imaging gas into liposomes and lipoplexes was performed according to our  
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44 previous report [16]. Briefly, prepared liposomes and lipoplexes were added to 5 mL  
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46 sterilized vials, filled with perfluoropropane gas (Takachiho Chemical Industries Co., Ltd.,  
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48 Tokyo, Japan), capped and then pressured with 7.5 mL of perfluoropropane gas. To enclose  
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1 US imaging gas into the liposomes and lipoplexes, the vial was sonicated using a bath-type  
2 sonicator (AS ONE Co., Osaka, Japan) for 5 min. The particle sizes and zeta potentials of  
3 liposomes and lipoplexes were determined by a Zetasizer Nano ZS instrument (Malvern  
4 Instrument, Ltd., Worcestershire, UK).  
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#### 10 11 12 13 *2.4. Harvesting of mouse peritoneal macrophages*

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16 Mouse peritoneal macrophages were harvested and cultured according to our previous  
17 report [16]. Briefly, the macrophages were harvested from mice at 4 days after  
18 intraperitoneal injection of 2.9% thioglycolate medium (1 mL). The collected macrophages  
19 were washed and suspended in RPMI-1640 medium supplemented with 10% FBS, 100  
20 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine, and plated on culture  
21 plates. After incubation for 2 hr at 37 °C in 5% CO<sub>2</sub>, non-adherent cells were washed off  
22 with culture medium, and the macrophages were incubated for another 72 hr.  
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#### 34 35 36 *2.5. In-vitro gene transfection*

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39 After the macrophages were collected and incubated for 72 hr, the culture medium was  
40 replaced with Opti-MEM I containing bubble lipoplexes (5 µg pDNA). The macrophages  
41 were exposed to US (frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0  
42 W/cm<sup>2</sup>) for 20 seconds using a 6 mm diameter probe placed in the well at 5 min after addition  
43 of bubble lipoplexes. In the transfection using naked pDNA and BLs, at 5 min after addition  
44 of naked pDNA (5 µg) and BLs (60 µg total lipids) were added, and the macrophages were  
45 immediately exposed to US. US was generated using a Sonopore-4000 sonicator (NEPA  
46 GENE, Chiba, Japan). Then, 1 hr later, the incubation medium was replaced with  
47 RPMI-1640 and incubated for an additional 23 hr. Lipofectamine<sup>®</sup> 2000 (Invitrogen,  
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1 Carlsbad, CA, USA) was used according to the recommended procedures, and the exposure  
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3 time of Lipofectamine<sup>®</sup> 2000 was 1 hr, which is the same exposure time in other experiments  
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5 using lipoplexes. Following incubation for 24 hr, the cells were scraped from the plates and  
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7 suspended in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8). Then,  
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9 the cell suspension was shaken, and centrifuged at 10,000 × g, 4 °C for 10 min. The  
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11 supernatant was mixed with luciferase assay buffer (Picagene, Toyo Ink Co., Ltd., Tokyo,  
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13 Japan) and the luciferase activity was measured in a luminometer (Lumat LB 9507, EG&G  
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15 Berthold, Bad Wildbad, Germany). The luciferase activity was normalized with respect to  
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17 the protein content of cells. The protein concentration was determined with a Protein  
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19 Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). The level of  
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21 luciferase mRNA expression was determined by RT-PCR.  
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## 31 *2.6. Inhibitory experiments of endocytosis in-vitro*

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33 Endocytosis was inhibited by chlorpromazine (50 μM) as clathrin-mediated endocytosis  
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35 inhibitor [22], genistein (200 μM) as caveolae-mediated endocytosis inhibitor [23] and  
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37 5-(N-ethyl-N-isopropyl)amiloride (EIPA, 50 μM) as macropinocytosis inhibitor [24]. Each  
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39 endocytosis inhibitor was added to the macrophages at 30 min before the addition of  
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41 lipoplexes.  
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## 49 *2.7. Fluorescence photographs of pDNA in mouse peritoneal macrophages*

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51 To visualize the cellular association of pDNA by fluorescence microscopy (Biozero  
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53 BZ-8000, KEYENCE, Osaka, Japan), lipoplexes were constructed with  
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55 TM-rhodamine-labeled pDNA prepared by a Label IT Nucleic Acid Labeling Kit (Mirus Co.,  
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57 Madison, WI, USA).  
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3 2.8. *Evaluation of cytotoxic effects by MTT assay*  
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5 The cytotoxicity was evaluated by MTT assay. Briefly,  
6 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyltetrazolium bromide (MTT, Nacalai Tesque, Inc.,  
7 Kyoto, Japan) solution was added to each well and incubated for 4 hr. The resultant  
8 formazan crystals were dissolved in 0.04 M HCl-isopropanol and sonicated for 10 min in a  
9 bath sonicator. Absorbance values at 550 nm (test wavelength) and 655 nm (reference  
10 wavelength) were measured and the results were expressed as viability (%).  
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23 2.9. *In-vivo gene transfection*  
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25 Four-week-old ICR female mice were intravenously injected with 400  $\mu$ L bubble  
26 lipoplexes via the tail vein using a 26-gauge syringe needle at a dose of 50  $\mu$ g pDNA. At 5  
27 min after the injection of bubble lipoplexes, US (frequency, 1.045 MHz; duty, 50%; burst rate,  
28 10 Hz; intensity 1.0 W/cm<sup>2</sup>; time, 2 min) was exposed transdermally to the abdominal area  
29 using a Sonopore-4000 sonicator with a probe of diameter 20 mm. In the transfection using  
30 naked pDNA and BLs, at 4 min after intravenous injection of BLs (500  $\mu$ g total lipid), naked  
31 pDNA (50  $\mu$ g) was intravenously injected and US was exposed at 1 min after naked pDNA  
32 injection. At predetermined times after injection, mice were sacrificed and their organs  
33 collected for each experiment. The organs were washed twice with cold saline and  
34 homogenized with lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris, pH 7.8).  
35 The lysis buffer was added in a weight ratio of 5 mL/g for the liver or 4 mL/g for the other  
36 organs. After three cycles of freezing and thawing, the homogenates were centrifuged at  
37 10,000  $\times$  g, 4  $^{\circ}$ C for 10 min. The luciferase activity of resultant supernatant was determined  
38 by luciferase assay and the level of luciferase mRNA expression was determined by RT-PCR.  
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3 *2.10. In-vivo imaging*  
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5           At 6 hr after transfection, anesthetized mice were administrated D-luciferin (10 mg/300  
6           μL PBS) (Promega Co., Madison, WI, USA). At 10 min after injection of D-luciferin,  
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8           organs were excised and luminescent images were taken by NightOWL LB 981 NC  
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10           instrument (Berthold Technologies, GmbH, Bad Wildbad, Germany). The pseudocolor  
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12           luminescent images were generated, overlaid with organ images and the luminescence  
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14           representation was obtained using WinLight software (Berthold Technologies GmbH, Bad  
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16           Wildbad, Germany).  
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26 *2.11. Separation of mouse hepatic PCs and NPCs*  
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28           The separation of mouse hepatic PCs and NPCs was performed according to our  
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30           previous reports [19]. Briefly, at 6 hr after in-vivo transfection using bubble lipoplexes and  
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32           US exposure, each mouse was anesthetized with pentobarbital sodium (40-60 mg/kg) and the  
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34           liver was perfused with perfusion buffer (Ca<sup>2+</sup>, Mg<sup>2+</sup>-free HEPES solution, pH 7.2) for 10 min.  
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36           Then, the liver was perfused with collagenase buffer (HEPES solution, pH 7.5 containing 5  
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38           mM CaCl<sub>2</sub> and 0.05 % (w/v) collagenase (type I)) for 5 min. Immediately after the start of  
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40           perfusion, the vena cava and aorta were cut and the perfusion rate was maintained at 5  
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42           mL/min. At the end of perfusion, the liver was excised. The cells were dispersed in  
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44           ice-cold Hank's-HEPES buffer by gentle stirring and then filtered through cotton mesh sieves,  
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46           followed by centrifugation at 50 × g for 1 min. The pellets containing the hepatic PCs were  
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48           washed five times with Hank's-HEPES buffer by centrifuging at 50 × g for 1 min. The  
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50           supernatant containing the hepatic NPCs was similarly centrifuged 5 times and the resulting  
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52           supernatant was centrifuged twice at 300 × g for 10 min. Then, the PCs and NPCs were  
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1 resuspended separately in ice-cold Hank's-HEPES buffer.  
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### 5 *2.12. Isolation of mouse splenic CD11c<sup>+</sup> cells* 6 7

8 The isolation of mouse splenic CD11c<sup>+</sup> cells was performed according to our previous  
9 reports [25]. Briefly, At 6 hr after in-vivo transfection using bubble lipoplexes and US  
10 exposure, the splenic cells were suspended in ice-cold RPMI-1640 medium on ice. Red  
11 blood cells were removed by incubation with hemolytic reagent (0.15 M NH<sub>4</sub>Cl, 10 mM  
12 KHCO<sub>3</sub>, 0.1 mM EDTA) for 3 min at room temperature. The CD11c<sup>+</sup> cells were isolated by  
13 magnetic cell sorting with anti-mouse CD11c (N418) microbeads and auto MACS (Miltenyi  
14 Biotec, Inc., Auburn, CA, USA) following the manufacturer's instructions.  
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### 28 *2.13. Quantitative RT-PCR* 29 30

31 Total RNA was isolated from separated cells using a GenElute Mammalian Total RNA  
32 Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of mRNA was  
33 carried out using a PrimeScript<sup>®</sup> RT reagent Kit (Takara Bio Inc., Shiga, Japan). Real-time  
34 PCR was performed using SYBR<sup>®</sup> Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and  
35 Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN, USA) with primers.  
36 The primers for luciferase and gapdh cDNA were constructed as follows: primer for luciferase  
37 cDNA, 5'-TTCTTCGCCAAAAGCACTC-3' (forward) and  
38 5'-CCCTCGGGTGTAATCAGAAT-3' (reverse); primer for gapdh,  
39 5'-TCTCCTGCGACTTCAACA-3' (forward) and 5'-GCTGTAGCCGTATTCATTGT-3'  
40 (reverse) (Sigma-aldrich, St. Louis, MO, USA). The mRNA copy numbers were calculated  
41 for each sample from the standard curve using the instrument software ('Arithmetic Fit Point  
42 analysis' for the Lightcycler). The results were expressed as the ratio of luciferase mRNA  
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1 copy numbers to the housekeeping gene (gapdh) mRNA copy numbers.  
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#### 5 *2.14. Tissue distribution of radio-labeled pDNA*

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8 Lipoplexes constructed with <sup>32</sup>P-labeled pDNA ([α-<sup>32</sup>P]-dCTP, PerkinElmer, Inc., MA,  
9 USA) [26] were injected intravenously into mice. At predetermined times after injection,  
10 blood was collected from the vena cava under pentobarbital anesthesia. Then, mice were  
11 sacrificed and the organs were collected, rinsed with saline and weighed. The tissues were  
12 dissolved in Soluene-350 and the resultant lysates were decolorized with isopropanol and  
13 30% H<sub>2</sub>O<sub>2</sub>, and then neutralized with 5 N HCl. The radioactivity of <sup>32</sup>P-labeled pDNA was  
14 measured in scintillation counter (LSA-500, Beckman Coulter, Inc., CA, USA) after addition  
15 of Clear-Sol I solution.  
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#### 31 *2.15. Measurement of transaminase activity in the serum*

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34 At predetermined times after transfection, the serum was collected from the  
35 anesthetized mice. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST)  
36 activities in the serum were determined using Transaminase CII-Test Wako kit (Wako Pure  
37 Chemical Industries Ltd., Tokyo, Japan) according to manufacturer's instructions.  
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#### 47 *2.16. Antigen presenting assay*

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49 The evaluation of antigen presentation on MHC class I molecules in the splenic  
50 dendritic cells was performed by in-vitro antigen presentation assay using CD8-OVA1.3 cells,  
51 which are T-cell hybridomas with specificity for OVA. The CD11c<sup>+</sup> cells isolated from  
52 immunized mice were plated in a 96-well plate at various cells numbers and co-cultured with  
53 CD8-OVA1.3 cells (1 × 10<sup>5</sup>) for 20 hr. The antigen presentation on MHC class I molecules  
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1 was evaluated by IL-2 secreted from activated CD8-OVA1.3 cells measured by a commercial  
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3 IL-2 ELISA Kit (Bay bioscience Co., Ltd., Hyogo, Japan).  
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#### 8 *2.17. Evaluation of OVA-specific cytokine secretion from the splenic cells*

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10 At 2 weeks after the last immunization, the splenic cells collected from immunized  
11 mice were plated in 96-well plates and incubated for predetermined times at 37 °C in the  
12 presence or absence of OVA (100 µg). IFN-γ and IL-4 in the culture medium were  
13 measured by the commercial ELISA Kit, respectively (Bay bioscience Co., Ltd., Hyogo,  
14 Japan).  
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#### 26 *2.18. OVA-specific CTL assay*

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28 At 2 weeks after the last immunization, the splenic cells harvested from immunized  
29 mice were plated in 6-well plates and co-incubated with mitomycin C-treated E.G7-OVA  
30 cells or EL4 cells for 4 days. After co-incubation, non-adherent cells were collected, washed  
31 and plated in 96-well plates with target cells (E.G7-OVA cells or EL4 cells) at various  
32 effector/target (E/T) ratios. The target cells were labeled with <sup>51</sup>Cr by incubating with  
33 Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (PerkinElmer, Inc., MA, USA) in culture medium for 1 hr at 37 °C. At 4 hr after  
34 incubation, the plates were centrifuged and the resultant supernatant of each well was  
35 collected and the radioactivity of released <sup>51</sup>Cr was measured in a gamma counter. The  
36 percentage of <sup>51</sup>Cr release was calculated as follows: specific lysis (%) = [(experimental <sup>51</sup>Cr  
37 release - spontaneous <sup>51</sup>Cr release)/(maximum <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release)] × 100.  
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### 2.19. Therapeutic effects

C57BL/6 mice were immunized three times bi-weekly. 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  $\pm$  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 Turkey-Kramer test.

