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# **pH-Sensitive fusogenic polymer-modified liposomes as a carrier of antigenic proteins for activation of cellular immunity**

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## **Abstract**

**By modification of liposomes with poly(glycidol) derivatives such as succinylated poly(glycidol) and 3-methylglutarylated poly(glycidol), we have developed functional liposomes that generate fusion ability at mildly acidic pH. We investigated the feasibility of these polymer-modified liposomes as a carrier of antigenic proteins for induction of cellular immunity. These pH-sensitive fusogenic liposomes encapsulating ovalbumin (OVA) were applied to DC2.4 cells, a murine dendritic cell line. Observation with confocal laser scanning microscopy showed that these polymer-modified liposomes were taken up efficiently by the cells, thereafter delivering their contents into the cytosol, probably through fusion with endosomal membranes. Murine bone-marrow-derived dendritic cells treated with polymer-modified liposomes encapsulating OVA stimulated CD8-OVA1.3 cells more strongly than OT4H.1D5 cells, indicating that the liposomes induced MHC class I-restricted presentation. Furthermore, administration of the polymer-modified, OVA-loaded liposomes from nasal cavities of mice induced stronger cellular immune responses than the OVA-loaded plain liposomes. Because the ability of the polymer-modified liposomes to activate cellular immunity was comparable to that of Freund's complete adjuvant, which is a widely used**

**adjuvant, they potentially have use in production of efficient vaccines for immunotherapy.**

*Keywords:* mucosal vaccine / dendritic cell / pH-sensitive polymer / cytoplasmic delivery / fusogenic liposome

## Introduction

Efficient vaccination strategies have been desired for overcoming new pathogens and for evolution of resistance of microorganisms. In addition, efficient vaccine delivery systems have been required for achievement of cancer immunotherapy. Dendritic cells (DCs) are known as potent professional antigen-presenting cells; they play a crucial role in innate and adaptive immune responses [1–3]. The DCs recognize, take up, process, and present antigens to native and resting T cells for induction of an antigen-specific immune response. Antigenic proteins internalized *via* endocytosis are degraded to peptide fragments. These peptides are presented by binding to major histocompatibility complex (MHC) class II molecules, which mainly activate CD4<sup>+</sup> T lymphocytes, thereby inducing humoral immunity. On the other hand, antigenic proteins introduced into cytosol of DCs are degraded by proteasomes after ubiquitination. These fragmented peptides are presented by MHC class I molecules on the surface of DCs. They mainly activate CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) to induce cellular immunity. To attain efficient target-specific immunity, induction of the antigen-specific CTLs is important because they eliminate the infected cells and pathogens directly. Therefore, carrier systems that can introduce antigenic proteins efficiently into the cytosol of DCs are necessary to establish effective immunotherapy.

Numerous attempts have been undertaken to achieve delivery of antigens into the DC's cytosol. For example, Akagi et al. reported that nanoparticles of  $\gamma$ -poly(glutamic acid) introduced entrapped antigenic ovalbumin (OVA) into cytosol of DC and induced antigen-specific CTLs [4,5]. In addition, Fréchet and coworkers showed that acid-degradable, acrylamide-based nanoparticles achieved cytosolic delivery of OVA and presentation of OVA-derived peptides *via* the MHC class I pathway [6]. These nanoparticles might be taken up by DC *via* endocytosis and enhance transfer of their encapsulated antigen molecules from endosome and/or lysosome to cytosol by destabilization of the membranes of these acidic compartments through hydrophobic or electrostatic interactions [5,6].

One of the most effective strategies for efficient introduction of antigenic proteins into cytosol of DC might be to use membrane fusion especially for membrane-based nanoparticles, such as liposomes. To date, viral fusion proteins have been used frequently to provide liposomes with fusion ability [7,8]. Indeed, viral fusion protein-incorporated liposomes have been used to introduce encapsulated antigenic OVA into DC's cytosol and induced efficient cellular immunity [7,8]. However, viral proteins might provoke unexpected immune responses. Therefore, the use of synthetic carriers might be preferred for the delivery of antigens into DCs.

We have developed pH-sensitive liposomes, which generate fusion ability under weakly acidic conditions, by surface modification of egg yolk phosphatidylcholine (EYPC) liposomes with poly(glycidol) derivatives having carboxyl groups (Fig. 1) [9,10]. In fact, these polymer-modified liposomes delivered a membrane-impermeable fluorescent dye, calcein, into cytosol of HeLa cells after internalization *via* endocytosis and subsequent fusion with membrane of endosome or lysosome [9,10]. Especially, 3-methylglutarylated poly(glycidol) (MGluPG), which has hydrophobic side chains, exhibited higher fusion ability than succinylated poly(glycidol) (SucPG) [10].

Considering the excellent performance of these polymer-modified liposomes as a cytoplasmic delivery vehicle, we attempted to apply these liposomes to the production of potent vaccines, which deliver antigenic proteins into cytosol of DCs and activate cellular immune response through their administration *via* nasal mucosa, which affords some advantages, such as noninvasive needle-free administration and induction of both mucosal and systemic immune responses [11–14]. Correlation of fusogenic properties of the liposomes with their ability to activate cellular immunity was described.

## **Materials and Methods**

### *Materials.*

EYPC and L-dioleoyl phosphatidylethanolamine (DOPE) were kindly donated by NOF Co. (Tokyo, Japan). N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)dioleoyl phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Monophosphoryl lipid A (MPL), Freund's complete adjuvant (CFA) and OVA were purchased from Sigma (St. Louis, MO.). SucPG and MGluPG were prepared using poly(glycidol) with the number and weight average molecular weights of  $1.6 \times 10^4$  and  $2.5 \times 10^4$ , respectively, as previously reported [10,15]. Molar percentages of glycidol/carboxylated glycidol/*n*-decylamine-attached unit in the resultant SucPG and MGluPG were determined to be 18/74/8 and 9/81/10, respectively, using  $^1\text{H}$  NMR.

### *Cell lines culture.*

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (Harvard Medical School, USA) and were grown in RPMI 1640 supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine, 100  $\mu\text{M}$

nonessential amino acid, 50  $\mu$ M 2-mercaptoethanol (2-ME) and antibiotics at 37 °C [16]. EL4, a C57BL/6 mice-derived T lymphoma, was obtained from Tohoku University (Sendai, Japan). E.G7-OVA, which is a chicken egg OVA gene-transfected clone of EL4 and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) [17]. CD8-OVA1.3 cells, a T–T hybridoma against OVA<sub>257–264</sub>/H-2K<sup>b</sup> complex, were kindly provided by Dr. C.V. Harding [18], and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 50  $\mu$ M 2-ME, and antibiotics. OT4H.1D5 cells, a T–T hybridoma against OVA<sub>265–277</sub>/I-A<sup>b</sup> complex, were kindly provided by Dr. J.A. Kapp [19], and were cultured in RPMI 1640 medium supplemented with 10% FBS, 50  $\mu$ M 2-ME, and antibiotics.

#### *Animals.*

Female C57BL/6 mice (H-2<sup>b</sup>, 7 weeks old) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

#### *Generation of murine bone marrow-derived DCs.*

Bone marrow-derived dendritic cells (BMDCs) were prepared according to the method of Lutz et al. with slight modification [20]. Briefly, bone marrow cells flushed from the femurs and tibias of C57BL/6 mice were seeded at  $5 \times 10^6$  cells per sterile 100-mm bacterial grade culture dish in 10 ml of RPMI 1640 containing 10% FBS, 10 ng/ml recombinant murine granulocyte/macrophage colony-stimulating factor (GM-SCF, PeproTech EC Ltd.), 50  $\mu$ M 2-ME, and antibiotics. On day 5, another 10 ml of culture medium was added to the dish for medium replenishment. Nonadherent cells were harvested on days 6–8 as immature BMDCs.

#### *Preparation of liposomes.*

Liposomes were prepared by two kinds of methods, namely vortex and extrusion. Liposomes were prepared using vortex as follows: a dry thin membrane of a mixture of EYPC, DOPE, and SucPG or MGluPG (EYPC/DOPE = 1/1, mol/mol; lipids/polymer = 7/3, w/w) was dispersed in PBS by vortex to afford liposomes. Also, liposomes were prepared by extrusion method as follows: a dry thin membrane of a mixture of lipid and polymer was suspended by a brief sonication using a bath-type sonicator and the obtained liposome suspension was extruded through a polycarbonate membrane of pore sizes of 100 nm [9,10,15]. For liposomes encapsulating OVA, PBS

containing OVA (4 mg/ml) was used for hydration of the lipid/polymer membranes. Free OVA was removed by ultracentrifuge for vortex method and by gel filtration using a Sepharose 4B column for extrusion method. Unmodified liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture without polymer.

#### *Dynamic light scattering and zeta potential*

Diameters and zeta potentials of the liposomes were measured using a Nicomp 380 ZLS dynamic light scattering instrument (Particle Sizing Systems, Santa Barbara, CA) equipped with a 35 mW laser (632.8 nm wavelength). Zeta potentials were measured by equipped an Avalanche photodiode detector, and were detected at an 18.9 angle treated with 9.75 mV. Data was obtained as an average of more than three measurements on different samples.

#### *Fusion assay*

Fusion between plain EYPC liposomes and polymer-modified liposomes was detected by measuring resonance energy transfer between NBD-PE and Rh-PE [21, 22]. Polymer-modified liposomes containing NBD-PE and Rh-PE were prepared according

to the above procedure using the lipid/polymer membrane containing NBD-PE (0.6 mol%) and Rh-PE (0.6 mol%) and extrusion through a polycarbonate membrane with a pore size of 50 nm. Probe-free plain liposomes were also prepared according to the same procedure. The labeled liposomes (final concentration of lipid 0.125 mM) were mixed with fluorescent probe-free EYPC liposomes (final concentration of lipid 0.25 mM) in 25 mM MES and 125 mM NaCl solution of varying pH. Their fusion was followed by monitoring the fluorescence intensity ratio of NBD-PE to Rh-PE ( $R$ ). The excitation wavelength of NBD-PE was 450 nm and monitoring wavelengths for NBD-PE and Rh-PE were 520 nm and 580 nm, respectively. Percentage increase in  $R$  was defined as:

$$\text{Percentage increase in } R = (R_t - R_0) / (R_{100} - R_0) \times 100 \quad (1)$$

where  $R_0$  and  $R_t$  represent the initial and intermediary  $R$  values.  $R_{100}$  is the  $R$  of the labeled liposomes when the liposomes fused completely. The fluorescent lipid-labeled liposomes and the unlabeled liposomes were dissolved in methanol, dried by evaporation, and resuspended in MES buffer. The  $R$  value of the suspension was taken as  $R_{100}$  [21].

### *Cellular uptake*

The DC2.4 cells ( $1 \times 10^5$  cells) cultured for 2 days in a 12-well plate were washed with Hank's balanced salt solution (HBSS, Sigma) and then incubated in culture medium. The liposomes containing FITC-labeled OVA or the liposomes which lipids were substituted by Rh-PE (1 mol%) were added gently to the cells and incubated for 4 h at 37 °C. The cells were washed with HBSS three times, and then the detached cells using trypsin were applied to flow cytometry [23].

### *Microscopy*

The DC2.4 cells ( $2 \times 10^5$  cells) cultured for 2 days in 35-mm glass-bottom dishes were washed with HBSS, and then incubated in serum-free medium. The liposomes containing FITC-labeled OVA (50  $\mu$ g), in which lipids were substituted by Rh-PE (1 mol%), were added gently to the cells and incubated for 4 h at 37 °C. After the incubation, the cells were washed with HBSS three times and then replaced by serum-free medium. LysoTracker Red DND-99 (Molecular Probes) was used by the staining of intracellular acidic compartments according to the manufacturer's instructions. Briefly, LysoTracker Red was added to cells at the final concentration of 75 nM. After the 5 min-incubation, the cells were washed with HBSS three times. Confocal laser scanning microscopic (CLSM) analysis of these cells was performed using LSM 5

EXCITER (Carl Zeiss Co. Ltd.).

*In vitro antigen presentation assay*

The BMDCs were seeded in a 96-well culture plate at a density of  $2 \times 10^4$  cells/well and cultured for 12 h at 37 °C. Each well was washed twice with HBSS, and then the cells were treated with various liposomes containing OVA or OVA solution at various OVA concentrations. After 3 h incubation at 37 °C, the cells were washed three times with HBSS. Subsequently, the cells were co-cultured with  $2 \times 10^4$  CD8-OVA1.3 or OT4H.1D5 cells for 24 h at 37 °C. The response of stimulated CD8-OVA1.3 or OT4H.1D cells was assessed by determining the amount of IL-2 released into an aliquot of culture medium (100  $\mu$ l) using a murine IL-2 ELISA KIT (PeproTech EC Ltd.).