### 3. Results

#### 3.1. *In-vitro gene transfection properties by Man-PEG<sub>2000</sub> lipoplexes*

Polyethylene-glycol (PEG) modification of particles is necessary to enclose US imaging gas stably and to prepare the small-sized microbubbles for in-vivo administration [12]. Firstly, we developed mannose-conjugated PEG<sub>2000</sub>-modified lipids (Man-PEG<sub>2000</sub>-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<sub>2000</sub>-modified lipoplexes (Man-PEG<sub>2000</sub> lipoplexes) containing Man-PEG<sub>2000</sub> lipids. The particle sizes and zeta potentials of Man-PEG<sub>2000</sub> lipoplexes and non-modified PEG<sub>2000</sub>-lipoplexes (Bare-PEG<sub>2000</sub> 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<sub>2000</sub> lipoplexes were significantly higher than those by Bare-PEG<sub>2000</sub> lipoplexes (Figs. 2A and 2B). Then, the level of gene expression obtained by Man-PEG<sub>2000</sub> lipoplexes was suppressed to same extent as that by Bare-PEG<sub>2000</sub> lipoplexes in the presence of an excess of mannan (Fig. 2A). Moreover, this level of gene expression obtained by Man-PEG<sub>2000</sub> lipoplexes was also suppressed to same extent as that by Bare-PEG<sub>2000</sub> 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<sub>2000</sub> 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<sub>2000</sub> lipoplexes*

Since the degradation of pDNA by nuclease in the blood is one of the critical factors in

1 the in-vivo gene transfection by intravenously administration of lipoplexes, we investigated  
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3 the stability of Bare-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> lipoplexes against nucleases.  
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5 Following electrophoresis of naked pDNA and lipoplexes after incubation with DNase I,  
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7 although naked pDNA underwent the degradation by DNase I, lipoplexes did not undergo the  
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9 degradation and retained the complex forms (Supplementary Fig. 2). Then, we investigated  
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11 the gene expression characteristics of Man-PEG<sub>2000</sub> lipoplexes in the liver and spleen, which  
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13 are the targeted organs of mannose-modified carriers [27]. In this study, liver was separated  
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15 in the parenchymal cells (PCs) and non-parenchymal cells (NPCs), and spleen was separated  
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17 in the dendritic cells (CD11c<sup>+</sup> cells) and other cells (CD11c<sup>-</sup> cells). As shown in Figs. 2C  
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19 and 2D, following intravenous administration of Man-PEG<sub>2000</sub> lipoplexes, selective gene  
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21 expression was observed in the hepatic NPCs and the splenic CD11c<sup>+</sup> cells, which are the  
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23 APCs expressing mannose receptors abundantly [28-30].  
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### 34 *3.3. In-vitro gene transfection efficiency by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure*

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36 Although Man-lipoplexes showed the APC-selective gene transfection properties in  
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38 vivo, this level of gene expression was low compared with our previous reports [1,19,25].  
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40 To enhance the level of gene expression by sonoporation method, we developed Man-PEG<sub>2000</sub>  
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42 bubble lipoplexes (Fig. 1) by enclosing US imaging gas (perfluoropropane gas) into  
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44 Man-PEG<sub>2000</sub> lipoplexes. The lipid composition of lipoplexes is important for the stable  
45  
46 enclosure of US imaging gas. Following optimization of lipid composition, lipoplexes  
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48 constructed with the saturated lipids only, which have a high melting temperature (T<sub>m</sub>), were  
49  
50 enclosed US imaging gas stably (Supplementary Table 2). Following enclosure of US  
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52 imaging gas in lipoplexes, lipoplexes became cloudy and their particle sizes were increased  
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54 (from 150 nm to 550 nm, approximately) (Supplementary Fig. 3A and Supplementary Table  
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1 3). Then, since the zeta potentials of bubble lipoplexes were lower than that of bubble  
2 liposomes and same as that of lipoplexes (Supplementary Tables 1 and 3), it is considered that  
3 pDNA is attached on the surface of bubble liposomes. Moreover, the stability against  
4 nucleases observed in Man-PEG<sub>2000</sub> lipoplexes (Supplementary Fig. 2) was maintained after  
5 enclosure of US imaging gas into lipoplexes (Supplementary Fig. 3B).  
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8 The level of gene expression obtained by Man-PEG<sub>2000</sub> bubble lipoplexes and US  
9 exposure was 500-fold higher than that by Man-PEG<sub>2000</sub> lipoplexes in mouse cultured  
10 macrophages expressing mannose receptors abundantly, and also higher than that by  
11 non-modified bubble lipoplexes (Bare-PEG<sub>2000</sub> bubble lipoplexes, Fig. 1) and US exposure or  
12 conventional sonoporation method using naked pDNA and BLs (Fig. 3A). This enhanced  
13 gene expression was observed when bubble lipoplexes and US exposure were used for  
14 in-vitro gene transfer (Fig. 3B). The cellular association of pDNA obtained by transfection  
15 using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure was also 10-fold higher than that by  
16 Man-PEG<sub>2000</sub> lipoplexes, and also higher than that by Bare-PEG<sub>2000</sub> bubble lipoplexes and US  
17 exposure or conventional sonoporation method using naked pDNA and BLs (Fig. 3C and  
18 Supplementary Fig. 4A). Moreover, this level of gene expression obtained by Man-PEG<sub>2000</sub>  
19 bubble lipoplexes and US exposure was comparable to that by Lipofectamine<sup>®</sup> 2000, which is  
20 widely used as a gene transfection reagent (Fig. 3D). On the other hand, the cytotoxicity by  
21 Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure was lower than that by Lipofectamine<sup>®</sup>  
22 2000 (Fig. 3E).  
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#### 54 *3.4. Intracellular uptake properties of pDNA by Man-PEG<sub>2000</sub> bubble lipoplexes and US* 55 *exposure* 56 57

58 The gene expression obtained by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure was  
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1 significantly suppressed in the presence of an excess of mannan (Fig. 4A). Therefore, the  
2 interaction with mannose receptors on the cell membrane is involved in the gene transfection  
3 by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure, similar to the gene transfection by  
4 Man-PEG<sub>2000</sub> lipoplexes. On the other hand, unlike Man-PEG<sub>2000</sub> lipoplexes (Fig. 2B), the  
5 gene expression obtained by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure was not  
6 suppressed in the presence of chlorpromazine (Fig. 4B), which is a clathrin-mediated  
7 endocytosis inhibitor [22]. These results agreed with the results of cellular association of  
8 pDNA (Supplementary Fig. 4B), and indicated that pDNA delivered by Man-PEG<sub>2000</sub> bubble  
9 lipoplexes was directly introduced into the cytoplasm without mediating endocytosis by the  
10 gene transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure.  
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### 3.5. *In-vivo* gene transfection efficiency by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure

31 As shown in Figs. 5A and 5B, the level of gene expression obtained by Man-PEG<sub>2000</sub>  
32 bubble lipoplexes and US exposure was 500~800-fold higher than that by Man-PEG<sub>2000</sub>  
33 lipoplexes, and also higher than that by Bare-PEG<sub>2000</sub> bubble lipoplexes and US exposure or  
34 the conventional sonoporation method using naked pDNA and BLs in the liver and spleen,  
35 which are the targeted organs of mannose-modified carriers [27]. This enhanced gene  
36 expression in the liver and spleen was observed when bubble lipoplexes and US exposure  
37 were used for in-vivo gene transfer (Figs. 5C and 5D). Moreover, this gene expression  
38 obtained by Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure or Man-PEG<sub>2000</sub> bubble  
39 lipoplexes with US exposure in the liver and spleen remained higher than that by  
40 Bare-PEG<sub>2000</sub> lipoplexes or Man-PEG<sub>2000</sub> lipoplexes for at least 48 hr, respectively (Figs. 5E  
41 and 5F). In addition, the gene expression was also enhanced in the US-exposed organ  
42 specifically following gene transfection by direct US exposure to the targeted organ after  
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1 intravenous administration of Man-PEG<sub>2000</sub> bubble lipoplexes (Supplementary Fig. 5). On  
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3 the other hand, the increase of gene expression by bubble lipoplexes and US exposure was not  
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5 observed in other organ such as lung, kidney and heart (Figs. 5G and 5H).  
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### 8 9 10 *3.6. Targeted cell-selective gene transfection properties by Man-PEG<sub>2000</sub> bubble lipoplexes* 11 12 *and US exposure in vivo*

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15 We investigated the mannose receptor-expressing cell selectivity of gene expression by  
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17 transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. In the liver, the level of  
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19 gene expression in the hepatic NPCs expressing mannose receptors was significantly higher  
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21 than that in the hepatic PCs following gene transfection by Man-PEG<sub>2000</sub> bubble lipoplexes  
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23 and US exposure (Fig. 6A). This difference in gene expression between the NPCs and PCs  
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25 obtained by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure was similar to that by  
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27 Man-PEG<sub>2000</sub> lipoplexes, although the level of gene expression in the NPCs and PCs was  
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29 markedly higher. On the other hand, selective gene expression in the NPCs was not  
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31 observed by Bare-PEG<sub>2000</sub> bubble lipoplexes and US exposure.  
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39 In the spleen, the level of mRNA expression in the CD11c<sup>+</sup> cells, which are the splenic  
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41 dendritic cells expressing mannose receptors, was significantly higher than that in the CD11c<sup>-</sup>  
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43 cells following transfection by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure (Fig. 6B).  
44  
45 On the other hand, selective gene expression in the CD11c<sup>+</sup> cells was not observed by  
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47 Bare-PEG<sub>2000</sub> bubble lipoplexes and US exposure.  
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### 54 *3.7. In-vivo distribution properties of pDNA by Man-PEG<sub>2000</sub> bubble lipoplexes and US* 55 56 *exposure*

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

### 31 *3.8. The liver toxicity by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure*

32 We examined ALT and AST activities in the serum to investigate the liver toxicity by  
33 gene transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. ALT and AST  
34 activities in the serum were increased by gene transfection using Bare-PEG<sub>2000</sub> lipoplexes and  
35 Man-PEG<sub>2000</sub> lipoplexes. On the other hands, the increase of ALT and AST activities was  
36 not observed by gene transfection using Bare-PEG<sub>2000</sub> bubble lipoplexes and Man-PEG<sub>2000</sub>  
37 bubble lipoplexes with US exposure (Fig. 8).

### 38 *3.9. Antigen presentation on MHC class I molecules in immunized splenic dendritic cells*

39 To investigate the DNA vaccine effects by Man-PEG<sub>2000</sub> bubble lipoplexes and US  
40 exposure, we prepared Man-PEG<sub>2000</sub> bubble lipoplexes constructed with pDNA expressing  
41 OVA as a model antigen. Firstly, to investigate the antigen (OVA) presentation on MHC  
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1 class I molecules in the splenic dendritic cells (CD11c<sup>+</sup> cells) by Man-PEG<sub>2000</sub> bubble  
2 lipoplexes constructed with pCMV-OVA and US exposure, the splenic CD11c<sup>+</sup> cells isolated  
3 from once-immunized mice were co-incubated with CD8-OVA1.3 cells, which are T-cell  
4 hybridomas with specificity for OVA. Following measurement of IL-2 to evaluate the  
5 activation of T-cells, the IL-2 secretion from activated CD8-OVA1.3 cells co-incubated with  
6 the CD11c<sup>+</sup> cells isolated from mice immunized by Man-PEG<sub>2000</sub> bubble lipoplexes and US  
7 exposure was the highest of all (Fig. 9A). This result indicates that DNA vaccination by  
8 Man-PEG<sub>2000</sub> bubble lipoplexes constructed with pCMV-OVA and US exposure can induce  
9 significantly high CD8<sup>+</sup>-T lymphocyte activation.  
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### 26 *3.10. Antigen-specific cytokine secretion from immunized splenic cells*

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28 We evaluated the OVA-specific cytokine secretion from the splenic cells immunized by  
29 Man-PEG<sub>2000</sub> bubble lipoplexes constructed with pCMV-OVA and US exposure. Following  
30 optimization of immunization schedule, it was shown that a 2-week interval was necessary to  
31 achieve the same level of gene expression as former transfection in the spleen (Supplementary  
32 Fig. 6) and at least three-times immunization was necessary to effective anti-tumor effects by  
33 DNA vaccination using this method (Supplementary Fig. 7). Therefore, the immunization to  
34 mice was performed according to the protocol shown in Fig. 9B. As shown in Fig. 9C, in  
35 the presence of OVA, the highest amount of IFN- $\gamma$  (Th1 cytokine) was secreted from splenic  
36 cells harvested from mice immunized with Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure.  
37 On the other hand, no secretion of IFN- $\gamma$  was observed in any of the groups in the absence of  
38 OVA. Moreover, the secretion of IL-4 (Th2 cytokine) was not increased in any of the groups  
39 both in the presence or absence of OVA (Fig. 9C). These results suggest that immunization  
40 by Man-PEG<sub>2000</sub> bubble lipoplexes constructed with pCMV-OVA and US exposure  
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1 significantly enhances the differentiation of helper T cells to Th1 cells, which are pivotal cells  
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3 for the activation of cytotoxic T lymphocytes (CTL) with high anti-tumor activity, by  
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5 OVA-stimulation.  
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### 7 8 9 10 11 *3.11. Antigen-expressing cell specific CTL activity in immunized splenic cells*

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13 Next, we assessed the CTL activity in the splenic cells harvested from mice immunized  
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15 by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. Following experiments according to  
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17 the protocol shown in Fig. 9B, the splenic cells immunized by Man-PEG<sub>2000</sub> bubble  
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19 lipoplexes constructed with pCMV-OVA and US exposure showed the highest CTL activity in  
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21 all groups against E.G7-OVA cells which are the lymphoma cells expressing OVA (Fig. 9D).  
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23 In contrast, the CTL activity was not observed in EL4 cells which are the lymphoma cells not  
24  
25 expressing OVA in all groups (Fig. 9D). These results indicate that the splenic cells  
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27 immunized by Man-PEG<sub>2000</sub> bubble lipoplexes constructed with pCMV-OVA and US  
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29 exposure induce the OVA-expressing cell specific CTL activity.  
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### 39 *3.12. Therapeutic effects against antigen-expressing tumor by DNA vaccination*

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41 Finally, we investigated the anti-tumor effects by DNA vaccination using Man-PEG<sub>2000</sub>  
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43 bubble lipoplexes and US exposure. Following experiments according to the protocol  
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45 shown in Fig. 10A, significantly high anti-tumor effects against E.G7-OVA cells were  
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47 observed in mice immunized by Man-PEG<sub>2000</sub> bubble lipoplexes constructed with  
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49 pCMV-OVA and US exposure (Fig. 10B). However, in mice transplanted EL4 cells, no  
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51 anti-tumor effects were observed in any of the groups (Fig. 10C). Moreover, we investigated  
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53 the maintenance of DNA vaccine effects following administration of Man-PEG<sub>2000</sub> bubble  
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55 lipoplexes and US exposure. According to the protocol shown in Fig. 11A, E.G7-OVA cells  
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1 were re-transplanted into mice which first-transplanted tumors were completely rejected by  
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3 DNA vaccination using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. As results, high  
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5 anti-tumor effects were observed in mice following re-transplantation of E.G7-OVA cells (Fig.  
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8 11B); therefore it was demonstrated that DNA vaccine effects obtained by Man-PEG<sub>2000</sub>  
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10 bubble lipoplexes constructed with pCMV-OVA and US exposure were maintained for at least  
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13 80 days.  
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#### 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<sub>2000</sub> bubble lipoplexes, which had selectivity to the APCs and responded to US exposure. The gene delivery system using Man-PEG<sub>2000</sub> 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<sub>2000</sub>-modification is necessary to enclose US imaging gas stably [12], we prepared Man-PEG<sub>2000</sub> lipoplexes containing Man-PEG<sub>2000</sub> lipids. This Man-PEG<sub>2000</sub> 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<sub>2000</sub> 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