*Immunization.*

Mice were nasally immunized with 10  $\mu$ l aliquots of polymer-modified liposomes or unmodified liposomes containing 100  $\mu$ g of OVA on days 0 and 14. Other group of mice was nasally immunized with OVA solution and another group of mice was subcutaneously immunized with CFA/OVA emulsion.

### *CTL assay.*

Splenocytes from immunized mice were suspended in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, and 20 U/ml recombinant murine IL-2 (Peprotech, London, UK). Seven days after the second immunization, splenocytes were obtained from five mice, and the splenocytes were pooled and stimulated with mitomycin C-treated E.G7-OVA cells at a ratio of 10:1 for 5 days. The stimulated splenocytes were used as effector cells for the cytotoxicity assay. The CTL activity was evaluated at various effector cell to target cell (E.G7-OVA or EL4 cell) ratios (E/T ratios) using a lactate dehydrogenase (LDH) cytotoxicity detection assay (Takara Biomedicals, Tokyo, Japan).

## **Results and Discussion**

### **Characterization of liposomes**

We measured sizes and zeta potentials of prepared liposomes to characterize the liposomes. Table 1 presents particle sizes and zeta potentials of various liposomes prepared by vortex method or extrusion method. When the liposomes were prepared using the vortex method, unmodified liposomes were of about 1500 nm diameter. Although the SucPG-modified and MGluPG-modified liposomes were prepared

according to the same method, they showed much smaller diameters of around 400–500 nm, probably because negatively charged polymers bound on the liposome surface enhanced hydration of the membrane surface and increased the colloidal stability of the liposomes. When prepared using the extrusion method, all liposomes showed similar particle sizes of around 110 nm, which corresponds to the pore size of the membrane used for their extrusion.

Regarding zeta potentials of the liposomes, the unmodified liposomes were nearly zero, indicating that their surface was electrically neutral. However, the liposomes modified with SucPG or MGluPG showed negative zeta potentials of around -11 mV because of carboxylate anions on the polymer chains. This result demonstrates that these polymer-modified liposomes have electrically similar surface characteristics, irrespective of the polymers attached on their surface.

### **Fusogenic activities of liposomes**

In previous studies, we showed that modification with SucPG and MGluPG provides pH-dependent fusion ability to stable EYPC liposomes [9,10]. In this study, we used a mixture of EYPC and DOPE as liposomal lipids because inclusion of DOPE is shown to increase the fusion ability of liposomes [24]. We examined the fusion abilities

of these polymer-modified liposomes using resonance energy transfer between NBD-labeled and Rh-labeled lipids in the liposome membranes. The labeled liposomes were mixed with unlabeled plain EYPC liposomes and incubated for 1 h at various pH. Fusion between these liposomes was evaluated by monitoring the ratio of fluorescence intensity of NBD to that of Rh ( $R$  value).

Figure 2 portrays the percent increase in the  $R$  value during 1 h incubation as a function of pH. The unmodified liposomes exhibit a very low extent of the increase in  $R$  throughout the experimental pH region, indicating the lack of fusion ability for unmodified liposomes. The SucPG-modified liposomes also showed a very low extent of the increase in  $R$  at neutral pH, but the  $R$  value increased below pH 6, suggesting that the SucPG-modified liposomes generated fusion ability at weakly acidic pH. Although a similar pH-dependence of fusion was apparent for the MGlPG-modified liposomes, the extent of increase in the  $R$  value in the weakly acidic pH region was greater, indicating that the MGlPG-modified liposomes generated higher fusion ability than SucPG-modified liposomes. Because MGlPG has more hydrophobic side chains than SucPG, polymer chains of MGlPG might strongly disrupt the liposome membrane, resulting in more intensive fusion of the liposomes.

These results indicate clearly that SucPG and MGlPG provided fusion ability to

stable EYPC/DOPE liposomes and that the latter gave higher fusion ability than the former, which is consistent with our previous observation [10].

### **Association of liposomes with DCs**

To estimate the performance of the polymer-modified liposomes as delivery vehicles of antigenic proteins to cytoplasm of DC, we examined the association of the liposomes with DCs after their incubation. The DC2.4 cells were incubated with various liposomes labeled with Rh-PE for 4 h; then fluorescence intensities of the cells were measured using a flow cytometer. As presented in Figs. 3a and 3b, cells treated with the labeled liposomes exhibited higher fluorescence intensities than intact cells, indicating the association of liposomes to the treated cells. In addition, the cells treated with SucPG-modified and MGluPG-modified liposomes displayed stronger fluorescence of Rh-PE, suggesting more efficient association of these polymer-modified liposomes to DCs. To evaluate their ability to associate DCs further, the average of cellular fluorescence intensities was calculated (Fig. 3c). Compared with the cellular association of the unmodified liposomes, on average, 6.6 and 6.2 times higher amounts of liposome association were observed, respectively, for SucPG-modified and MGluPG-modified liposomes. Actually, DCs are known to have scavenger receptors, which recognize the

micro-organisms or apoptotic cells with an anionic component [25]. As described in a previous report, these polymer-modified liposomes are likely to be taken up by DCs through interaction of their anionic surfaces with scavenger receptors [23].

Comparison of liposomes prepared by the extrusion method to those prepared by the vortex method shows that the former exhibited slightly higher cellular association than the latter, indicating that DCs can engulf small liposomes more efficiently than large liposomes with diameters of 500–1500 nm, although the difference was slight.

### **Cytoplasmic delivery of antigenic protein to DCs**

We examined the ability of the polymer-modified liposomes to deliver OVA, which was used as an antigenic protein into cytosol of DCs. To monitor the intracellular distribution of the liposomes, the encapsulated OVA and the liposome membrane were labeled, respectively, using FITC and Rh-PE. The labeled liposomes encapsulating FITC-OVA were added to DC2.4 cells and incubated for 4 h. Then, the cells were washed with buffer and observed using CLSM (Fig. 4). When DC2.4 cells were treated with the unmodified liposomes, punctate fluorescence of FITC-OVA was observed in the cells. In addition, fluorescence of Rh was observed at the same places that FITC-OVA fluorescence appeared. This result suggests that OVA molecules loaded in

the unmodified liposomes were still trapped in the endosomes and/or the lysosomes. For cells treated with the SucPG liposomes, the fluorescence of FITC-OVA was also mostly punctate and overlapped with Rh-PE fluorescence. In contrast, for cells treated with MGluPG liposomes, diffuse fluorescence of FITC-OVA was observed in the cells, while fluorescence of Rh-PE remained punctate, indicating that the labeled OVA molecules were transferred into cytosol from endosome, where liposomal membrane remained trapped. Probably, the highly fusogenic MGluPG liposomes generate strong fusion ability in the weakly acidic environments of endosome and fuse with endosomal membrane, resulting in the release of entrapped OVA molecules into cytosol [9,10]. The SucPG liposomes only slightly induced the transfer of OVA into cytosol. Therefore, it is implied that their fusion ability is insufficient to induce fusion or destabilization of endosomal membrane for efficient transfer of OVA molecules into cytosol (Fig. 2).