1 contributed by the reduced interaction with the cell membrane and the reduction of endosomal  
2 escape efficiency by PEG<sub>2000</sub>-modification [36,37]. In the sonoporation method, Tachibana  
3 et al. demonstrated that a transient pore is created on the cell membrane followed by the  
4 degradation of microbubbles [38]. Then, nucleic acids, such as pDNA, siRNA and  
5 oligonucleotides, are introduced into the cell through the generated pore [13,15,16].  
6 Consequently, since the nucleic acids are directly introduced into cytoplasm in the  
7 sonoporation method [13,14], it is considered that the low level of transfection efficiency  
8 obtained by Man-PEG<sub>2000</sub> lipoplexes can be overcome by applying sonoporation method.  
9 As shown in Figs. 3 and 4, a large amount of pDNA is directly introduced into the cytoplasm  
10 and high level of gene expression is observed by gene transfection using Man-PEG<sub>2000</sub> bubble  
11 lipoplexes and US exposure. Therefore, by delivering pDNA to the APCs using  
12 Man-PEG<sub>2000</sub> bubble lipoplexes, it is suggested that high level of gene expression in the APCs  
13 can easily achieve by following US exposure in this gene transfection method.  
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34 In this study, the level of gene expression obtained by transfection using Man-PEG<sub>2000</sub>  
35 bubble lipoplexes and US exposure was higher than that obtained by Man-PEG<sub>2000</sub> lipoplexes  
36 or Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure in the liver and spleen (Fig. 5).  
37 Moreover, gene expression by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure was observed  
38 selectively in the hepatic NPCs and the splenic dendritic cells (Fig. 6), known as mannose  
39 receptor-expressing cells [28-30]. Although this selectivity of gene expression was the same  
40 as that obtained by mannosylated lipoplexes reported previously by our group [1,25], this  
41 level of gene expression was markedly higher. It is considered that this enhanced and  
42 cell-selective gene expression is contributed by the increase of interaction with mannose  
43 receptor-expressing cells by mannose modification (Supplementary Fig. 1), by the  
44 improvement of delivering efficiency of nucleic acids to the targeted organs (Fig. 7) and by  
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1 the direct introduction of nucleic acids into the cytoplasm of targeted cells followed by US  
2 exposure to Man-PEG<sub>2000</sub> bubble lipoplexes (Figs. 3C, 4B and Supplementary Fig. 4).  
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4 Moreover, the enhanced gene expression was not observed in the lung, kidney and spleen  
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6 (Figs. 5G and 5H). It is guessed that the reason why the enhanced gene expression was not  
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8 observed in the lung is because US is not spread to the thoracic cavity by the diaphragm, and  
9  
10 the reason why the enhanced gene expression was not observed in the kidney and heart was  
11  
12 because the distributed amounts of bubble lipoplexes were markedly small. In addition,  
13  
14 since the particle size of bubble lipoplexes (approximately 500 nm) is suitable for delivery to  
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16 the liver and spleen, compared with stabilized liposomes (approximately 100 nm) [39], the  
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18 gene transfection system using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure is a suitable  
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20 method for the selective delivery of nucleic acids into the mannose receptor-expressing cells  
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22 in the liver and spleen.  
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31 On the other hand, the liver toxicity followed by gene transfection using Man-PEG<sub>2000</sub>  
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33 bubble lipoplexes and US exposure was lower than that by Man-PEG<sub>2000</sub> lipoplexes (Fig. 8).  
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35 It was reported that the CpG motifs in the pDNA sequence are recognized to Toll-like receptor  
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37 9 (TLR9) in the endosomes [40,41]; therefore it has been considered that the production of  
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39 proinflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$  and IL-12, could be induced in the  
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41 lipofection method using liposomes and emulsions, and these cytokines cause liver injury [42].  
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43 However, in the gene transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure, a  
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45 large amount of pDNA was directly introduced into the cytoplasm not-mediated endocytosis  
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47 (Figs. 3C, 4B and Supplementary Fig. 4). Therefore, it is considered that pDNA is not  
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49 recognized to TLR9 in the endosomes, and consequently liver toxicity followed by  
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51 transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure is low.  
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59 In the previous study [16], we developed combination-use method using mannosylated  
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1 lipoplexes [1] and BLs [12] with US exposure to achieve targeted cell-selective gene transfer.  
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3 However, this combination-use method is complicated because of the necessity of twice  
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5 injection of mannosylated lipoplexes and BLs, therefore it is difficult to apply for medical  
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7 treatments using multiple injection. Moreover, it is considered that the difference of in-vivo  
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9 distribution characteristics between mannosylated lipoplexes and BLs might be decreased its  
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11 transfection efficacy. On the other hand, this transfection method using Man-PEG<sub>2000</sub> bubble  
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13 lipoplexes and US exposure is handy because of using only once injection of Man-PEG<sub>2000</sub>  
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15 bubble lipoplexes and external US exposure. In addition, this method using Man-PEG<sub>2000</sub>  
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17 bubble lipoplexes and US exposure overcame the difference of in-vivo distribution of  
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19 formulations, which might lead to the decrease of transfection efficiency. In fact, the level  
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21 of gene expression by this method was higher than that by combination-use method reported  
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23 previously in the targeted organs (liver and spleen) (Fig. 5) and targeted cells (hepatic NPC  
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25 and splenic dendritic cells) (Fig. 6); therefore this gene transfection method using  
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27 Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure is more suitable for APC-selective gene  
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29 transfer in vivo.  
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39 Since APC-selective and efficient gene expression was observed by transfection using  
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41 Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure, effective therapeutic effects are to be  
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43 expected by applying this transfection method to DNA vaccine therapy, which the targeted  
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45 cells are the APCs, using tumor specific antigen-coding pDNA [31,32]. However, since the  
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47 level of gene expression by transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US  
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49 exposure was reduced sequentially (Supplementary Fig. 6), multiple transfections are  
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51 essential to obtain more effective vaccine effects against cancer (Supplementary Fig. 7). On  
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53 the other hand, a 2-week interval was needed to achieve the same level of gene expression by  
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55 lipoplexes or bubble lipoplexes with US exposure as former transfection in the spleen  
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1 (Supplementary Figs. 7B and 7C). Meyer et al. reported that the optimal transfection  
2 interval was necessary to achieve high gene expression by the second transfection using  
3 lipofection methods because of IFN- $\gamma$  secretion by intravenous administration of lipoplexes  
4 [43]; therefore it is considered that a similar phenomenon is contributed to the sonoporation  
5 methods using microbubbles constructed with phospholipids. Based on these findings, we  
6 performed the optimization of immunization times (Supplementary Fig. 7) and determined the  
7 optimal immunization schedule as shown in Figs. 9B, 10A and 11A.  
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18 In DNA vaccine therapy, unlike cancer immunotherapy using tumor-specific antigenic  
19 peptides, the peptides expressed as gene products in the cells act as the internal antigen.  
20 Since the internal antigens are presented on MHC class I molecules, the strong activation of  
21 CTL and high anti-tumor effects are expected in DNA vaccination therapy [44,45]. In this  
22 study, by applying this gene transfection method to DNA vaccine therapy using  
23 OVA-expressing pDNA, i) the presentation of OVA-peptides on MHC class I molecules of  
24 splenic dendritic cells, ii) OVA-specific Th1 cytokine secretion from splenic cells by OVA  
25 stimulation and iii) marked activation of CTL against OVA-expressing tumor were observed  
26 by gene transfection using Man-PEG<sub>2000</sub> bubble lipoplexes constructed with pCMV-OVA and  
27 US exposure (Fig. 9). Moreover, high and long-term anti-tumor effects against  
28 OVA-expressing tumor were observed in mice transfected by Man-PEG<sub>2000</sub> bubble lipoplexes  
29 constructed with pCMV-OVA and US exposure (Figs. 10 and 11). It is considered that these  
30 results are contributed by APS-selective and efficient gene transfection efficiency using  
31 Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. Although more detailed examination by  
32 pDNA encoding other tumor-specific antigens, such as gp100 in melanoma or PSA in prostate  
33 cancer [45], is necessary, this transfection method by Man-PEG<sub>2000</sub> bubble lipoplexes and US  
34 exposure might be available for DNA vaccine therapy.  
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1 The gene transfection method using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure  
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3 was enabled selective and efficient gene transfer to the mannose receptor-expressing cells in  
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5 the liver such as Kupffer cells and hepatic endothelial cells, which are components of the  
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7 APCs (Fig. 6A). Therefore, this method is also suitable for anti-inflammatory therapy  
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9 targeted to Kupffer cells and hepatic endothelial cells, known to play a key role in  
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11 inflammation [46,47]. In spite of low liver toxicity, since this gene transfection system  
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13 showed NPC-selective and efficient gene expression in the liver (Fig. 8), better therapeutic  
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15 effects could be expected by Man-PEG<sub>2000</sub> bubble lipoplexes constructed with various types  
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17 of nucleic acids, such as NF- $\kappa$ B decoy [48], ICAM-1 antisense oligonucleotides [49], with  
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19 low doses of nucleic acids. Moreover, organ-specific gene expression was observed in  
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21 US-exposed organ by exposing US to the organ directly after intravenous administration of  
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23 Man-PEG<sub>2000</sub> bubble lipoplexes (Supplementary Fig. 5); therefore the beforehand knockdown  
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25 of inflammatory factors such as NF- $\kappa$ B or ICAM-1 by Man-PEG<sub>2000</sub> bubble lipoplexes and  
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27 US exposure might be available for the prevention of ischemia reperfusion injury, a major  
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29 problem in living donor liver transplantation.  
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## 5. Conclusion

In this study, we developed the gene transfection method using Man-PEG<sub>2000</sub> 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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**Figure Captions**

**Fig. 1.** Structure of Bare-PEG<sub>2000</sub> bubble lipoplex containing NH<sub>2</sub>-PEG<sub>2000</sub>-DSPE and Man-PEG<sub>2000</sub> bubble lipoplex containing Man-PEG<sub>2000</sub>-DSPE used in this study.

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

**Fig. 3.** Enhancement of gene expression by Man-PEG<sub>2000</sub> 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<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes

with US exposure, Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (5 µg pDNA) at 24 hr after transfection. Significant difference; \*  $p < 0.05$ ; \*\*, <sup>††</sup>  $p < 0.01$ . (B) The level of luciferase expression obtained by Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with or without US exposure (5 µg pDNA) at 24 hr after transfection. \*\*  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> lipoplex, <sup>††</sup>  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> lipoplex + US, <sup>‡‡</sup>  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> bubble lipoplex. (C) Representative fluorescent images of cellular association of pDNA obtained by Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (5 µg pDNA) at 2 hr 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<sub>2000</sub> bubble lipoplexes (5 µg pDNA) and US exposure with that by Lipofectamine 2000. \*\*  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> lipoplexes, <sup>††</sup>  $p < 0.01$ , compared with Lipofectamine 2000 (0.1 µg). (E) Comparison of cell viability by transfection using Man-PEG<sub>2000</sub> 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 ( $n = 4$ ).

**Fig. 4.** Effects of mannan and chlorpromazine on gene expression by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure in vitro. (A) The level of luciferase expression obtained by Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (5 µg pDNA) in the absence or presence of 1 mg/mL mannan at 24 hr after transfection. \*\*  $p < 0.01$ , compared with the corresponding group of -mannan. (B) The level of luciferase expression by Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (5 µg pDNA) in the absence or presence of

50  $\mu$ M chlorpromazine at 24 hr after transfection. Each value represents the mean + SD ( $n = 4$ ).

**Fig. 5.** Enhancement of mannose receptor-expressing cells-selective gene expression by Man-PEG<sub>2000</sub> 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<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure, Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu$ g pDNA) in the liver (A) and spleen (B) at 6 hr after transfection. Significant difference; \*\*, <sup>††</sup>  $p < 0.01$ . (C, D) The level of luciferase expression obtained by Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with or without US exposure (50  $\mu$ g pDNA) in the liver (C) and spleen (D) at 6 hr after transfection. \*\*  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> lipoplex, <sup>††</sup>  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> lipoplex + US, <sup>‡‡</sup>  $p < 0.01$ , compared with Man-PEG<sub>2000</sub> bubble lipoplex. (E, F) Time-course of luciferase expression in the liver (E) and spleen (F) after transfection by Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu$ g pDNA). Each value represents the mean  $\pm$  SD ( $n = 4$ ). \*\*  $p < 0.01$ , compared with Bare-PEG<sub>2000</sub> bubble lipoplex + US, <sup>†</sup>  $p < 0.05$ ; <sup>††</sup>  $p < 0.01$ , compared with Bare-PEG<sub>2000</sub> lipoplex. (G) In-vivo imaging photographs of luciferase expression in the isolated organs at 6 hr after transfection by Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu$ g pDNA). (H) The level of luciferase expression in each organ at 6 hr after transfection by Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu$ g pDNA). \*\*  $p < 0.01$ , compared with the corresponding group of Bare-PEG<sub>2000</sub> lipoplex, <sup>††</sup>  $p < 0.01$ , compared with

the corresponding group of Man-PEG<sub>2000</sub> lipoplex, <sup>##</sup>  $p < 0.01$ , compared with the corresponding group of Bare-PEG<sub>2000</sub> bubble lipoplex + US. Each value represents the mean + SD ( $n = 4$ ).

**Fig. 6.** Hepatic and splenic cellular localization of luciferase expression by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. (A) Hepatic cellular localization of luciferase expression at 6 hr after transfection by Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu$ g pDNA). \*\*  $p < 0.01$ , compared with the corresponding group of PCs. (B) Splenic cellular localization of luciferase mRNA expression at 6 hr after transfection by Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu$ g pDNA). \*\*  $p < 0.01$ , compared with the corresponding group of CD11c<sup>-</sup> cells. Each value represents the mean + SD ( $n = 4$ ).

**Fig. 7.** Tissue distribution of pDNA by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. Tissue distribution after intravenous administration of (A) Bare-PEG<sub>2000</sub> bubble lipoplexes and (B) Man-PEG<sub>2000</sub> bubble lipoplexes (50  $\mu$ g 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  $\pm$  SD ( $n = 3$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ , compared with the corresponding group of -US exposure.

**Fig. 8.** Liver toxicity by gene transfection using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. Time-course of serum transaminase activities after transfection by Bare-PEG<sub>2000</sub>

lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> 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 ( $n = 4$ ).

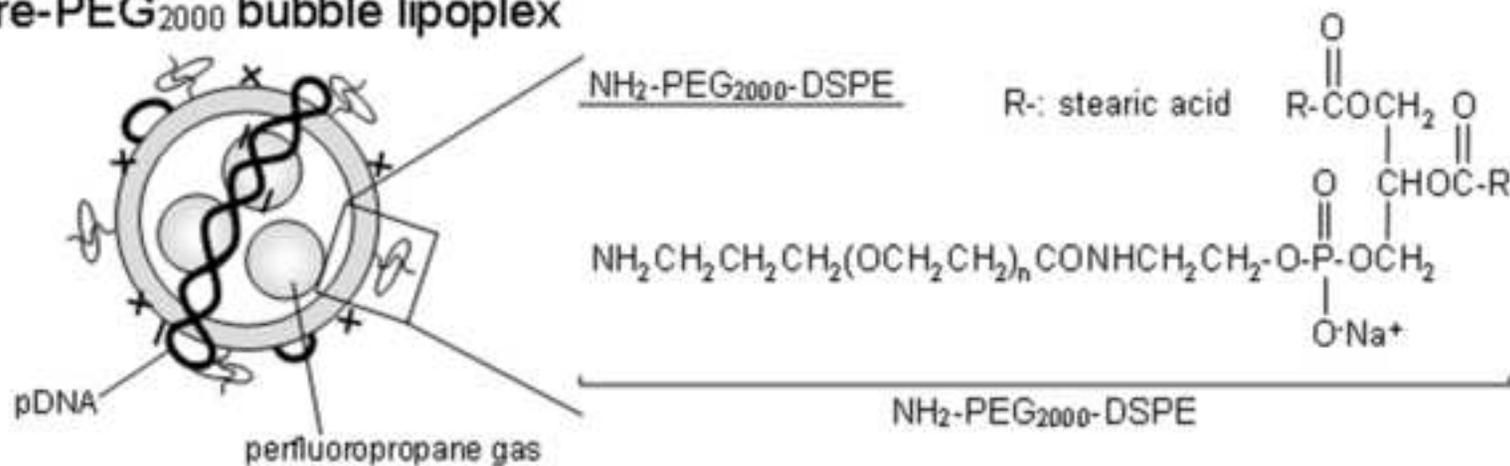
**Fig. 9.** Cytokine secretion characteristics and CTL activities by DNA vaccination using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. (A) OVA presentation on MHC class I molecules in the splenic CD11c<sup>+</sup> cells at 24 hr after transfection by Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> 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<sup>+</sup> 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- $\gamma$  and IL-4 secretion from the splenic cells immunized three times biweekly by Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> 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 hr in the absence or presence of 100 µg OVA, IFN- $\gamma$  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<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> 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  $^{51}\text{Cr}$  release assay. Each value represents the mean  $\pm$  SD ( $n = 4$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ , compared with the corresponding group of N.T.. N.T., non-treatment.

**Fig. 10.** Anti-tumor effects by DNA vaccination using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. (A) Schedule of immunization for experiments of therapeutic effects. (B, C) Anti-tumor effects by immunization using Bare-PEG<sub>2000</sub> lipoplexes, Man-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (50  $\mu\text{g}$  pDNA). Two weeks after last immunization, (B) E.G7-OVA cells or (C) EL4 cells ( $1 \times 10^6$  cells) were transplanted subcutaneously into the back of mice ( $n = 8-11$ ). The tumor volume was evaluated (each value represents the mean  $\pm$  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.

**Fig. 11.** Maintenance of Anti-tumor effects by DNA vaccination using Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. At 80 days after first transplantation of E.G7-OVA cells to immunized mice three times by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure, E.G7-OVA cells ( $1 \times 10^6$  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  $\pm$  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.

### Bare-PEG<sub>2000</sub> bubble lipoplex



### Man-PEG<sub>2000</sub> bubble lipoplex

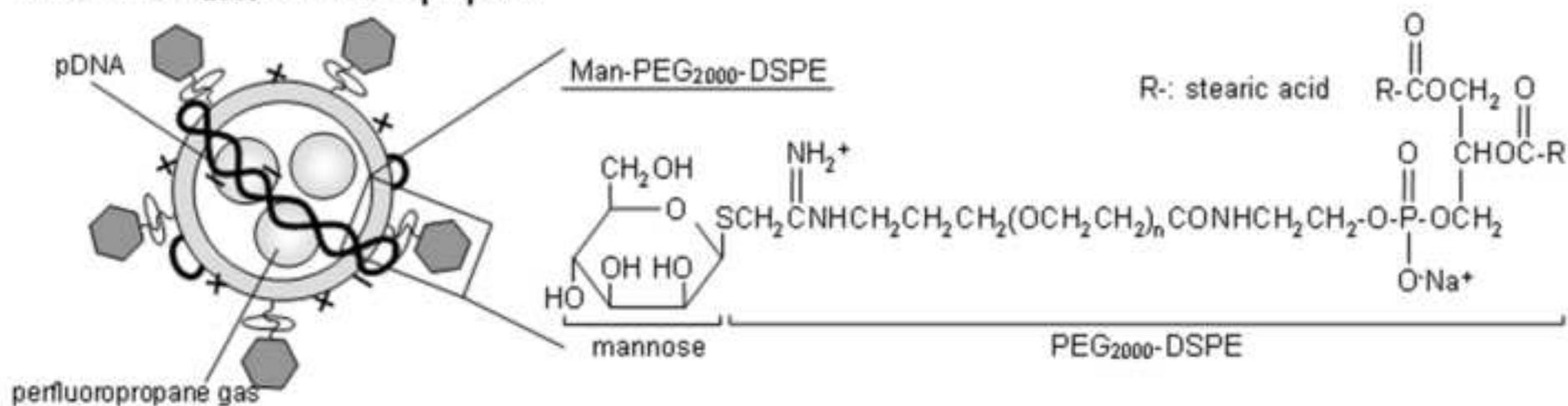
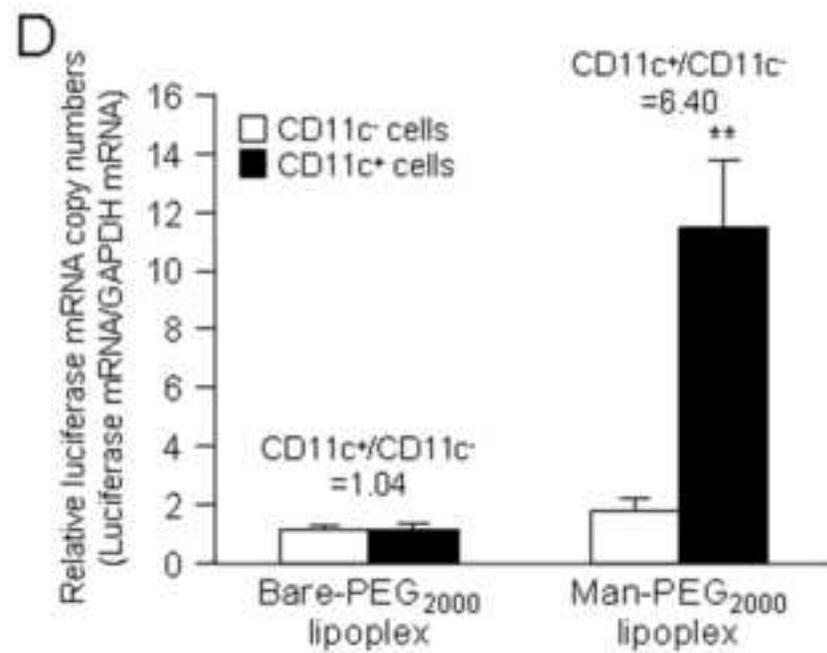
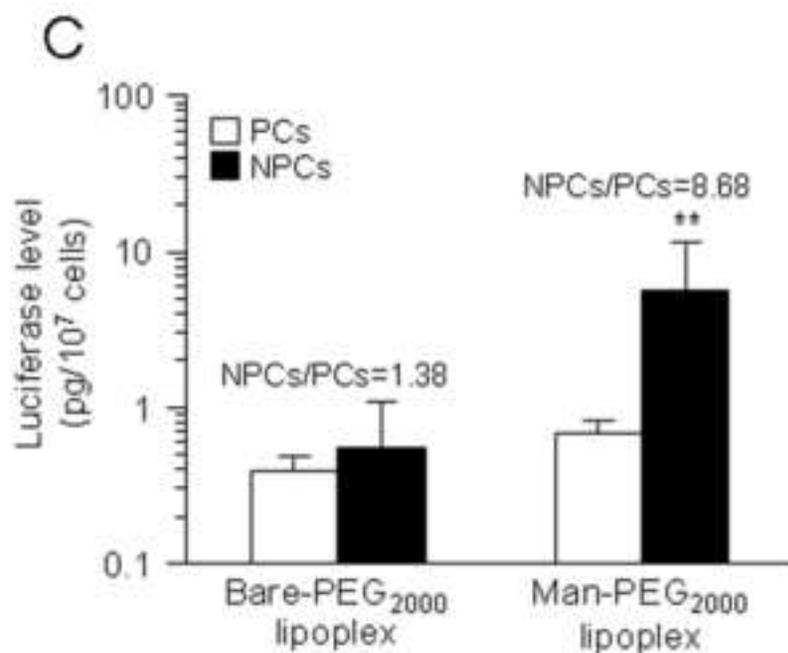
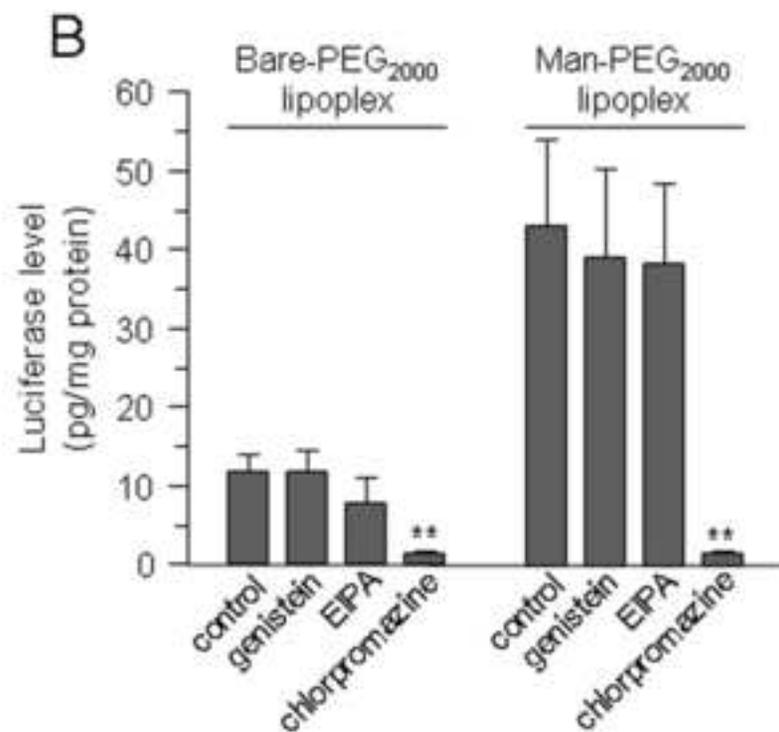
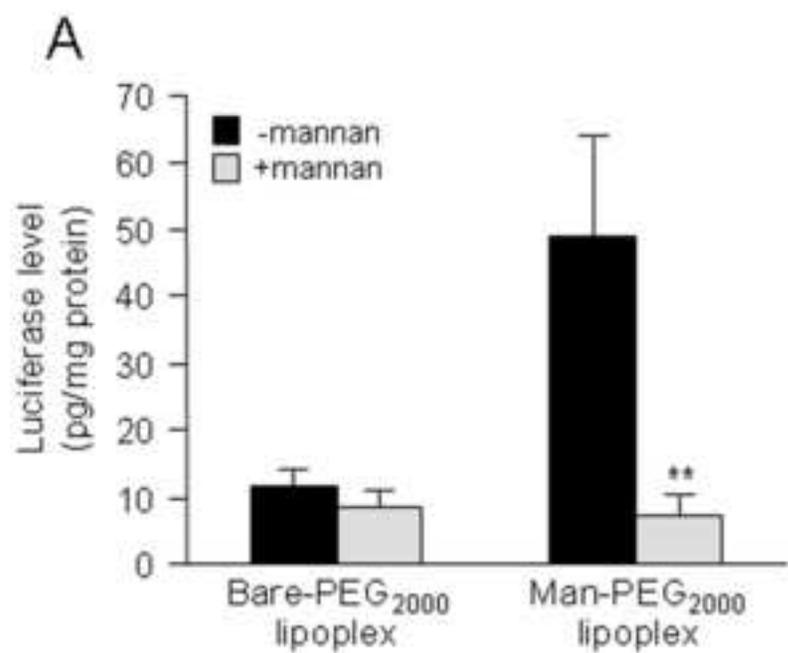
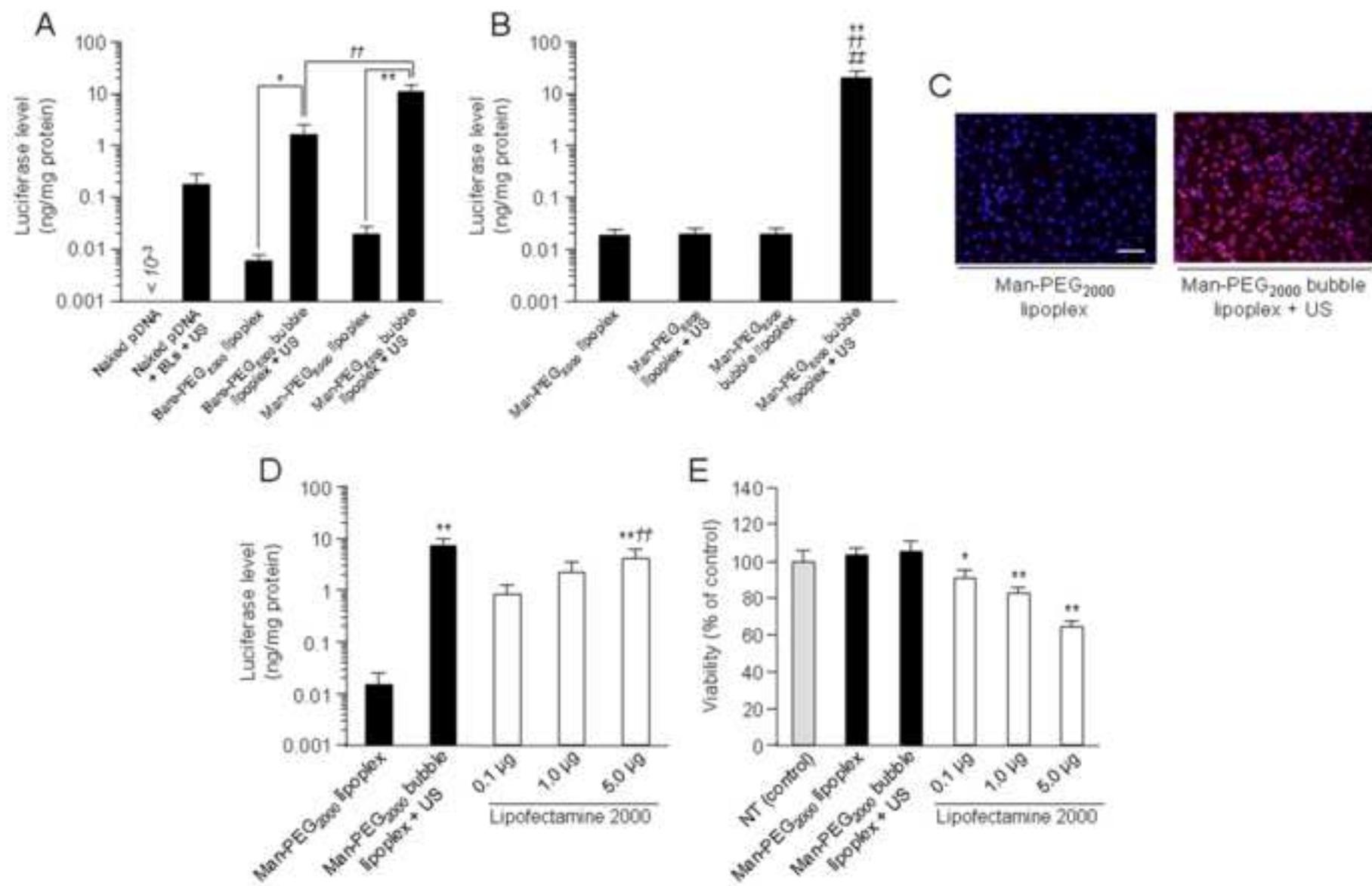