### **Induction of cellular immune responses**

We examined the ability of these polymer-modified liposomes to activate cellular immunity. Although immunization is generally conducted *via* subcutaneous injection of antigen, we exploited the administration of antigen through nasal mucosa because antigen administration can be achieved using a noninvasive, needleless method in this

immunization. It can also induce both mucosal and systemic immunities [11–13].

For this study, the C57BL/6 mice were immunized nasally twice with OVA-loaded liposomes containing MPL in the membrane as an adjuvant. One week later, splenocytes were collected from the immunized mice and stimulated with mitomycin C-treated E.G7-OVA cells. Then their toxicity toward E.G7-OVA cells, which are OVA-presenting recombinant cells derived from EL4 cells, was measured to estimate the induction of OVA-specific CTLs.

Figure 5a shows cellular toxicities of the stimulated splenocytes recovered from mice that had been immunized with various OVA-loaded liposomes or free OVA. When free OVA was used for the immunization, CTL activity was almost identical to that of the case of untreated mice, indicating that free OVA has no ability to induce cellular immunity under experimental conditions. In addition, administration of the OVA-loaded unmodified liposomes increased the CTL activity only slightly. In contrast, when the polymer-modified liposomes containing OVA were used for immunization, higher CTL activities were observed. Especially, MGluPG-modified liposomes more efficiently induced activation of CTLs than the SucPG-modified liposomes did. Indeed, the same stimulated splenocytes did not exhibit cellular toxicity toward EL4 cells (Fig. 5b). Therefore, immunization with these polymer-modified liposomes containing OVA

induced activation of OVA-specific CTLs. Considering that these polymer-modified liposomes showed a similar degree of cellular association with DCs (Fig. 3), it is likely that the difference in fusion ability between these liposomes caused their different abilities for cellular immune activation.

### **Estimation of antigen presentation**

The highly fusogenic MGlucPG-modified liposomes induced cellular immune response efficiently. Because the cellular immunity is activated through antigen presentation on the MHC class I molecules of antigen presenting cells (APCs), such as DCs, we expected that the liposome-mediated delivery of OVA molecules into cytosol of APCs caused the antigen presentation on the MHC class I molecules, engendering activation of cellular immunity. To confirm this mechanism, we further examined whether the MGlucPG-modified liposomes actually possess the capability of inducing antigen presentation on the MHC class I on DCs.

The OVA-loaded liposomes or free OVA were added to BMDCs; then BMDCs were co-cultured with CD8-OVA1.3 or OT4H.1D5 cells, which respectively recognize MHC class I/peptide complexes or MHC class II/peptide complexes. Subsequently, MHC class-restricted antigen presentation was evaluated by detection of IL-2 secretion in

supernatants of the co-cultured medium. Figure 6 shows IL-2 production levels of the co-cultured medium of BMDCs treated with the OVA-loaded liposomes and free OVA. As depicted in Fig. 6a, interaction with BMDCs and OVA-loaded MGLuPG-modified liposomes strongly enhanced IL-2 release from CD8-OVA1.3 cells. The BMDCs treated with either OVA-loaded in the plain liposomes or free OVA also promoted IL-2 release from the CD8-OVA1.3 cells, but the promotion occurred to a much lesser degree than the case of the OVA-loaded MGLuPG-modified liposomes. This result suggests that the OVA-loaded MGLuPG-modified liposomes induced antigen presentation through the MHC class I molecules on BMDCs more efficiently than free or plain liposome-encapsulated OVA. In contrast, BMDCs treated with these OVA-loaded liposomes or free OVA enhanced IL-2 release from OT4H.1D5 only slightly, suggesting that the OVA-loaded MGLuPG-modified liposomes have no ability to induce MHC class II-mediated antigen presentation. These results demonstrate that the MGLuPG-modified liposomes can induce antigen presentation through MHC class I molecules.

### **Influence of liposome size on cellular immunity activation**

Finally, we examined the influence of liposome size on their ability to activate cellular immunity. First, MGLuPG liposomes with diameters around 500 nm and 110 nm

were prepared, respectively, using vortex and extrusion methods (Table 1). We compared the respective efficiencies of the intracellular OVA delivery of these liposomes with different sizes. The amounts of OVA encapsulated in the liposomes were estimated to be 0.77  $\mu\text{g}$  per 1 nmol lipid for the large liposomes and 0.2  $\mu\text{g}$  per 1 nmol lipid for the small liposomes. When the same amount of FITC-OVA was added to DC2.4 cells as encapsulated in the liposomes, approximately double the amount of FITC-OVA was taken up by the cells using the large liposomes than those using the small liposomes (Fig. 7a).

We also examined the intracellular distribution of OVA molecules delivered by these MGLuPG liposomes of different sizes. The DC2.4 cells were incubated with these MGLuPG liposomes encapsulating FITC-OVA for 4 h and then observed using CLSM (Fig. 7b). Strong and punctate fluorescence of FITC-OVA was observed in cells treated with large MGLuPG liposomes. Punctate fluorescence of FITC-OVA is mostly overlapped with fluorescence of LysoTracker Red. Therefore, most FITC-OVA molecules remained trapped in the endosomes and lysosomes. The punctate fluorescence of FITC-OVA was observed in the large liposome-treated cells even after 24 h-incubation, indicating that FITC-OVA molecules were retained in endosome and lysosome for 24 h (data not shown). In contrast, cells treated with the small MGLuPG

liposomes displayed diffuse fluorescence of FITC-OVA, suggesting that these small liposomes delivered the antigenic protein efficiently into the cytosol.

The large MGluPG liposomes prepared by vortex showed less endosomal escape, compared to the small extruded MGluPG liposomes, although these liposomes were modified with the same fusogenic polymer MGluPG. Because the large liposome should have a multilamellar structure, only the outermost layer of the bilayer membranes with low curvature might contact with the target membrane, resulting in low efficiency of fusion. In fact, we evaluated fusion ability of the large MGluPG liposome by the same method used for evaluation of the small MGluPG liposome fusion (Fig. 2) and observed only 15% increase in R value after 4 h incubation at pH 4, indicating a low fusion ability of the large MGluPG liposome (data not shown).