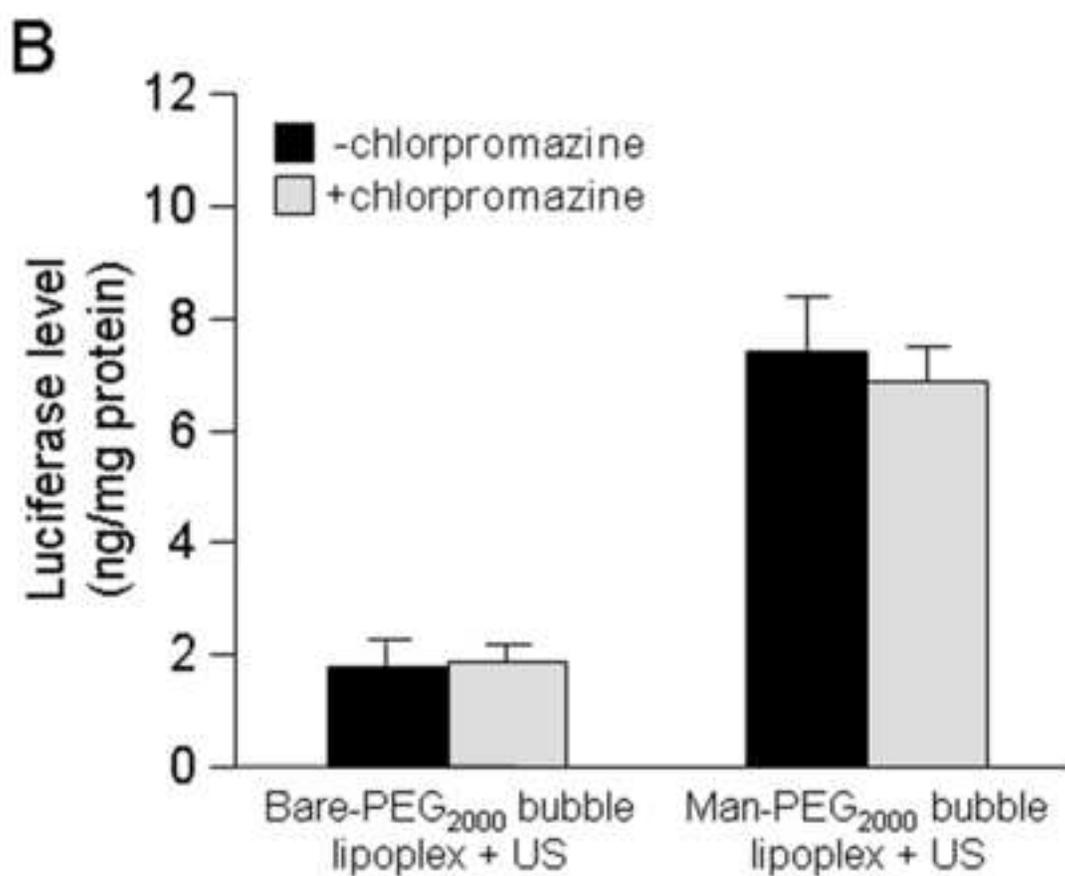
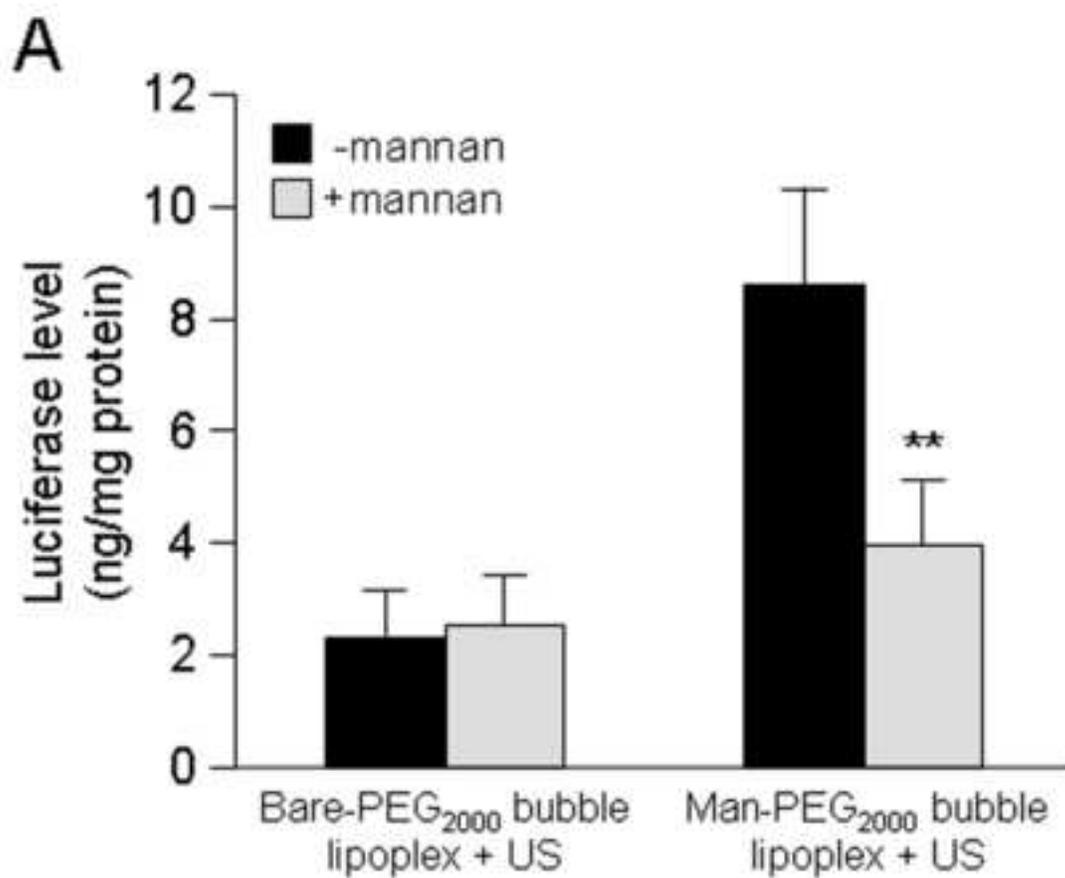
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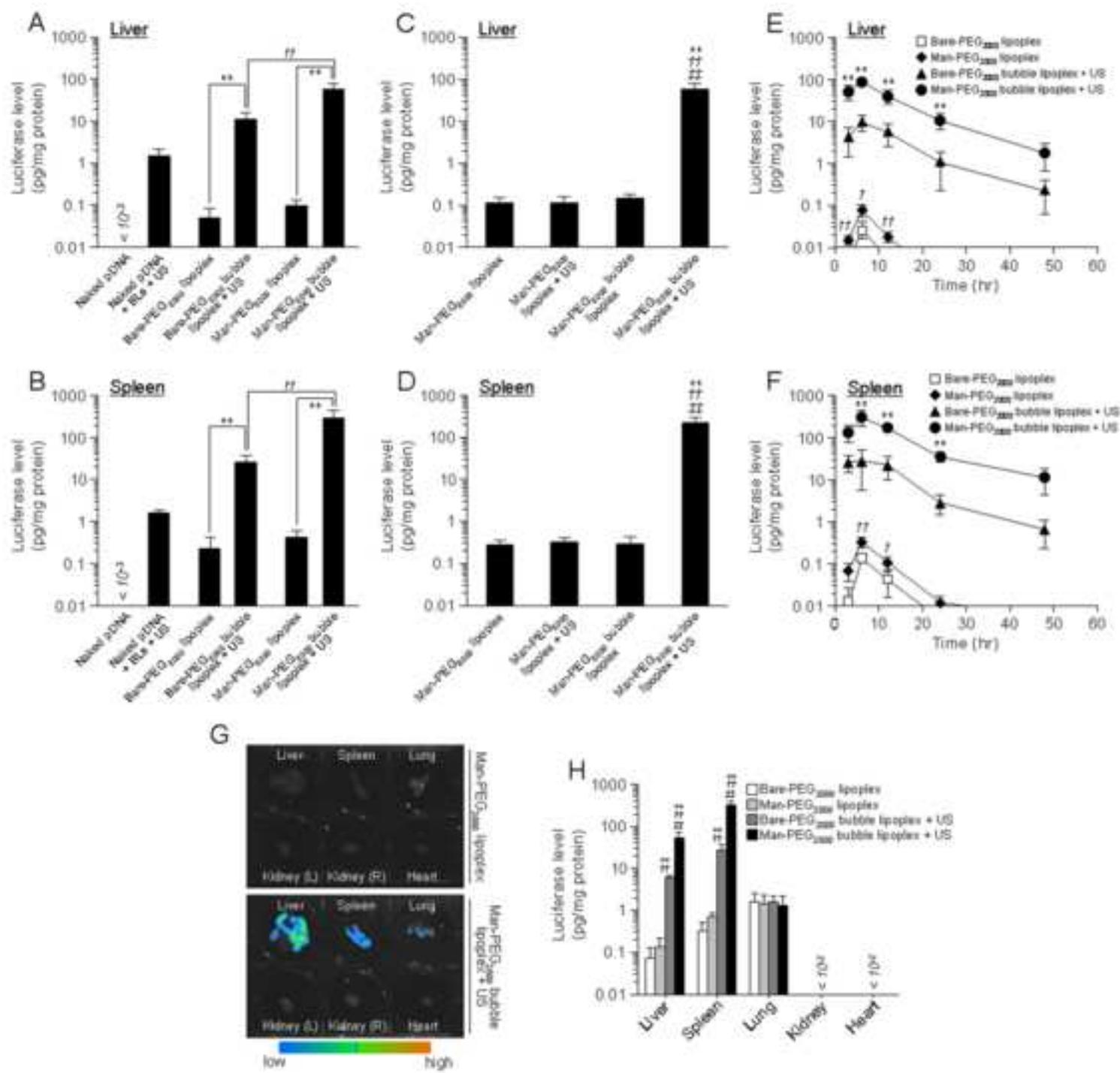
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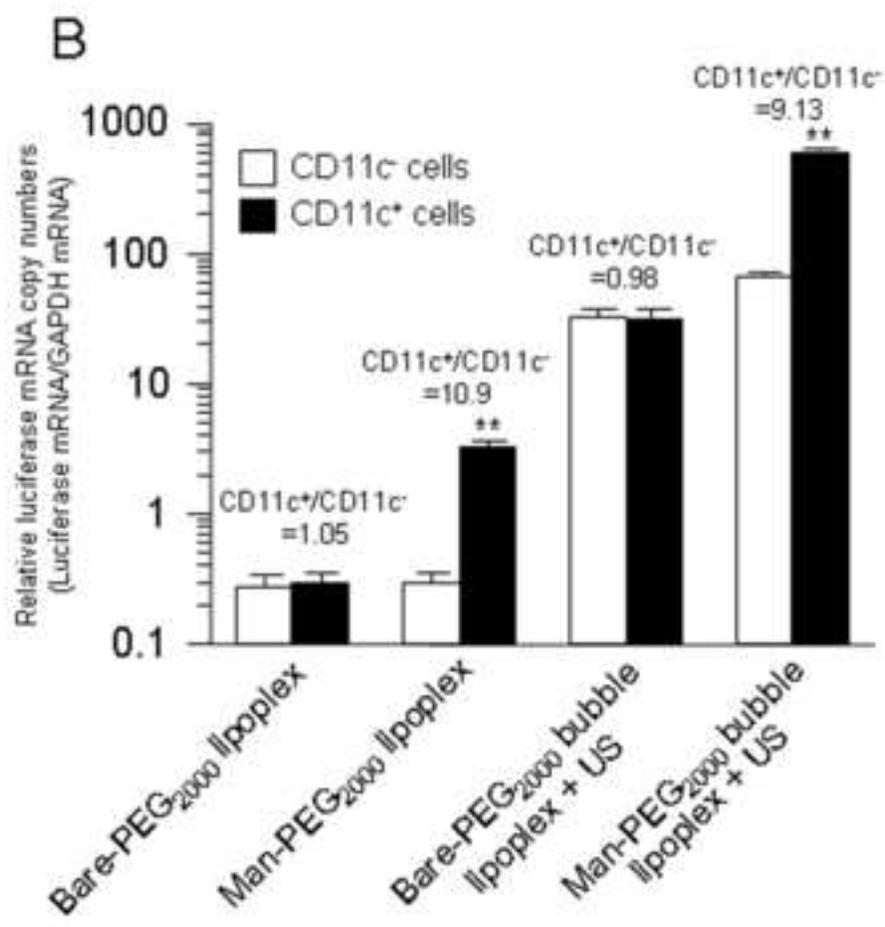
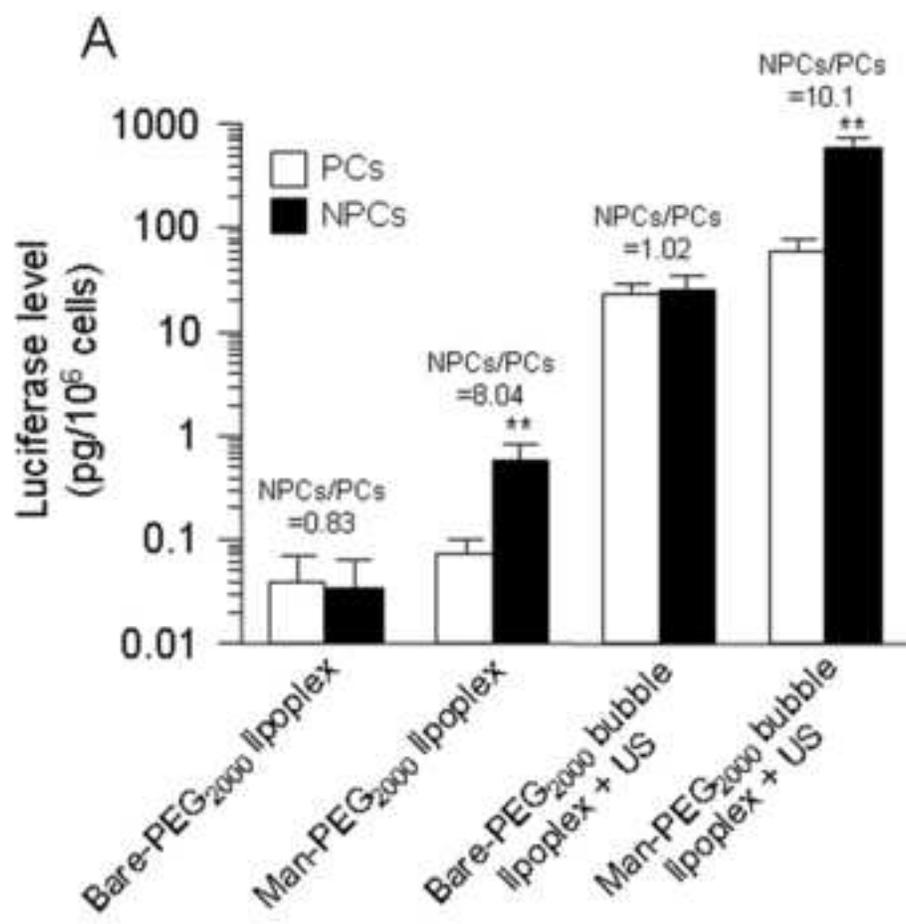


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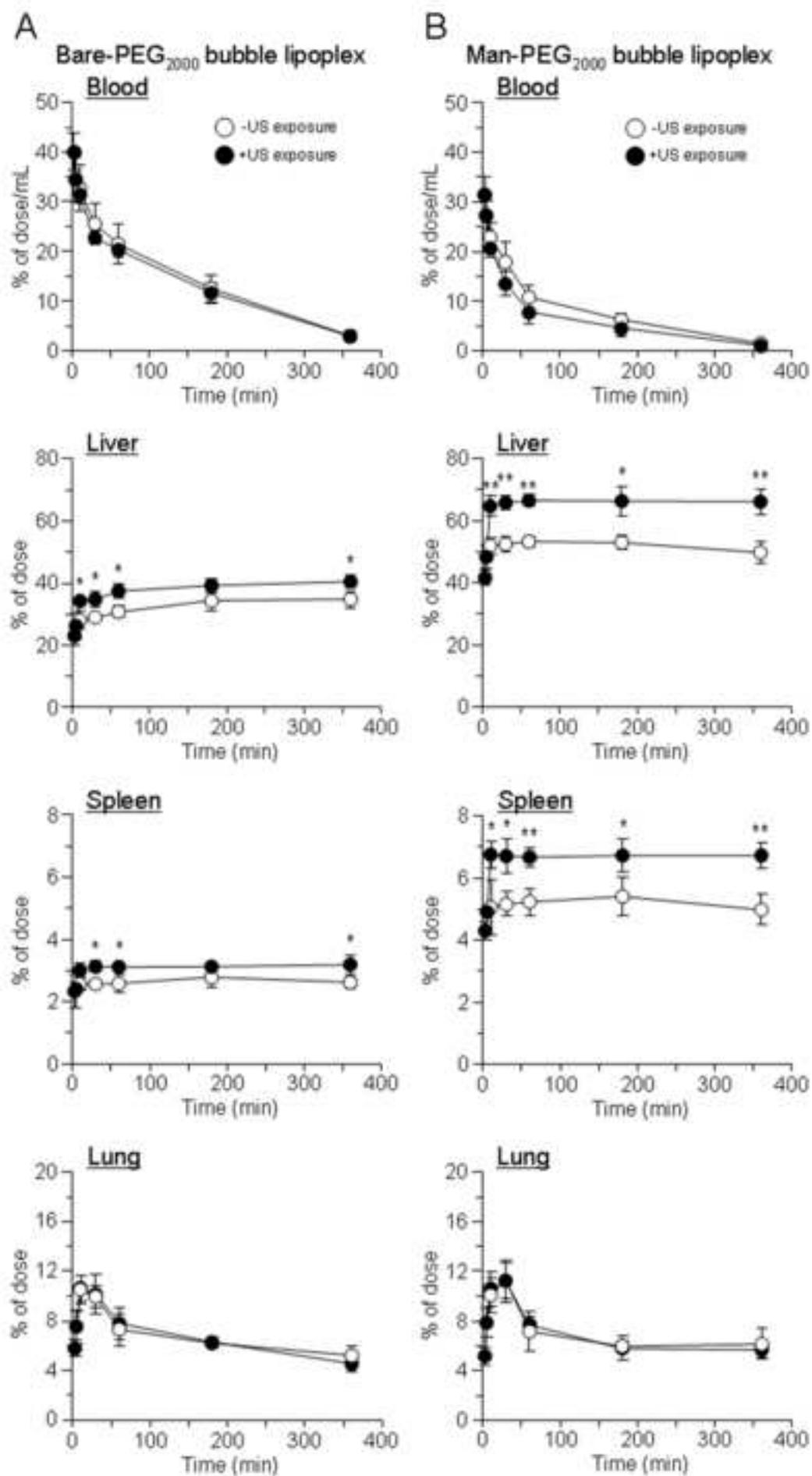


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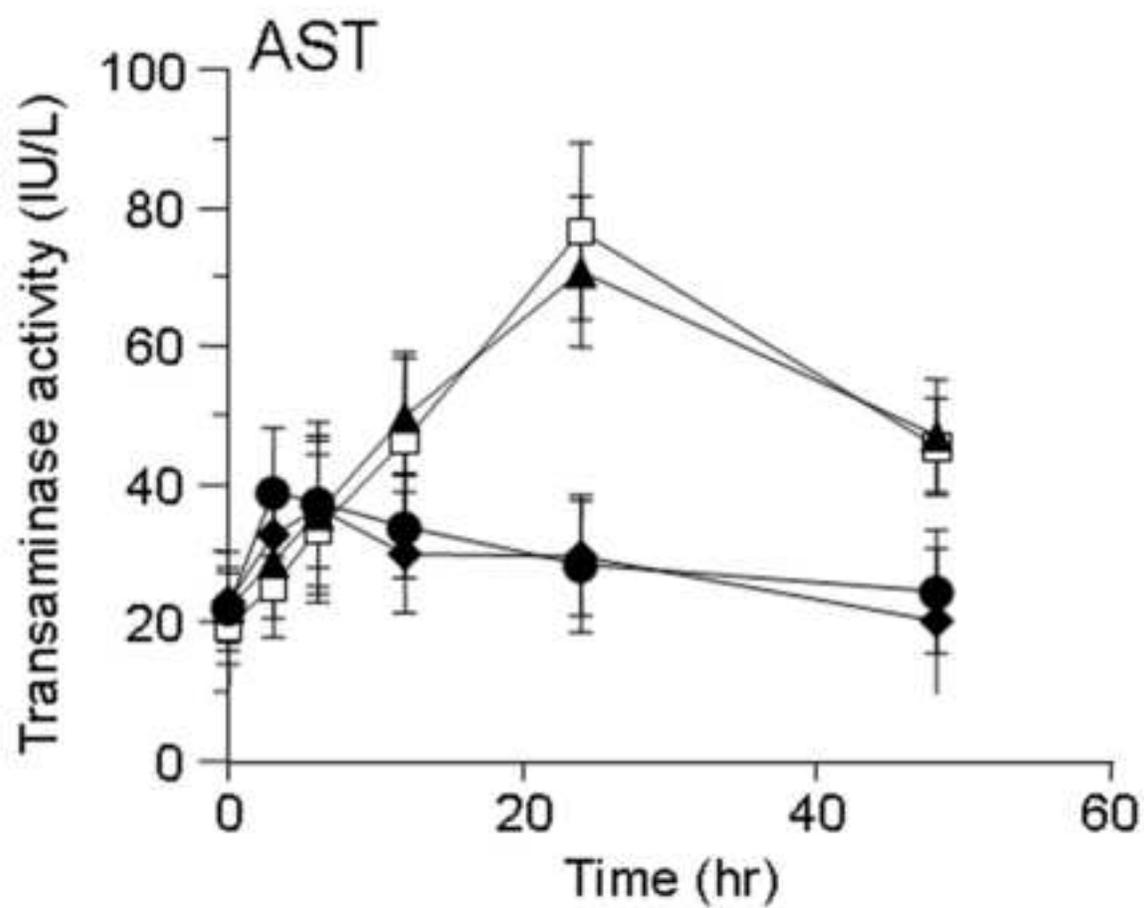
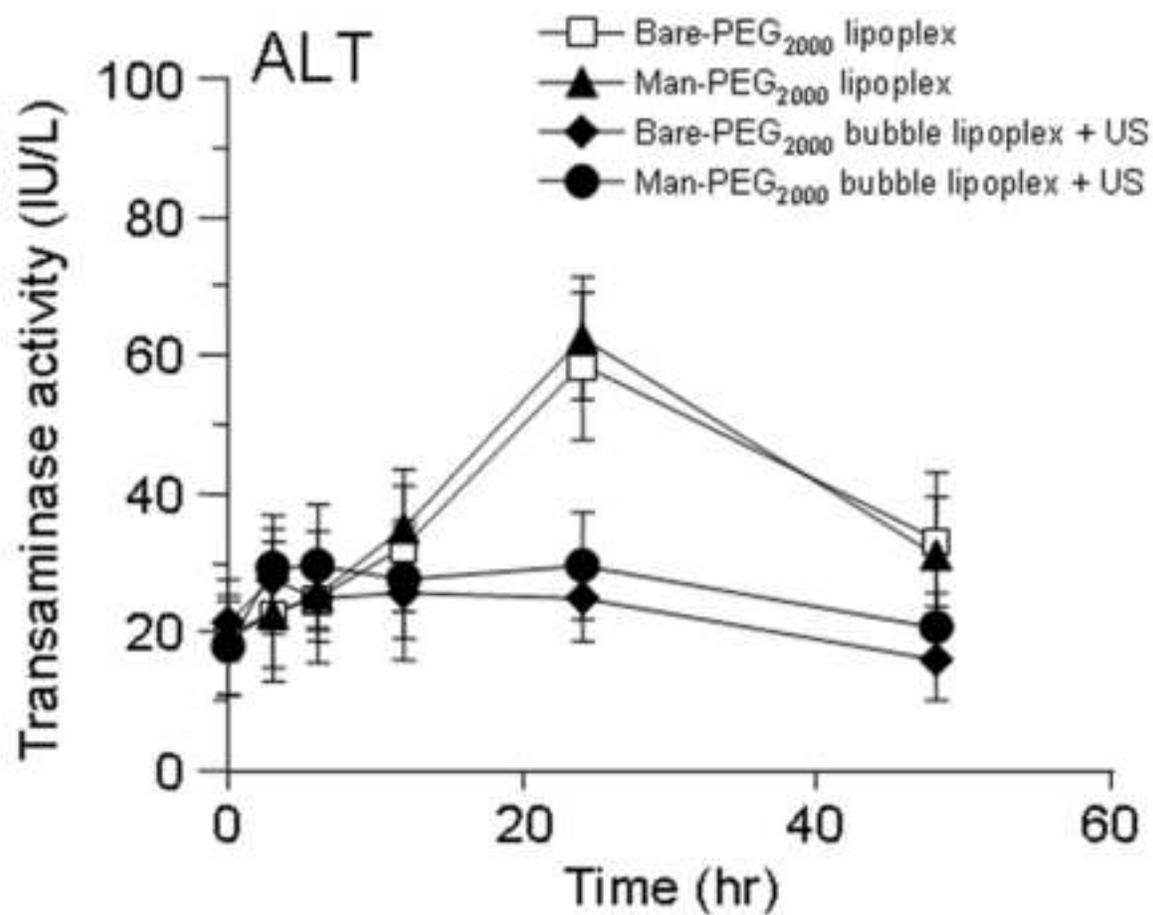
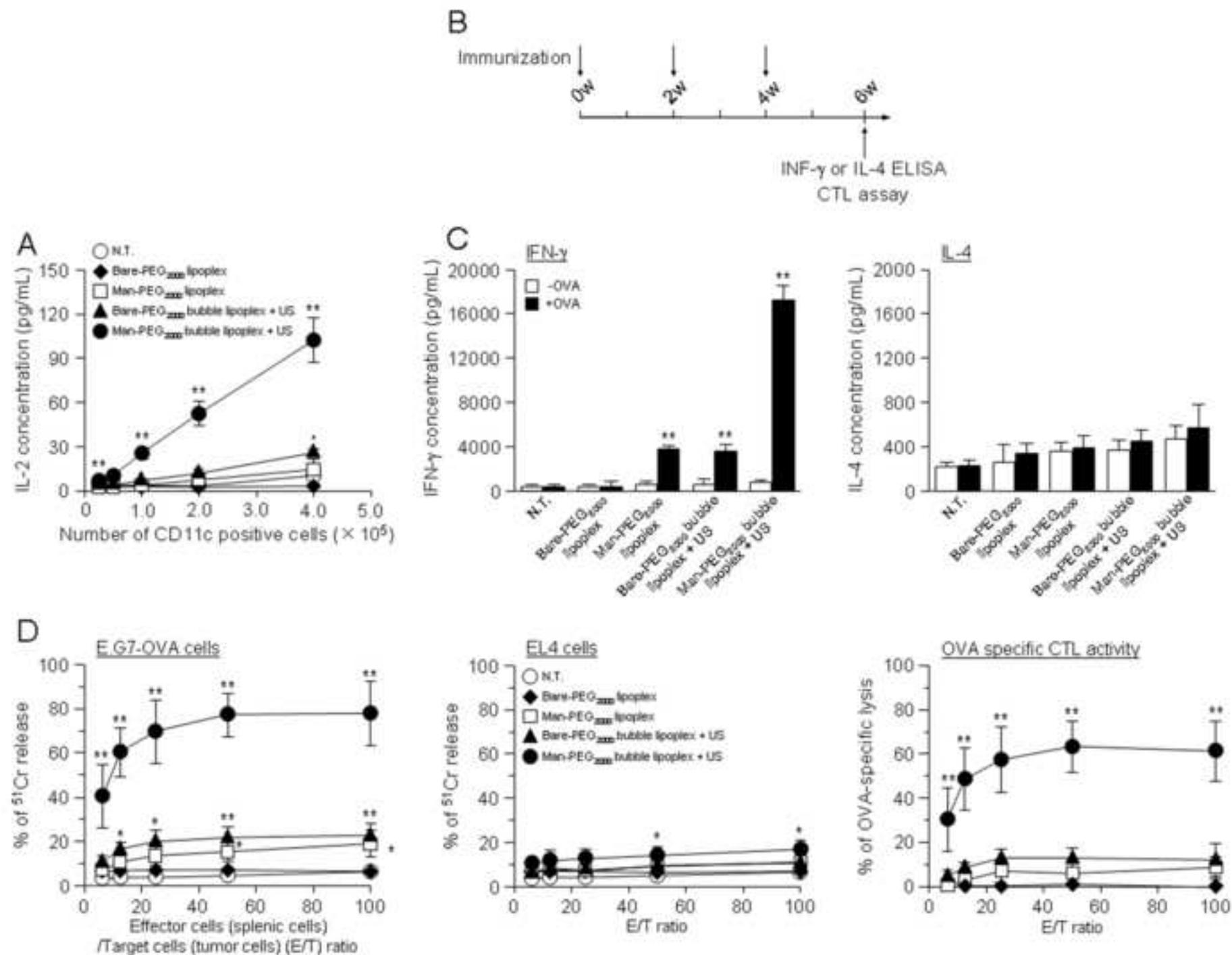
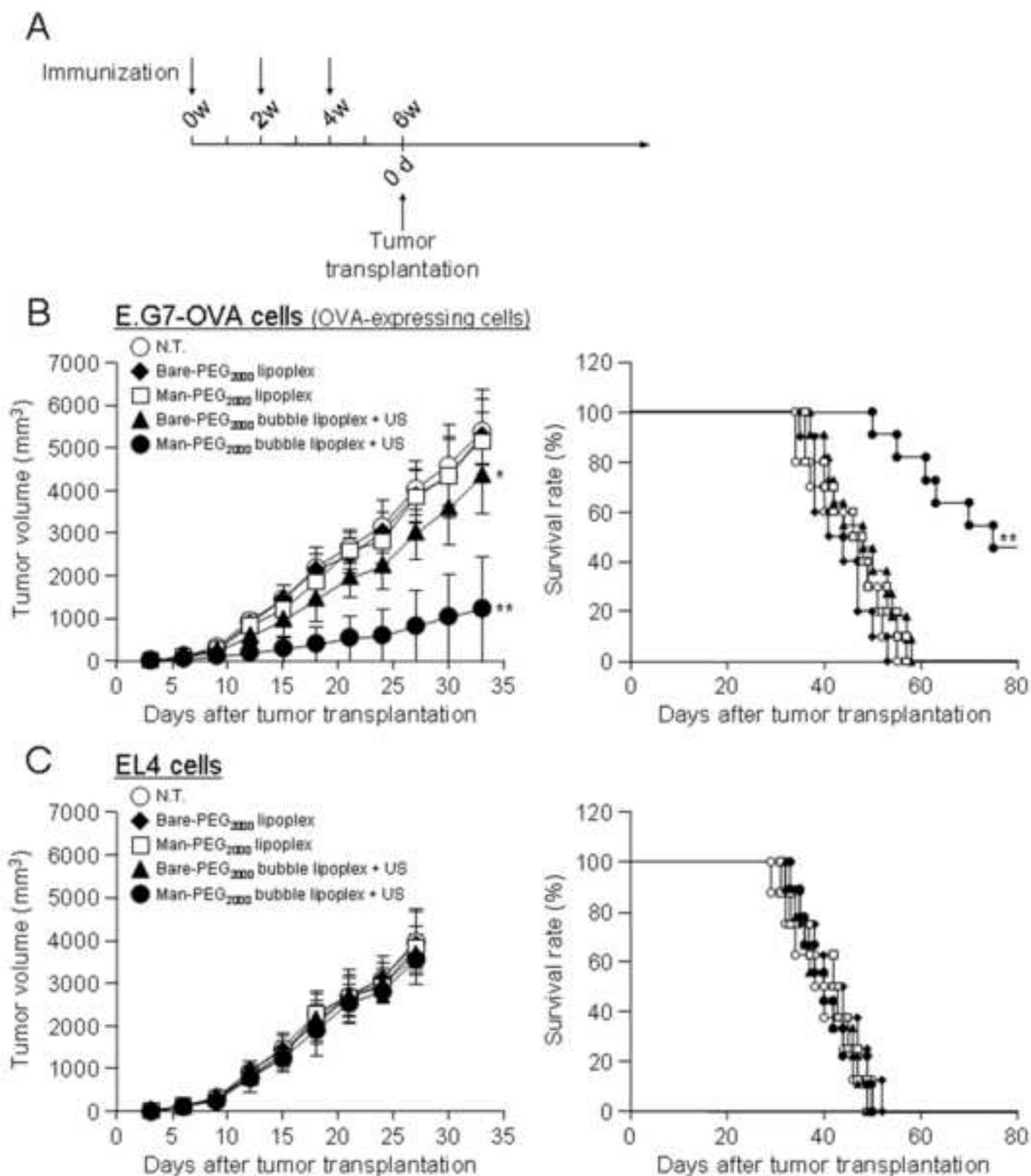
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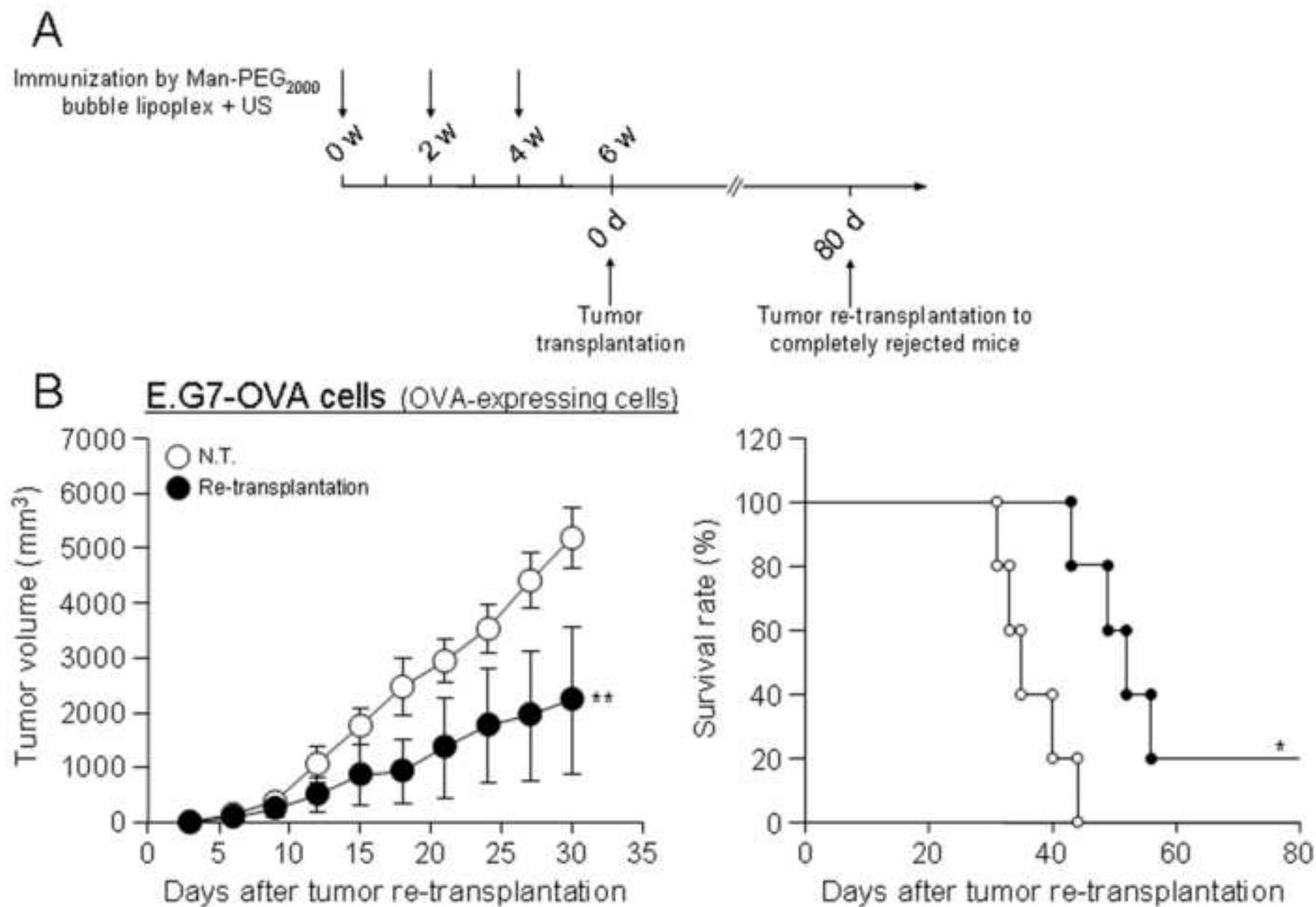