These liposomes encapsulating OVA were administered nasally; then CTL responses were evaluated as described above (Fig. 7c). Splenocytes of the immunized mice exhibited high toxicity toward OVA-presenting E.G7-OVA cells but almost no toxicity toward their parent EL4 cells, indicating that both liposomes were able to induce OVA-specific CTLs, irrespective of their size difference. Moreover, the CTLs derived with these OVA-loaded liposomes showed an almost identical level of toxicity to E.G7-OVA cells within the error bars. The large MGluPG liposomes delivered more

OVA molecules to cells than the small liposomes. However, the latter introduced OVA molecules into the cytosol more efficiently than the former. Consequently, these liposomes might achieve CTL activation to a similar degree.

To evaluate the potential of MGluPG liposomes as an adjuvant, we compared their induced CTLs activity with that of CFA, which is widely used for induction of immune responses [26]. The result is also presented in Fig. 7c. Apparently, CTL activity induced by CFA is of a comparable level to that of MGluPG liposomes.

## **Conclusions**

For this study, by modification of liposomes with carboxylated poly(glycidol) derivatives, such as SucPG and MGluPG, we prepared pH-sensitive fusogenic liposomes that can deliver antigenic proteins into the cytosol of DCs. We then investigated their ability to activate cellular immune response. Indeed, these polymer-modified liposomes were shown to have capabilities of delivering antigenic OVA into cytosol of DCs and inducing antigen presentation through the MHC class I molecules on DCs. Nasal administration of these liposomes encapsulating OVA activated the antigen-specific cellular immunity in mice. Especially, highly fusogenic MGluPG liposomes exhibited high ability for CTL activation, which is comparable to

the widely used adjuvant CFA. Despite its high ability to activate immune response, CFA is known to induce inflammatory reactions at the site of administration. Consequently, CFA is not applicable to humans. In contrast, MGluPG liposomes comprise phospholipid and biocompatible poly(glycidol) derivatives [27]. For that reason, MGluPG liposomes and their relevant liposomes might be promising antigen carriers for establishment of cancer immunotherapy and mucosal vaccination.

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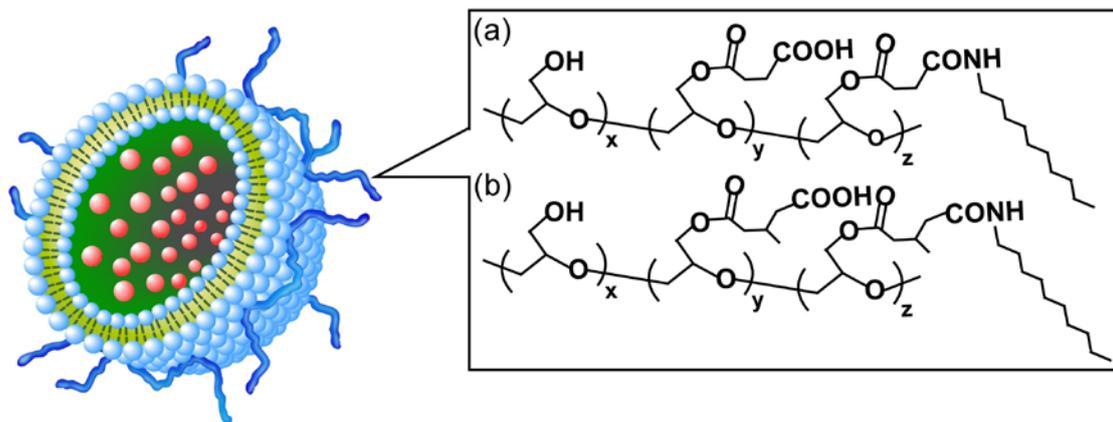
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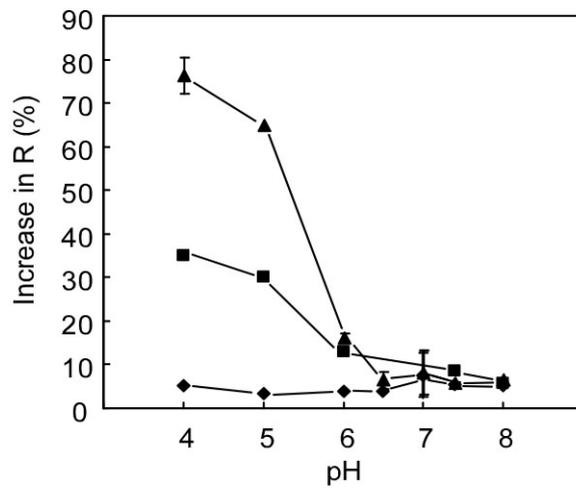
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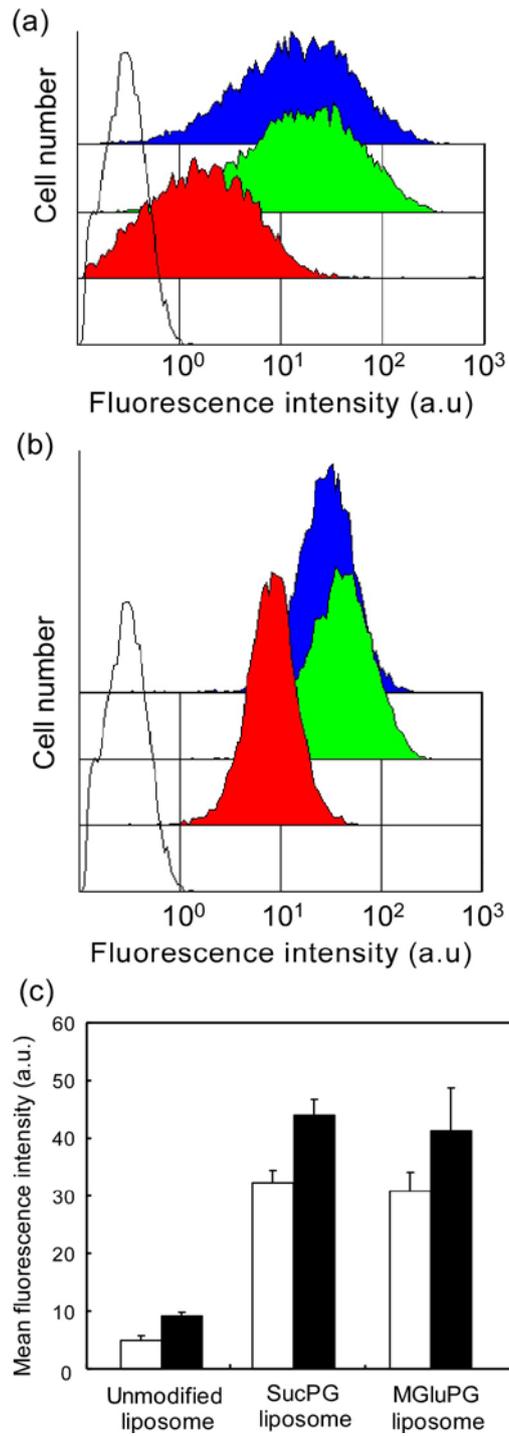
*Yuba et al., Figure 1.*

Liposome	Method	Zeta potential/mV (SD)	Particle size/nm (SD)
Unmodified	Vortex	-0.51 (0.73)	1560 (817)
Unmodified	Extrusion	-0.92 (0.42)	116 (26.0)
SucPG	Vortex	-11 (2.4)	431 (112)
SucPG	Extrusion	-12 (2.5)	105 (18.0)
MGLuPG	Vortex	-11 (3.5)	523 (78.0)
MGLuPG	Extrusion	-11 (2.4)	110 (23.0)

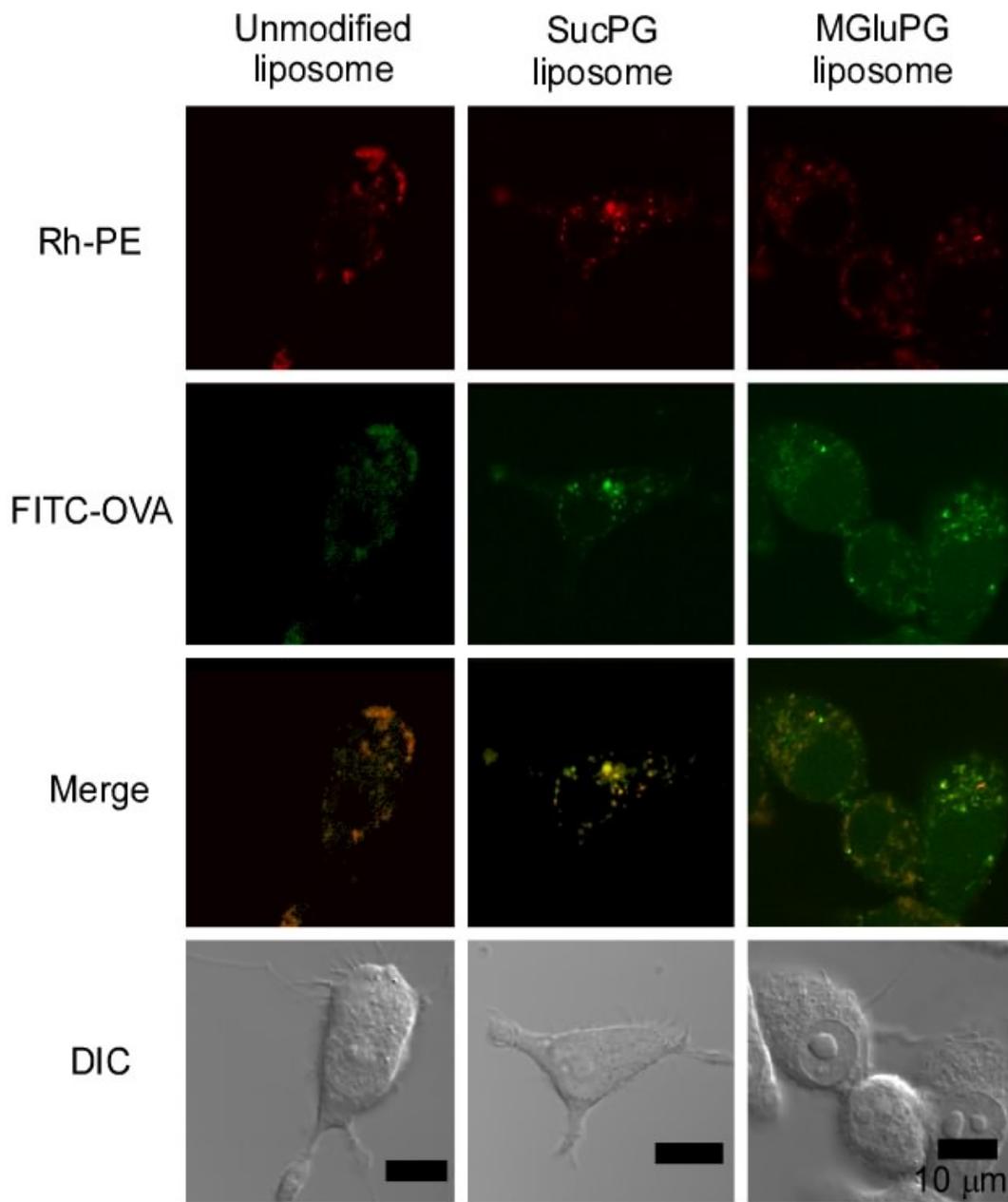
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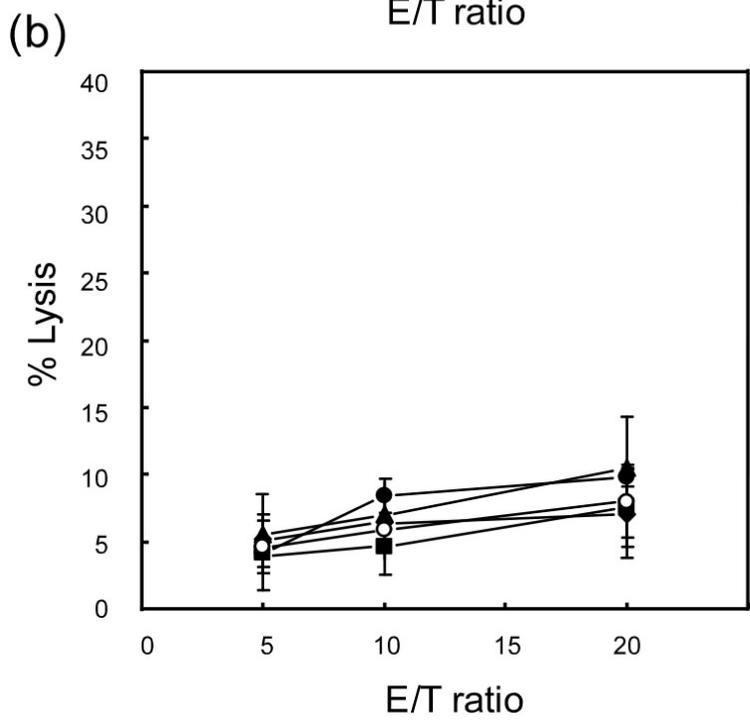
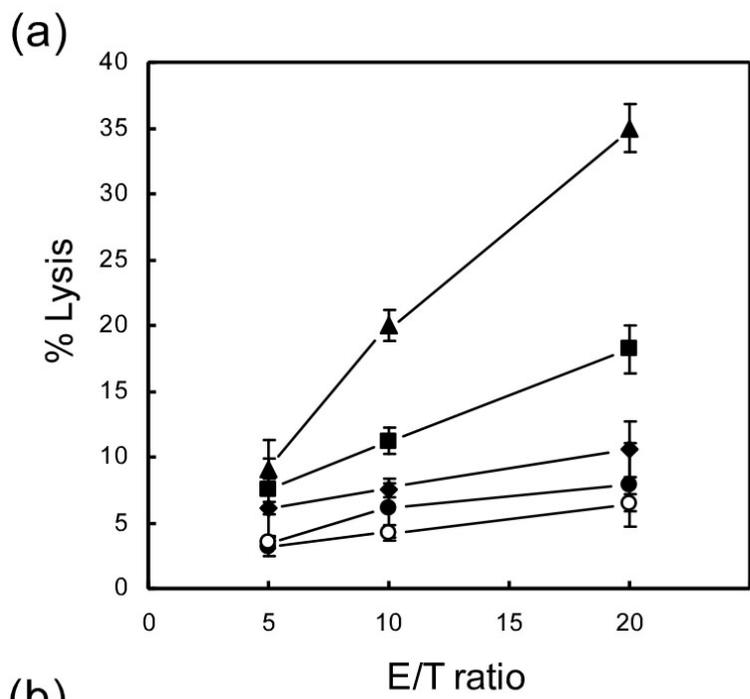
*Yuba et al., Figure 2.*



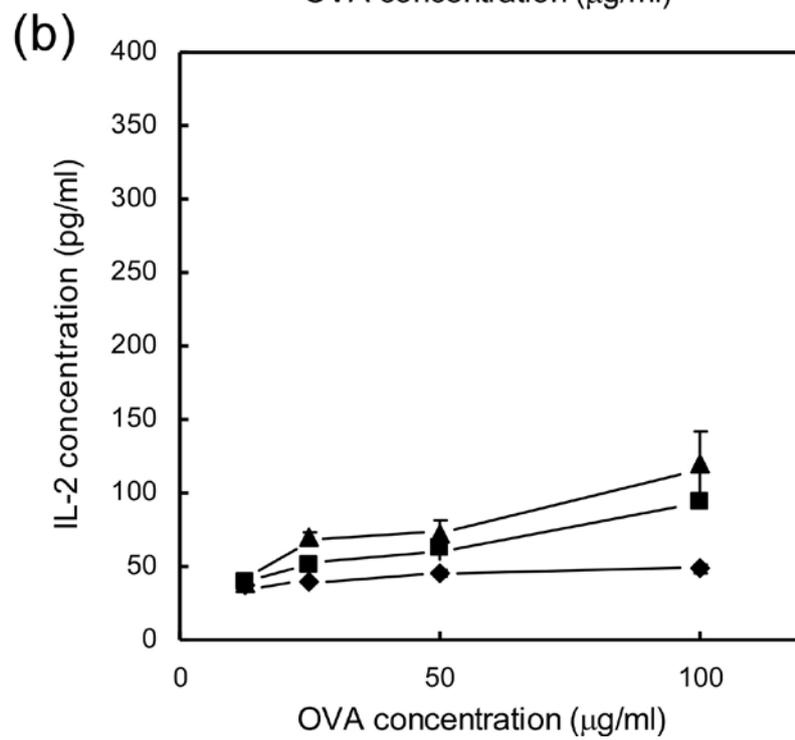
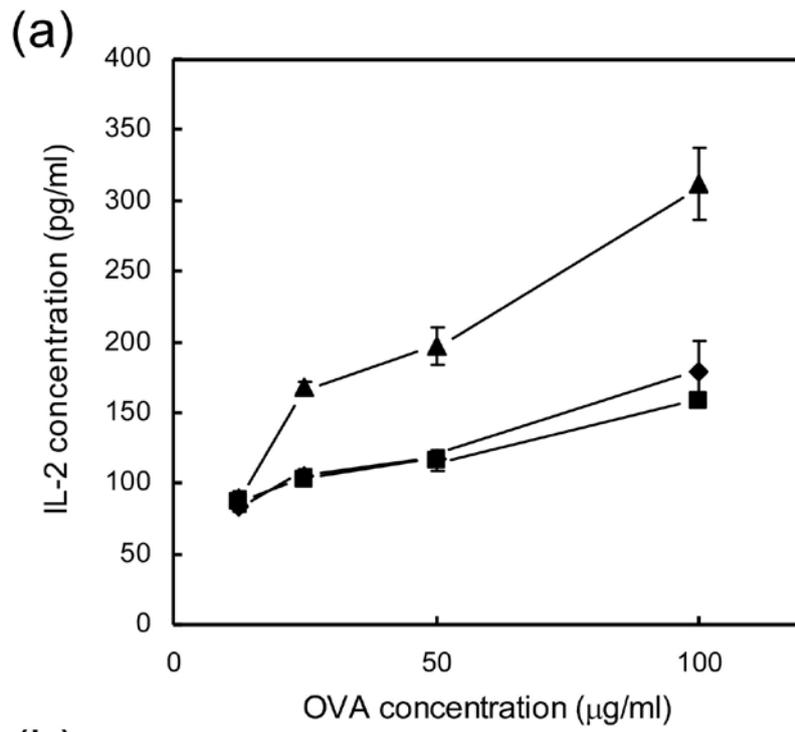
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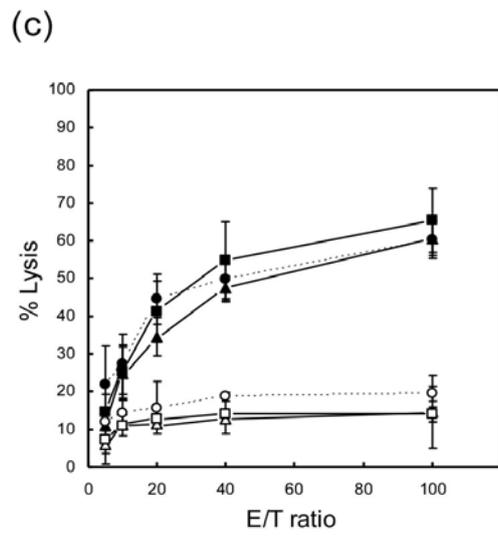
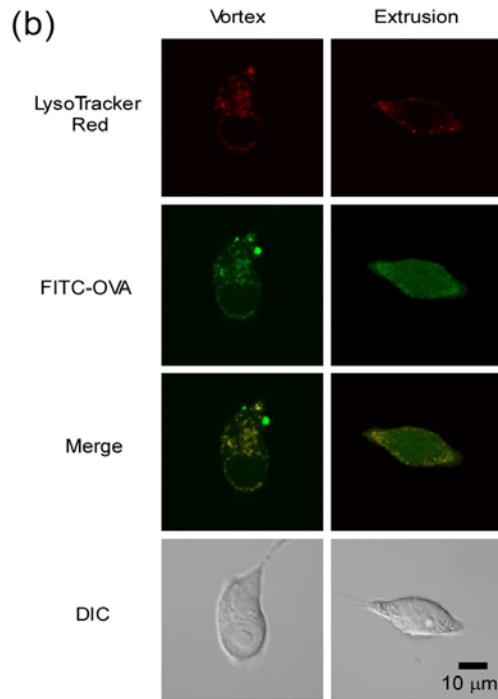
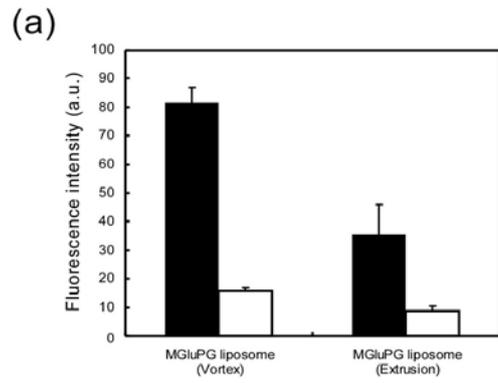
*Yuba et al., Figure 4.*



*Yuba et al., Figure 5.*



*Yuba et al., Figure 6.*



*Yuba et al., Figure 7.*

## Figure captions

**Figure 1.** Structures of SucPG (a), MGluPG (b) and pH-sensitive polymer-modified liposome.

**Table 1.** Particle size and zeta potential of various liposomes.

**Figure 2.** pH-dependent fusogenic properties of liposomes. Percent increase in *R* values for fluorescent lipid-labeled unmodified (diamonds), SucPG-modified (squares) and MGluPG-modified (triangles) EYPC/DOPE liposomes after 1 h-incubation with the unlabeled plain EYPC liposomes at varying pH was shown. Measurements were performed in 25 mM MES and 125 mM NaCl at 37 °C. Each point is the mean  $\pm$  SD (n = 3). Liposomes prepared by extrusion method were used.

**Figure 3.** Evaluation of cellular association of various liposomes labeled with Rh-PE using flow cytometry. DC2.4 cells were treated with SucPG-modified (green), MGluPG-modified (blue) and unmodified (red) liposomes prepared by vortex method (a) or extrusion method (b). Fluorescence intensity of untreated cells (black lines) is also shown. (c) Mean fluorescence intensity of the liposome-treated DC2.4 cells. Each point is the mean  $\pm$  SD (n = 3). Cellular treatment was performed by incubating with liposomes prepared by vortex method (open) or extrusion method (closed) in serum-free

medium at 37 °C for 4 h. Concentration of liposomal lipids was 0.1 mM.

**Figure 4.** CLSM images of DC2.4 cells treated with various liposomes labeled with Rh-PE and loaded with FITC-OVA. Liposomes prepared by extrusion method were used. DC2.4 cells were incubated with liposomes at 37 °C for 4 h in the serum-free medium, washed with PBS and observed with CLSM. Scale bar represents 10  $\mu$ m. Concentration of liposomal lipids for cellular treatment was 0.1 mM.

**Figure 5.** OVA-specific cytotoxic T cell responses in spleen at Day 21 after nasal immunization with OVA solution (●), polymer-unmodified liposomes (◆), SucPG liposomes (■) and MGluPG liposomes (▲). Liposomes prepared by vortex method were used. Cytotoxic activity was measured by a LDH assay at indicated E/T ratios. E.G7-OVA cells (a) and EL4 cells (b) were used as target cells. T cell responses from mice without treatment (○) were also shown as a negative control.

**Figure 6.** Presentation of OVA-derived epitope peptides *via* MHC molecules in BMDCs. BMDCs were incubated with free OVA (diamonds), and OVA-loaded MGluPG-modified (triangles) and unmodified (squares) liposomes at varying OVA concentrations for 3 h. Liposomes prepared by extrusion method were used. Concentrations of IL-2 in the medium after co-culture of OVA-treated BMDCs with (a) CD8-OVA1.3 (specific for OVA<sub>257-264</sub>/H-2K<sup>b</sup> complex) and (b) OT4H.1D5 (specific for

OVA<sub>265-277</sub>/I-A<sup>b</sup> complex) cells for 24 h as a function of OVA concentration during the BMDCs treatment were shown. Each point represents means  $\pm$  SD.

**Figure 7.** (a) Effect of liposome size on delivery of FITC-OVA by MGLuPG liposomes.

DC2.4 cells were treated with FITC-OVA-loaded liposomes prepared by extrusion or vortex method in the serum-free medium at 4 °C (open) and 37 °C (closed) for 4 h, and then cellular association of FITC-OVA was estimated using flow cytometry. Each point

is the mean  $\pm$  SD (n = 3). OVA concentration was 50  $\mu$ g/ml. (b) CLSM images of

DC2.4 cells treated with FITC-OVA-loaded liposomes prepared by vortex method or extrusion method. DC2.4 cells were incubated with MGLuPG-modified liposomes encapsulating FITC-OVA (50  $\mu$ g of OVA/ml) in the serum-free medium at 37 °C for 4

h. (c) Effect of liposome size on OVA-specific cytotoxic T cell response. Mice were nasally immunized with OVA-loaded MGLuPG liposome prepared by vortex method

(triangles) or by extrusion method (squares). Mice were also immunized with CFA/OVA emulsion subcutaneously (circles). Cytotoxic activity of splenocytes of the

immunized mice was measured by a LDH assay at indicated E/T ratio. E.G7-OVA cells (closed symbols) and EL4 cells (open symbols) were used as target cells.