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Biomaterials

Supplementary Data

## **Development of an ultrasound-responsive and mannose-modified gene carrier for DNA vaccine therapy**

Keita Un<sup>a,b</sup>, Shigeru Kawakami<sup>a</sup>, Ryo Suzuki<sup>c</sup>, Kazuo Maruyama<sup>c</sup>,  
Fumiyoshi Yamashita<sup>a</sup> and Mitsuru Hashida<sup>a,d\*</sup>

<sup>a</sup>Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

<sup>b</sup>The Japan Society for the Promotion of Science (JSPS), Chiyoda-ku, Tokyo 102-8471, Japan

<sup>c</sup>Department of Biopharmaceutics, School of Pharmaceutical Sciences, Teikyo University, 1091-1 Suwarashi, Sagamiko, Sagamihara, Kanagawa 229-0195, Japan

<sup>d</sup>Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Yoshida-ushinomiya-cho, Sakyo-ku, Kyoto 606-8302, Japan

**\*Corresponding author:**

Mitsuru Hashida, Ph.D.,

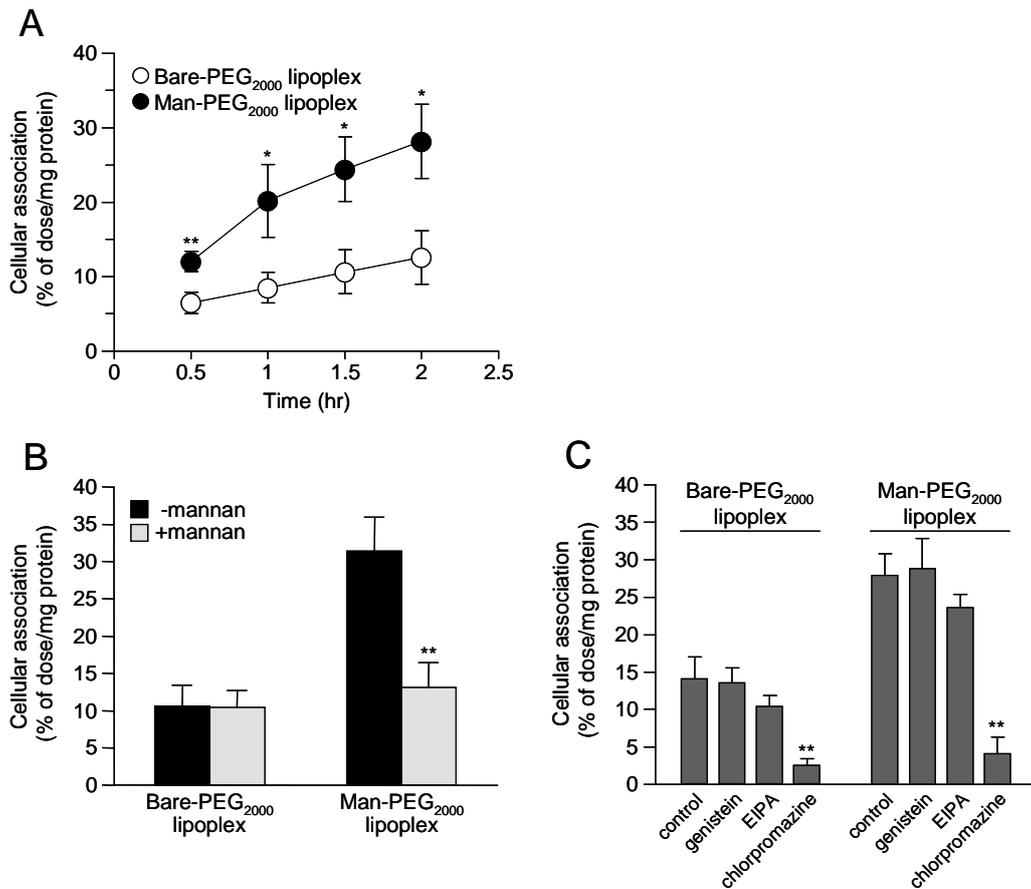
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Graduate School of Pharmaceutical Sciences, Kyoto University,

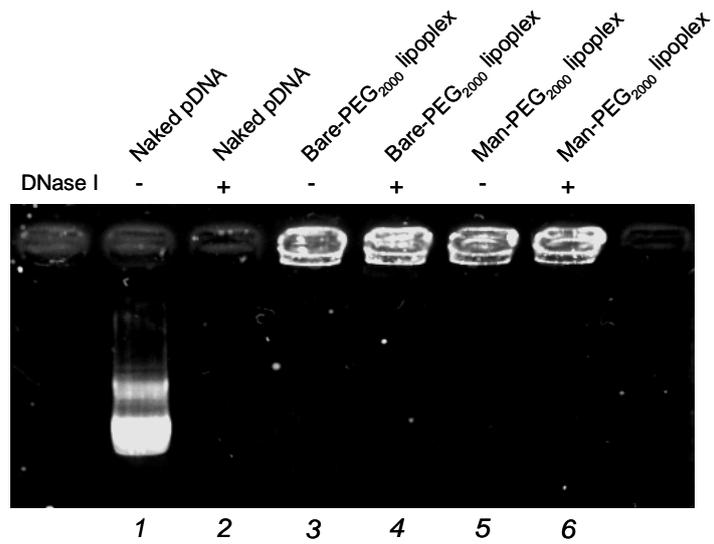
46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan.

Phone: +81-75-753-4545. Fax: +81-75-753-4575.

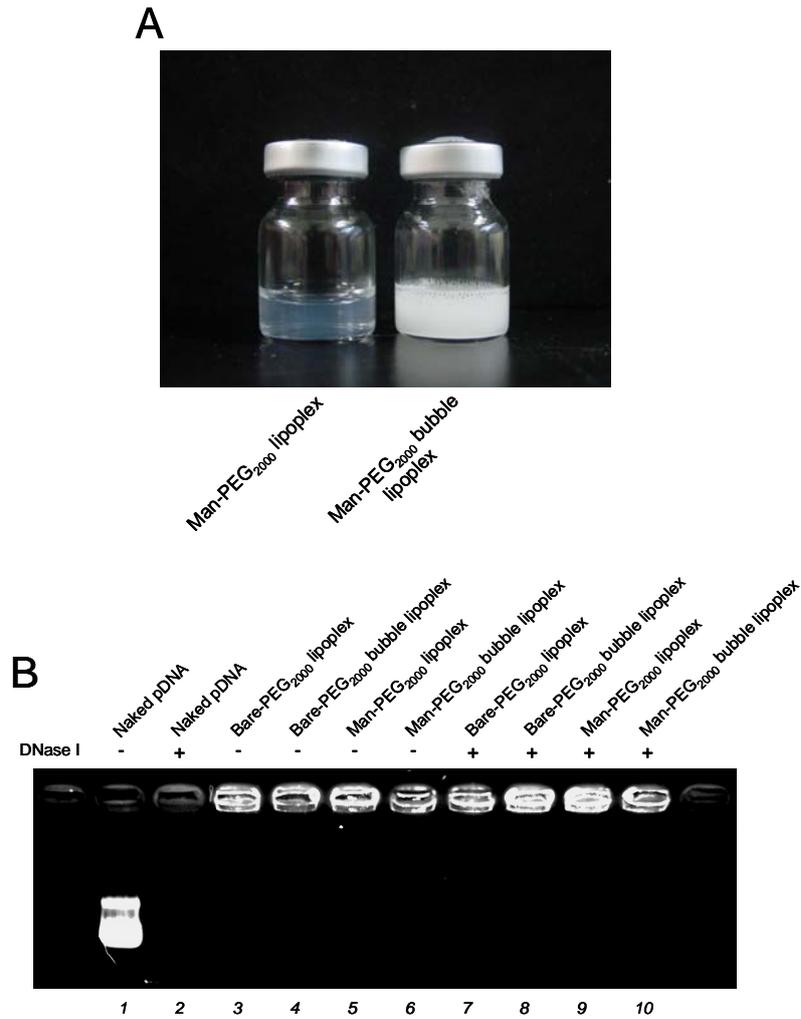
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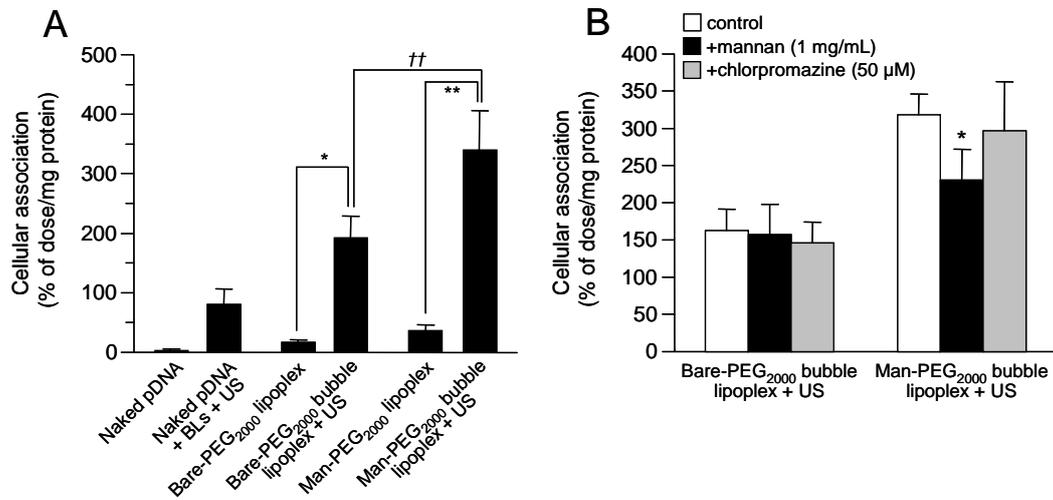
**Supplementary Fig. 1.** The cellular association of pDNA obtained by Bare-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> lipoplexes in mouse cultured macrophages. (A) Time-course of the cellular association of pDNA obtained by Bare-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> lipoplexes constructed with <sup>32</sup>P-labeled pDNA (5 μg pDNA). Each value represents the mean ± SD (*n* = 3). \* *p* < 0.05; \*\* *p* < 0.01, compared with the corresponding group of Bare-PEG<sub>2000</sub> lipoplex. (B) Cellular association of pDNA obtained by Bare-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> lipoplexes constructed with <sup>32</sup>P-labeled pDNA (5 μg pDNA) in the absence or presence of 1 mg/mL mannan at 2 hr after addition of each lipoplex. Each value represents the mean + SD (*n* = 3). \*\* *p* < 0.01, compared with the corresponding group of -mannan. (C) Inhibition of cellular association of pDNA obtained by Bare-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> lipoplexes constructed with <sup>32</sup>P-labeled pDNA (5 μg pDNA) in addition of various endocytosis inhibitors at 2 hr after addition of each lipoplex. Each value represents the mean + SD (*n* = 3). \*\* *p* < 0.01, compared with the corresponding group of control.



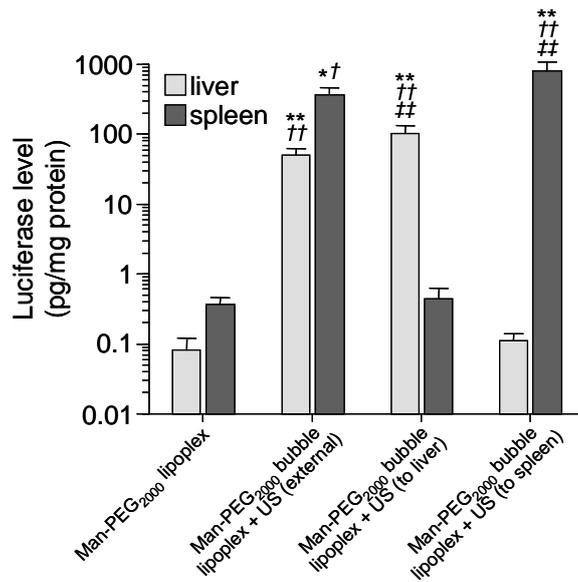
**Supplementary Fig. 2.** DNase I protection assay. Naked pDNA, Bare-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> lipoplexes were treated with DNase I at 37 °C for 15 min, and subjected to agarose gel electrophoresis. The samples were as follows: naked pDNA (lane 1 and 2); Bare-PEG<sub>2000</sub> lipoplexes (lane 3 and 4); Man-PEG<sub>2000</sub> lipoplexes (lane 5 and 6); non-treated with DNase I (lane 1, 3 and 5); treated with DNase I (lane 2, 4 and 6).



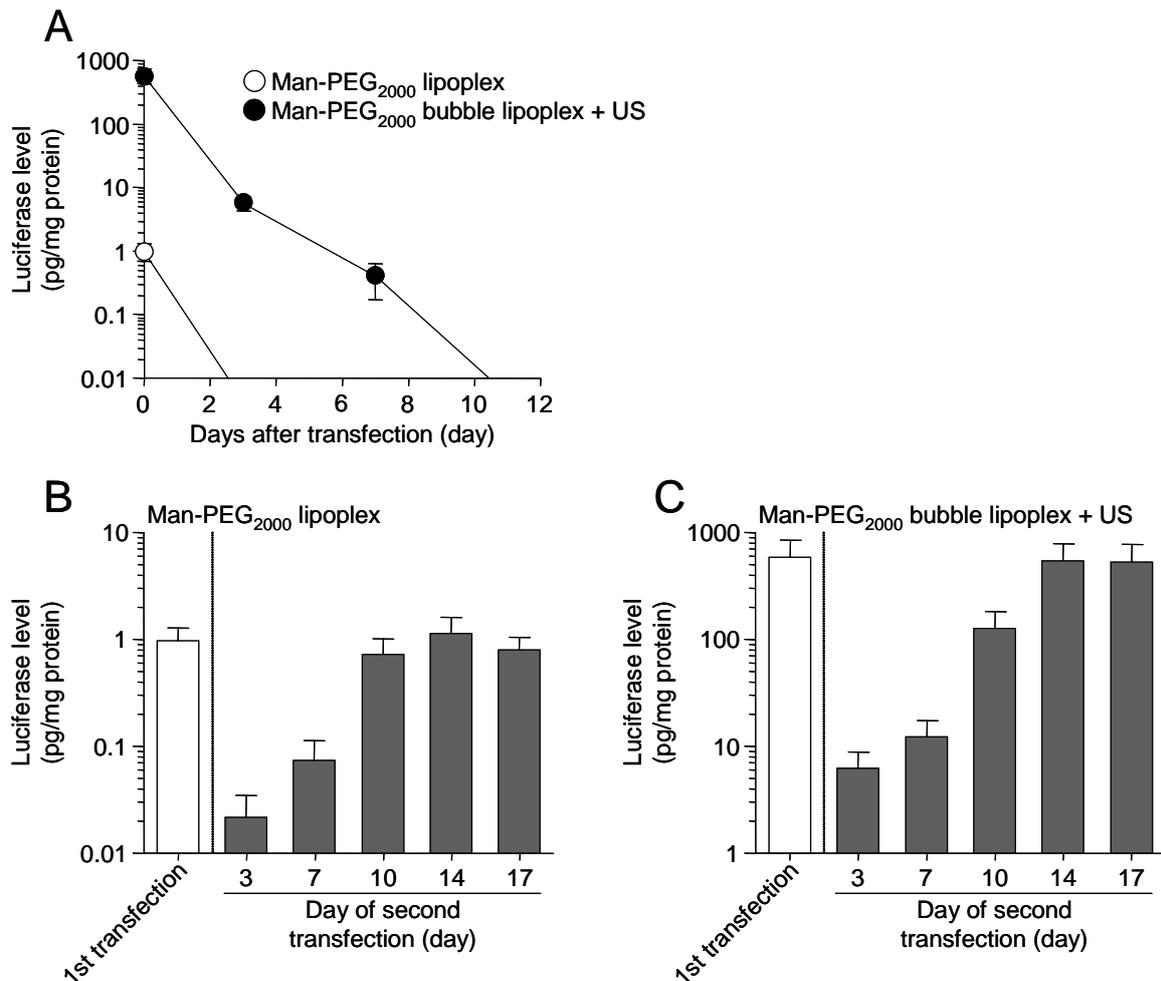
**Supplementary Fig. 3.** Photographs of Man-PEG<sub>2000</sub> bubble lipoplexes and stability of Bare-PEG<sub>2000</sub> bubble lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes against nuclease. (A) Man-PEG<sub>2000</sub> bubble lipoplexes (right) were prepared by sonication of Man-PEG<sub>2000</sub> lipoplexes (left) with supercharged perfluoropropane gas. (B) DNase I protection assay. Naked pDNA and lipoplexes were treated with DNase I, and then subjected to agarose gel electrophoresis. The samples were as follows: naked pDNA (lane 1 and 2); Bare-PEG<sub>2000</sub> lipoplex (lane 3 and 7); Bare-PEG<sub>2000</sub> bubble lipoplex (lane 4 and 8); Man-PEG<sub>2000</sub> lipoplex (lane 5 and 9); Man-PEG<sub>2000</sub> bubble lipoplex (lane 6 and 10); non-treated with DNase I (lane 1 and 3-6); treated with DNase I (lane 2 and 7-10).



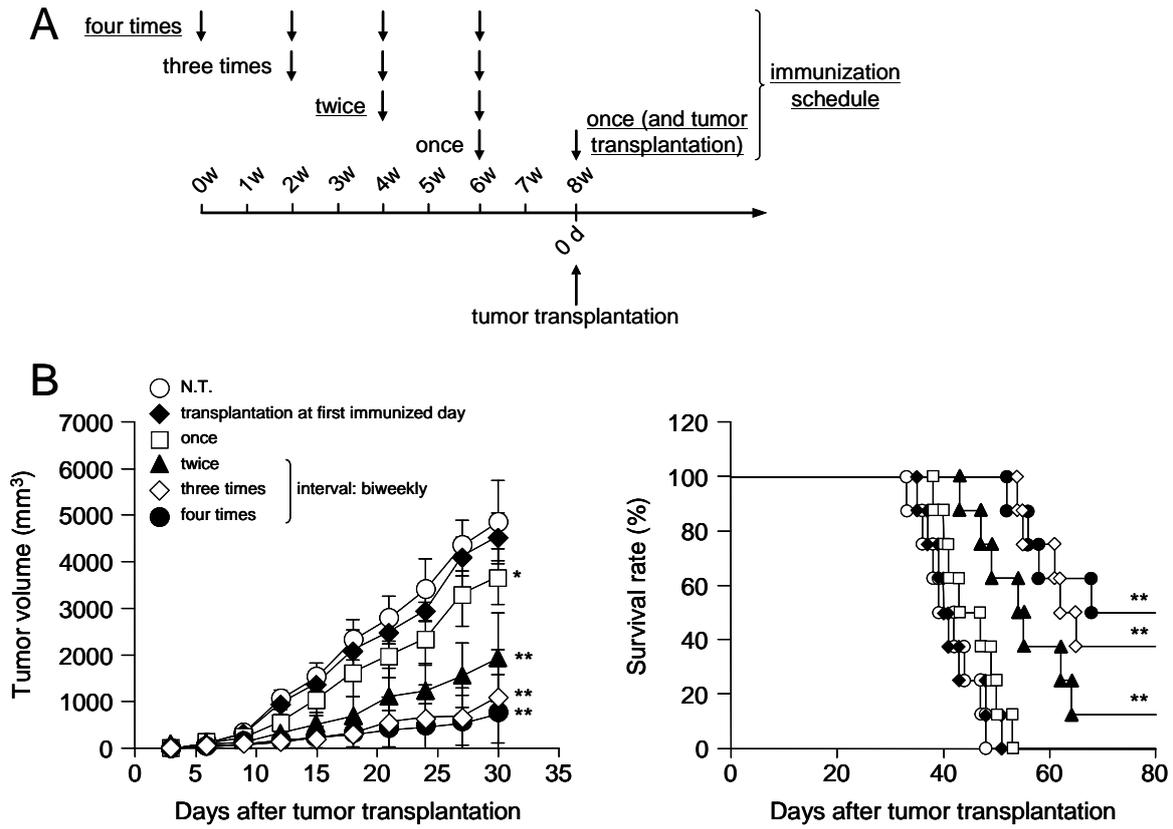
**Supplementary Fig. 4.** Enhancement of cellular association of pDNA by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure in vitro. (A) Cellular association of <sup>32</sup>P-labeled pDNA by transfection using naked pDNA, naked pDNA + BLs with US exposure, Bare-PEG<sub>2000</sub> lipoplexes, Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure, Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (5 μg pDNA) at 2 hr after addition of each lipoplex. Each value represents the mean + SD (*n* = 3). Significant difference; \* *p* < 0.05; \*\*, †† *p* < 0.01. (B) Cellular association by Bare-PEG<sub>2000</sub> bubble lipoplexes with US exposure and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (5 μg <sup>32</sup>P-labeled pDNA) at 2 hr after addition of each lipoplex. Each value represents the mean + SD (*n* = 3). \* *p* < 0.05, compared with the corresponding group of control.



**Supplementary Fig. 5.** The level of luciferase expression by Man-PEG<sub>2000</sub> bubble lipoplexes and direct US exposure. Four-week-old ICR female mice received an intravenous injection of 400  $\mu$ L Man-PEG<sub>2000</sub> bubble lipoplexes using a 26-gauge syringe needle at a dose of 50  $\mu$ g pDNA. After injection of Man-PEG<sub>2000</sub> bubble lipoplexes, mice were operated the abdominal area under anesthesia. At 5 min after injection of Man-PEG<sub>2000</sub> bubble lipoplexes, the organ was exposed directly to US using a Sonopore-4000 sonicator. US exposure conditions for the liver: frequency, 1.045 MHz; duty, 50%; burst rate, 10 Hz; intensity 1.0 W/cm<sup>2</sup>; time, 2 min by using a 20 mm diameter probe, and for the spleen: frequency, 2.062 MHz; duty, 50%; burst rate, 10 Hz; intensity 4.0 W/cm<sup>2</sup>; time, 2 min using a 6 mm diameter probe. At 6 hr after injection, mice were sacrificed and their organs collected for each experiment. The level of luciferase expression in the liver and spleen was determined at 6 hr after injection of Man-PEG<sub>2000</sub> bubble lipoplexes. Each value represents the mean + SD ( $n = 4$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ , compared with the corresponding group of Man-PEG<sub>2000</sub> lipoplex, †  $p < 0.05$ ; ††  $p < 0.01$ , compared with the corresponding group of non-exposed US in each organ. †††  $p < 0.01$ , compared with the corresponding group of externally exposed US.



**Supplementary Fig. 6.** Repeated transfection by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. (A) Time-course of gene expression in the spleen by Man-PEG<sub>2000</sub> lipoplexes and Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure. Each value represents the mean  $\pm$  SD ( $n = 4$ ). (B, C) The effect of transfection interval between first and second transfection on the level of gene expression by repeated transfection using (B) Man-PEG<sub>2000</sub> lipoplexes and (C) Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure in the spleen. Mice were received the second transfection at 3, 7, 10, 14 and 17 days after first transfection. Each value represents the mean  $\pm$  SD ( $n = 4$ ). The level of gene expression was sequentially decreased after transfection using Man-PEG<sub>2000</sub> lipoplexes or Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure (Supplementary Fig. 6A). Therefore, it is considered that multiple transfusions are essential to obtain more effective vaccine effects against cancer. However, following optimization of transfection interval, the levels of gene expression obtained by Man-PEG<sub>2000</sub> lipoplexes or Man-PEG<sub>2000</sub> bubble lipoplexes with US exposure in the second transfection were lower than that in first transfection when second transfection was performed at 3rd or 7th day after first transfection (Supplementary Figs. 6B and 6C). In this study, the level of gene expression in the second transfection reached to the same level as gene expression in the first transfection when second transfection was performed at 14th day after first transfection (Supplementary Figs. 6B and 6C).



**Supplementary Fig. 7.** Optimization of immunization times by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure. (A) Schedule of optimization of immunization times by Man-PEG<sub>2000</sub> bubble lipoplexes (50 μg pDNA) and US exposure. (B) Effects of the immunization times by Man-PEG<sub>2000</sub> bubble lipoplexes and US exposure on DNA vaccine effects. Mice were immunized once, twice, three times and four times biweekly. At the immunized day or 2 weeks after last immunization, E.G7-OVA cells ( $1 \times 10^6$  cells) were transplanted subcutaneously into the back of mice. The tumor volume was calculated as follows: (long diameter)  $\times$  (short diameter)<sup>2</sup>  $\times$  0.50. Each value represents the mean  $\pm$  SD ( $n = 8$ ) and the survival was monitored up to 80 days after tumor transplantation ( $n = 8$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ , compared with the corresponding group of N.T.. N.T., non-treatment.

Particle sizes and zeta potentials of liposomes and lipoplexes.

	Particle size (nm)	Zeta-potential (mV)
Bare-PEG <sub>2000</sub> liposome (DSTAP:DSPC:NH <sub>2</sub> -PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	96.7±2.2	61.9±1.4
Man-PEG <sub>2000</sub> liposome (DSTAP:DSPC:Man-PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	95.6±2.5	63.0±2.4
Bare-PEG <sub>2000</sub> lipoplex (DSTAP:DSPC:NH <sub>2</sub> -PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	155.3±6.7	42.3±2.5
Man-PEG <sub>2000</sub> lipoplex (DSTAP:DSPC:Man-PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	157.7±9.9	44.6±2.4

**Supplementary Table 1.** Particle sizes and zeta potentials of liposomes and lipoplexes. Each value represents the mean ± SD ( $n = 3$ ).

Optimal lipid composition of lipoplexes to enclose the US imaging gas stably.

cationic lipid	neutral lipid	enclosing of US imaging gas
DSTAP	DSPC	+
	DPPC	+
	DOPE	-
	DOPC	-
DOTAP	DSPC	-
	DPPC	-
	DOPE	-
	DOPC	-
DOTMA	DSPC	-
	DPPC	-
	DOPE	-
	DOPC	-

**Supplementary Table 2.** Optimal lipid composition of lipoplexes to enclose US imaging gas stably. To optimize the lipid composition of lipoplexes to enclose US imaging gas (perfluoropropane gas) stably, lipoplexes constructed with several types of phospholipids were prepared and evaluated whether US imaging gas was enclosed into prepared lipoplex stably or not. The enclosing of US imaging gas was identified by the cloudiness of lipoplex solution. The lipid composition of lipoplexes was cationic lipid:neutral lipid:Man-PEG<sub>2000</sub>-DSPE (7:2:1 (molar ratio)), and the charge ratio of pDNA (pCMV-Luc) and liposomes was 1.0:2.3 (-: +). US imaging gas was enclosed as described in *Materials and Methods* section. +; enclosing US imaging gas stably, -; not enclosing US imaging gas.

**Cationic lipids:**

DSTAP; 1,2-stearoyl-3-trimethylammoniumpropane (18/0 (carbon chain/the number of double bonds), saturated, T<sub>m</sub> > 50 °C),

DOTAP; 1,2-dioleoyl-3-trimethylammoniumpropane (18/1, unsaturated, T<sub>m</sub> < 0 °C),

DOTMA; 1,2-di-*o*-octadecenyl-3-trimethylammonium propane (18/1, unsaturated, T<sub>m</sub> < 0 °C).

**Neutral lipids:**

DSPC; 1,2-distearoyl-*sn*-glycero-3-phosphocholine (18/0, saturated, T<sub>m</sub> = 55 °C),

DPPC; 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (16/0, saturated, T<sub>m</sub> = 41 °C),

DOPE; 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18/1, unsaturated, T<sub>m</sub> = -16 °C),

DOPC; 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18/1, unsaturated, T<sub>m</sub> = -20 °C).

Particle sizes and zeta potentials of bubble liposomes and bubble lipoplexes.

	Particle size (nm)	Zeta-potential (mV)
Bare-PEG <sub>2000</sub> bubble liposome (DSTAP:DSPC:NH <sub>2</sub> -PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	504 ± 7.4	61.4 ± 2.6
Man-PEG <sub>2000</sub> bubble liposome (DSTAP:DSPC:Man-PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	505 ± 21	60.8 ± 1.8
Bare-PEG <sub>2000</sub> bubble lipoplex (DSTAP:DSPC:NH <sub>2</sub> -PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	568 ± 13	45.3 ± 3.1
Man-PEG <sub>2000</sub> bubble lipoplex (DSTAP:DSPC:Man-PEG <sub>2000</sub> :DSPE=7:2:1 (mol))	569 ± 12	44.8 ± 2.1

**Supplementary Table 3.** Particle sizes and zeta potentials of bubble liposomes and bubble lipoplexes. Each value represents the mean ± SD (*n* = 3).

## Supplementary Materials and Methods

### *Gel electrophoresis of pDNA.*

15  $\mu\text{L}$  of naked pDNA and lipoplexes (1.5  $\mu\text{g}$  pDNA) were incubated with 5  $\mu\text{L}$  DNase I solution (10  $\mu\text{g}/\text{mL}$  in DNase I buffer consisted of 100 mM Tris, 25 mM  $\text{MgCl}_2$  and 5 mM  $\text{CaCl}_2$ ) for 15 min at 37  $^\circ\text{C}$ . The digestion by DNase I was terminated by addition of 100 mM EDTA (5  $\mu\text{L}$ ). Electrophoresis was carried out with 1% agarose gel prepared with  $1 \times$  TAE buffer at a constant voltage of 100 V for 30 min. The band of DNA was detected by ethidium bromide and visualized using a LAS-3000 imaging system (FUJIFILM Co., Tokyo, Japan).

### *Evaluation of cellular association of pDNA.*

The cultured macrophages were plated on a 12-well culture plate at a density of  $5 \times 10^5$  cells/ $3.8 \text{ cm}^2$ . After 72 hr incubation, the macrophages were washed twice with Hank's balanced salt solution (HBSS, pH 7.4), and the culture medium was replaced with 1 mL HBSS containing lipoplexes constructed with  $^{32}\text{P}$ -labeled pDNA ( $[\alpha\text{-}^{32}\text{P}]\text{-dCTP}$ , PerkinElmer, Inc., MA, USA). At predetermined time after incubation, the solution was immediately removed and the macrophages were washed five times with ice-cold HBSS. 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, Inc., MA, USA). Then, the resultant lysates were decolorized with isopropanol and 30%  $\text{H}_2\text{O}_2$ , and neutralized with 5 N HCl. The radioactivity of  $^{32}\text{P}$ -labeled pDNA was measured in scintillation counter (LSA-500, Beckman Coulter, Inc., CA, USA) after addition of Clear-Sol I solution. The protein concentration was determined with a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan).