pH-sensitive fusogenic 3-methyl-glutarylated hyperbranched poly(glycidol) (MGlu-HPG)-conjugated liposome induces antigen-specific cellular and humoral immunity

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Running title: Adjuvant activity of polymer-modified liposome
Abstract

We examined the ability of a novel liposome, surface-modified by 3-methyl-glutarylated hyperbranched poly(glycidol) (MGlu-HPG), to enhance antigen-specific immunity \textit{in vitro} and \textit{in vivo} and to function as a vaccine carrier. Murine bone marrow-derived dendritic cells took up ovalbumin (OVA) encapsulated in MGlu-HPG-modified liposomes more effectively than free OVA or OVA encapsulated in unmodified liposomes. Immunization of mice with OVA-containing MGlu-HPG-modified liposomes induced antigen-specific splenocyte proliferation and production of IFN-\(\gamma\) more strongly than did immunization with free OVA or OVA encapsulated in unmodified liposomes. The immune responses induced by OVA encapsulated in MGlu-HPG-modified liposome were significantly suppressed by addition of anti-MHC class I and class II monoclonal antibodies, indicating the involvement of antigen presentation via MHC class I and II. Furthermore, delayed-type hypersensitivity responses and OVA-specific antibodies were induced more effectively in mice immunized with OVA encapsulated by MGlu-HPG-modified liposomes than with unencapsulated OVA or OVA encapsulated in unmodified liposomes. These results suggested that MGlu-HPG-modified liposomes effectively induced both cell-mediated and humoral immune responses. Collectively, this study is
the first to demonstrate the induction of both cell-mediated and humoral immune
responses *in vivo* by MGlu-HPG–modified liposomes.

**Keywords:** Polymer-modified liposome, Vaccine carrier, Dendritic cells, Antibody,
Cellular immunity
Introduction

For the development of new and improved vaccines against diseases of public health importance, several approaches to rational design have been developed such as the selection of the most immunologically effective pathogen antigen (14, 17). One promising strategy for the development of new vaccines is the search for novel adjuvants to enhance the potency and longevity of antigen-specific immune responses (19). Adjuvants include immunostimulants that directly enhance immune responses to vaccines and vehicles that present antigens to the immune system through controlled release and delivery.

Liposomes consist of an aqueous core entrapped by one or more bilayers composed of natural or synthetic lipids that can protect the liposome contents, such as antigenic proteins, by providing a permeability barrier that defines an internal compartment (6, 10). Liposomes have been explored as potential antigen carriers for more than 20 years (2, 8, 11, 23, 24, 26). In spite of their efficient delivery into cultured cells in vitro, conventional liposomes are only weakly immunostimulatory in vivo, thus limiting their practical application (7, 9, 20). Numerous attempts have been made to improve the performance of liposomes as vaccine adjuvants by, for example, enhancing intracellular antigen delivery (3-5, 13, 15, 16, 18, 22-25, 28-31). A variety
of strategies have been adopted to achieve this, including liposome modification with
bacteria-derived lipids (5, 23, 24) and viral fusion proteins such as influenza
hemagglutinin (3, 4) and Sendai virus fusion protein (13, 28). These modified liposomes
were able to deliver antigenic proteins through membrane fusion with
antigen-presenting cells, leading to efficient antigen presentation via the MHC class I
pathway (3-5, 13, 23, 24, 28). However, there are biological safety concerns about these
systems since they contain components derived from viruses and bacteria.

Another approach to enhancing intracellular antigen delivery of vaccines is
functionalization of liposomes with synthetic molecules. For example,
membrane-penetrating peptides such as octaarginine have been used to endow
liposomes with membrane-destabilizing activity (16). These liposomes were able to
deliver antigen into the cytosol of dendritic cells (DCs) and induce cellular immunity.
pH-sensitive polymers have also been used to give liposomes membrane-fusing activity,
which might enhance the delivery of antigenic proteins into antigen-presenting cells and
induce cellular immunity (15, 22, 29-31).

We recently developed a novel functional liposome by surface modification of
stable liposomes with carboxylated poly(glycidol) derivatives having a linear or
hyperbranched backbone structure, designated as 3-methyl-glutarylated poly(glycidol)
or hyperbranched poly(glycidol) (MGlu-PG or MGlu-HPG), for effective antigen delivery into DCs (21, 29-31). These polymers become fusogenic only in a weakly acidic environment, which allows them to remain stable under neutral conditions and to specifically target tissues such as acidic inflammatory/tumorigenic sites or acidic cellular compartments. Murine DC-like DC2.4 cells efficiently took up antigen encapsulated by MGlu-PG– or MGlu-HPG–modified liposomes, and early delivery of antigen from the acidic endosome into the cytosol was also observed (29, 30). MGlu-HPG–modified liposomes promoted cytoplasmic delivery of antigenic proteins more efficiently than MGlu-PG–modified liposomes (29). Since the activation of antigen-specific cytotoxic T lymphocytes (CTLs) is elicited by the translocation of antigen into the cytosol (1), it is conceivable that the early cytosolic delivery by these polymer-modified liposomes can enhance cellular immunity. Administration of MGlu-PG liposomes induced antigen-specific cellular immune responses in mice (30). However, the immunological responses of animals immunized with antigens encapsulated by MGlu-HPG–modified liposomes have not yet been analyzed in detail.

This study confirmed the utility of MGlu-HPG–modified liposomes as effective antigen carriers for the development of novel vaccines. We first showed high uptake of MGlu-HPG–modified liposomes by primary DCs freshly isolated from C57BL/6 mice.
mice. We also documented the modified liposome’s stimulatory effects on antigen-specific immunity in C57BL/6 mice immunized with ovalbumin (OVA) encapsulated by MGlu-HPG–modified liposomes.

MATERIALS AND METHODS

Preparation of 3-methyl-glutarylated hyperbranched poly(glycidol)-modified liposomes. MGlu-HPG–modified liposomes were prepared as previously reported (29). Briefly, MGlu-HPG was prepared by mixing HPG with a degree of polymerization of 60 (HPG60), LiCl, and 3-methylglutaric anhydride in pyridine. 1-Aminodecane was combined with about 10% of carboxyl groups as anchor moieties for fixation of polymer to the liposome membrane. A dry, thin membrane composed of a mixture of egg yolk phosphatidylcholine (EYPC), L-dioleoyl phosphatidylethanolamine (DOPE), and MGlu-HPG60 (EYPC/DOPE = 1/1 mol/mol; lipid/polymer = 7/3 w/w) was suspended by a brief sonication and the resulting liposomal suspension was extruded through a polycarbonate membrane with a pore size of 100 nm. For liposomes encapsulating OVA or OVA labeled with fluorescein isothiocyanate (FITC), the lipid/polymer membranes were hydrated in phosphate-buffered saline (PBS) containing OVA or OVA labeled with FITC.
OV A or OVA labeled with FITC were removed by gel filtration using Sepharose 4B columns. Unmodified liposomes were also prepared according to the above procedure using a dry membrane of a lipid mixture without polymer. The concentration of OVA or OVA labeled with FITC encapsulated in liposomes was equalized in each sample based on results obtained with the Coomassie Protein Assay Reagent (Thermo Scientific Pierce; Rockford, IL).

Mice. Five- to ten-week-old female C57BL/6NCrSlc mice (Japan SLC Inc., Shizuoka, Japan) were maintained at the animal facilities of RIKEN BSI (Saitama, Japan), according to institutional guidelines. The mice were kept in an air-conditioned room and fed standard laboratory food and water *ad libitum*. All experiments were approved by the RIKEN Animal Experiments Committee.

Uptake by bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDCs) were generated according to a previously reported protocol, with slight modifications (7). Briefly, bone marrow cells were collected by flushing the femurs and tibias of 6- to 10-week-old female C57BL/6 mice. The cells were cultured at $1 \times 10^6$/ml in 6-well plates in RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with L-glutamine, 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 5 ng/ml of recombinant murine
granulocyte/macrophage colony-stimulating factor (GM-CSF; Prospec-Tany Technogene LTD, Rehovot, Israel), and 100 μg/ml of penicillin, streptomycin and gentamicin (GIBCO, Invitrogen). On days three and five of culture, plates were swirled gently and two-thirds of the conditioned medium, including the non-adherent cells, was removed. Fresh GM-CSF-containing culture medium was added to the cells. On day seven or eight, the non-adherent cells were harvested after vigorous pipetting of the DC clusters.

For detecting the uptake of OVA-containing MGlu-HPG–modified liposomes, BMDCs (1 × 10⁶ in 100 μl) were incubated for 1 h at 4°C or 37°C with 1 μg/ml of free OVA, or with liposomes (with or without the polymer modification) containing FITC-labeled OVA. After incubation, the cells were washed three times with cold PBS containing 0.1% bovine serum albumin (BSA; Iwai Chemicals, Tokyo, Japan) and 0.01% sodium azide, and the percentage of FITC-positive cells was analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA).

Immunization and isolation of blood samples and spleens. Six-week-old C57BL/6 mice were administered 3.3 mg/kg of free OVA or OVA-containing liposomes, with or without the polymer modification on day 0 and 14. Blood samples were collected from the tail vein once a week for antibody titration. On day 28, whole
blood samples were collected by heart puncture under deep anesthesia with diethyl ether and the spleens were harvested.

The spleens were homogenized in 3 ml RPMI 1640 supplemented with L-glutamine, 10% FBS, and 100 μg/ml of penicillin, streptomycin, and gentamycin by using glass homogenizer on ice and processed as single cell by passing the cells through a strainer (100 μm pore size; Becton Dickinson). The red blood cells were removed by suspension in a lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA₂Na). Splenocytes were washed with complete RPMI and counted using a trypan blue exclusion method.

**In vitro cytokine release measured by enzyme-immunosorbent assay (ELISA).**

Splenocytes (2 × 10⁶ in 2 ml) were incubated for 72 h with 1–25 μg/ml of free OVA or OVA-containing liposomes with or without the polymer modification. After incubation, the concentration of IFN-γ was analyzed by ELISA.

NUNC MaxiSorp 96-well ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated with affinity-purified rat anti-mouse IFN-γ (eBioscience; 100 μl, 1 μg/ml) in 0.1 M NaHCO₃, incubated overnight at 4°C, and washed with 1× PBS containing 0.05% Tween 20 (PBS-T). The plates were blocked with 3% BSA in PBS for 1 h at room temperature and washed with PBS-T. Standard recombinant IFN-γ diluted in PBS containing 1% BSA was added to the wells, and the plates were
incubated for 1 h at room temperature and then washed with PBS-T. A biotin-labeled rat anti-mouse IFN-γ (eBioscience; 100 μl, 1 μg/ml) was added to the wells, after which the plates were incubated for 1 h at room temperature and then washed with PBS-T. Horseradish-peroxidase (HRP)-conjugated avidin (eBioscience; 100 μl, 1 μg/ml) was added to the wells, and the plates were incubated for 1 h at room temperature and then washed with PBS-T. Finally, colorimetric signals were generated using 1-Step Ultra TMB-ELISA solution (Thermo Scientific Pierce). After 15 min, the reaction was stopped with 1 N H₂SO₄ and the absorbance at 450 nm was measured using a Wallac ARVO multilabel counter (Perkin Elmer, Foster City, CA). The concentration of IFN-γ in the samples was interpolated on the basis of standard curves.

**Splenocyte proliferation assay.** Splenocytes (2 × 10⁶ in 2 ml) were incubated for 96 h with 1–25 μg/ml of free OVA or OVA-containing liposomes with or without the polymer modification. For examination of MHC restriction, 1–10 ng/ml of anti-mouse H-2Db monoclonal antibody (MAb) (MHC class I; eBioscience, San Diego, CA) or anti-mouse I-A/I-E MAb (MHC class II; eBioscience) was added to the cultures. Mouse IgG₂a and rat IgG₂b antibodies were used as isotype controls for MHC class I and II, respectively. Splenocyte proliferation was determined using the FITC BrdU Flow Kit (Becton Dickinson, San Diego, CA), according to the manufacturer’s instructions.
Briefly, 5-bromo-2-deoxyuridine (BrdU; 5 μM) was added for the last 2 h of incubation. After washing, cells were fixed, permeabilized and treated with DNase I, as described in the assay kit. Cells were washed and analyzed on a FACSCalibur (Becton Dickinson) using CellQuest software (Becton Dickinson).

**Antibody titration.** Specific antibody titers against OVA were determined by performing an endpoint ELISA. NUNC MaxiSorp 96-well ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated with 5 μg/ml of OVA in 0.1 M NaHCO₃ (Nacalai Tesque, Kyoto, Japan), incubated overnight at 4°C, and washed with PBS-T. The plates were blocked with 3% BSA in PBS for 1 h at room temperature and washed with PBS-T. After serial dilution in 1% BSA in PBS, starting at an 80-fold dilution, serum samples (100 μl) were added to the wells and the plates were incubated for 1 h at room temperature. The plates were washed with PBS-T and 100 μl of peroxidase-conjugated goat anti-mouse IgG (heavy chain- and light chain-specific, diluted 32000-fold in 1% BSA in PBS; Jackson Immuno Research Laboratory Inc., West Grove, WA) was added to each well. The plates were incubated for 1 h at room temperature and then washed with PBS-T. Colorimetric signals were generated using 1-Step Ultra TMB-ELISA solution (Thermo Scientific Pierce). The reaction was stopped with 1 N H₂SO₄ after 15 min, and the absorbance at 450 nm was measured.
using a Wallac ARVO multilabel counter (Perkin Elmer). The antibody titer was determined as the highest dilution below the cut-off value indicated by the mean OD value of the background ± 2 SD.

**Induction and measurement of DTH responses.** Six-week-old C57BL/6 mice were immunized by subcutaneous injection of 10 µg OVA with PBS, unmodified liposome, or the polymer-modified liposome. One week after immunization, mice were challenged by subcutaneous injection of 30 µg OVA with the same compound into the left footpad and PBS only into the right footpad. Footpad swelling was measured 24 h later. DTH response was calculated as the difference in thickness between the right and left footpads.

**Statistical analysis.** All data are expressed as the mean ± SEM. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with post-hoc analysis followed by Bonferroni’s test.

**RESULTS**

Association of MGlu-HPG–modified liposome containing OVA with DCs.

Our previous study showed that MGlu-HPG–modified liposomes effectively delivered antigen into DC2.4 cells, a murine DC-like line (29). To examine the ability of
MGlu-HPG–modified liposomes to deliver antigenic proteins into primary DCs, 

BMDCs derived from freshly isolated bone marrow cells from C57BL/6 mice were 

incubated with FITC-labeled free OVA (FITC-OVA) or with liposomes containing 

FITC-labeled OVA without the polymer modification (lipo-FITC-OVA) or with the 

graphite modification (MGlu-HPG-lipo-FITC-OVA) for 1 h at 4°C or 37°C. After 

incubation, the fluorescence intensities in the BMDCs were analyzed by flow cytometry 

(Fig. 1). FITC-OVA, lipo-FITC-OVA, or MGlu-HPG-lipo-FITC-OVA were not taken 

up by BMDCs (6.48 ± 1.30%, 8.61 ± 1.54%, and 9.25 ± 2.13% positive cells, 

respectively) at 4°C. At 37°C, an increased number of BMDCs displayed fluorescence 

following treatment with MGlu-HPG-lipo-FITC-OVA or lipo-FITC-OVA compared to 

cells treated with free FITC-OVA, and the fluorescence was of greater intensity. The 

population of FITC-positive BMDCs incubated with MGlu-HPG-lipo-FITC-OVA 

(18.73 ± 3.43% positive cells) was approximately 2.5-fold higher than for BMDCs 

incubated with OVA (7.30 ± 1.82% positive cells) at 37°C (P < 0.05). The uptake by 

BMDCs treated with MGlu-HPG-lipo-FITC-OVA tended to be greater than that in 

BMDCs treated with lipo-OVA (12.52 ± 2.63% positive cells) at 37°C. These results 

demonstrated a high uptake of MGlu-HPG–modified liposomes by primary DCs, further 

supporting the physiological relevance of our previous findings with the murine DC line.
Response of splenocytes from OVA-immunized mice to in vitro stimulation with MGlù-HPG–modified liposomes containing OVA. Our data showed that MGlù-HPG–modified liposomes are efficiently taken up by antigen-presenting cells (APCs). It was important to determine whether the antigen would be presented to T cells, leading to cytokine production and cell proliferation. Splenocytes collected from C57BL/6 mice intraperitoneally immunized with OVA were cultured for 72 h or 96 h with either 5 μg/ml of free OVA, OVA-containing liposomes without the polymer modification (lipo-OVA), or OVA-containing liposomes with the polymer modification (MGlù-HPG-lipo-OVA). IFN-γ production was detected by ELISA (Fig. 2A) and cell proliferation was analyzed by BrdU uptake analysis (Fig. 2B). Splenocytes from OVA-immunized mice produced IFN-γ in the presence of OVA, lipo-OVA and MGlù-HPG-lipo-OVA, but not in the PBS control group. The production of IFN-γ was higher in splenocytes stimulated with MGlù-HPG-lipo-OVA than in splenocytes stimulated with OVA or the unmodified OVA-containing liposomes: we detected a statically significant difference (P < 0.05) between those in splenocytes stimulated with the MGlù-HPG-lipo-OVA and OVA. Proliferation of the splenocytes in response to stimulation with OVA, lipo-OVA, or MGlù-HPG-lipo-OVA showed the same pattern as
for the production of IFN-γ. In contrast, splenocytes from PBS-immunized mice did not produce IFN-γ or proliferate in response to the stimulation with OVA, lipo-OVA, or MGlù-HPG-lipo-OVA. These results suggested that MGlù-HPG–modified liposomes efficiently induce OVA-specific immune responses via uptake by primary splenocytes.

Responses of splenocytes from mice immunized with OVA to stimulation with OVA-containing MGlù-HPG–modified liposomes. To investigate the induction of cellular immune responses in mice by MGlù-HPG-lipo-OVA, C57BL/6 mice were intraperitoneally immunized with OVA, lipo-OVA, or MGlù-HPG-lipo-OVA. Splenocytes were collected from the immunized mice and cultured for 72 or 96 h in the absence or presence of 1, 5, or 25 μg/ml of OVA. Splenocytes from mice that were given OVA, lipo-OVA, or MGlù-HPG-lip-OVA secreted IFN-γ after in vitro stimulation with OVA. No IFN-γ was secreted by splenocytes from the negative control mice that had been treated with PBS. IFN-γ secretion in response to stimulation with OVA increased in a dose-dependent manner. At all concentrations of OVA, splenocytes from MGlù-HPG-lipo-OVA–immunized mice secreted significantly more IFN-γ than splenocytes from mice that had been immunized with free OVA or lipo-OVA (Fig. 3A). This result indicated that MGlù-HPG-lipo-OVA is able to efficiently induce an immune response in mice.
The proliferative response of primary splenocytes was examined by BrdU uptake analysis. Splenocyte proliferation in response to *in vitro* stimulation with OVA was observed in splenocytes from mice that had been immunized with OVA, lipo-OVA, or MGlù-HPG-lipo-OVA, but not in splenocytes from mice given PBS (Fig. 3B).

**MHC class I and II restriction in splenocytes stimulated with OVA-containing MGlù-HPG-modified liposomes.** To confirm that the OVA-induced proliferation of splenocytes from mice immunized with MGlù-HPG-lipo-OVA was induced by APCs (DCs) through MHC class I and II antigen presentation pathways, each pathway was separately blocked using specific MAbs (H-2D\(^b\) for MHC class I and anti-mouse I-A/I-E for MHC class II). Splenocytes from C57BL/6 mice intraperitoneally immunized with 5 \(\mu\)g/ml of MGlù-HPG-lipo-OVA were incubated *in vitro* with 5 \(\mu\)g/ml of OVA in the absence or presence of 1 or 10 ng/ml of either anti-mouse MHC class I MAb or anti-mouse MHC class II MAb. After 96 h, cell proliferation was determined by BrdU uptake analysis. As shown in Fig. 4, splenocytes from mice immunized with MGlù-HPG-lipo-OVA proliferated in response to OVA (16.41 ± 0.76% positive cells), and this proliferation was not affected by the isotype control antibodies for anti-MHC class I and II MAbs (17.47 ± 0.16% and 15.72 ± 0.73% positive cells, respectively). In contrast, OVA-specific proliferation was significantly suppressed by 10 ng/ml
anti-MHC class I MAb (9.71 ± 1.99% positive cells), although it was not affected by 1 ng/ml anti-MHC class I MAb (17.37 ± 2.08% positive cells). Similarly, the anti-MHC class II MAb blocked the proliferation of the splenocytes by up to half in a dose-dependent manner. These results suggest that the proliferative responses stimulated by OVA may be mediated by antigen presentation via MHC class I and II molecules and that MGluc-HPG-lipo-OVA can potentially enhance both cell-mediated and humoral immunity.

**OVA-specific antibody stimulated by MGluc-HPG–modified liposomes containing OVA.** To investigate the induction of humoral immune responses, serum samples were collected once a week from C57BL/6 mice intraperitoneally immunized with OVA, lipo-OVA, or MGluc-HPG-lipo-OVA, and OVA-specific antibody titers were measured using an endpoint ELISA. On day 28, the OVA-specific antibody titer in mice immunized with MGluc-HPG-lipo-OVA was more than 500-fold higher than in mice immunized with free OVA and was about 5-fold higher than in mice immunized with lipo-OVA (Fig. 5A). The trend of high antibody production in mice immunized with MGluc-HPG-lipo-OVA was observed as early as one week after the first immunization and continued throughout the experimental period (Fig. 5B).

**Induction of DTH by MGluc-HPG–modified liposomes containing OVA.** To
investigate whether the MGlu-HPG–modified liposome induces an OVA-specific cellular immune response in mice, mouse footpad DTH assay was used, with the footpad thickness measured by micrometer. The DTH response in C57BL/6 mice sensitized and challenged with the MGlu-HPG–modified liposome differed significantly from the PBS-, OVA-, or unmodified liposome-sensitized group (Fig. 6). This result clearly demonstrated that MGlu-HPG–modified liposome enhanced OVA-specific cell-mediated immune responses in mice.

DISCUSSION

This study investigated MGlu-HPG–modified liposome as a potential carrier of antigenic proteins. MGlu-HPG–modified liposomes containing OVA were taken up by primary BMDCs more efficiently than were free OVA or unmodified liposomes containing OVA. These results were in agreement with our previous finding that the polymer-modified liposomes were taken up efficiently by the murine DC2.4 cell line (30, 31). The uptake of MGlu-HPG–modified liposomes by DCs resulted in the efficient induction of antigen-specific immune responses. Splenocytes from OVA-immunized mice responded more strongly to in vitro stimulation with MGlu-HPG-lipo-OVA than with OVA alone or OVA in unmodified liposomes (Fig. 2).
Antigen presentation by DCs following the uptake of antigen-containing MGlu-HPG–modified liposomes may stimulate antigen-specific T cells, resulting in cytokine production and clonal expansion. Cytokine production by activated T cells is frequently able to stimulate other immune cells; for example, IFN-γ enhances the cytotoxicity of CTL (16) and NK cells (1). Thus, the increased production of IFN-γ by splenocytes using MGlu-HPG–modified liposomes could strongly enhance antigen-specific immunity. Splenocytes collected from MGlu-HPG-lipo-OVA–immunized mice showed more potent OVA-specific cellular responses than splenocytes obtained from mice immunized with free OVA or unmodified liposomes containing OVA (Fig. 3), which confirmed the effective induction of antigen-specific immune responses by encapsulating antigen in MGlu-HPG–conjugated liposome in vivo as well as in vitro (Fig. 2.). The proliferative response involved antigen presentation via both the MHC class I and II antigen presentation pathways (Fig. 4). Finally, antigen-specific antibody production and DTH responses were increased in MGlu-HPG-lipo-OVA–immunized mice, indicating that there was an enhancement of antigen-specific humoral and cellular immunity. The enhancement of antigen-specific antibody production by immunization with MGlu-HPG-lipo-OVA was observed.
Carboxylated poly(glycidol) derivatives were known as modifiers of liposomes to improve their fusogenicity of membranes (12, 21, 27). This fusogenic activity may be one reason for the high uptake of antigen encapsulated in polymer-modified liposomes by APC. Previous studies showed that polymer-modified liposomes are incorporated not only by DC2.4 cells but also by HeLa cells (21). MGlu-HPG–modified liposomes become fusogenic to lipid membranes only in weakly acidic environments, probably due to the protonation of carboxyl groups and concomitant conformational changes of the polymer chains (29). Antigens encapsulated within MGlu-HPG-modified liposomes may escape rapidly from the acidic endosome into the cytosol within MHC class I-expressing APCs. Since antigens in the cytosol are presented to CD8-positive CTLs via MHC class I, it is conceivable that the activation of antigen-specific CTLs, which is required for an effective immune response, is enhanced by the rapid escape of antigen from the endosome to the cytosol by encapsulating with MGlu-HPG-conjugated liposome (29). Induction of OVA-specific CTL and Th1 responses indicate the strong induction of cell-mediated immunity. Our preliminary data showed that MGlu-HPG-conjugated liposomes tend to induce CD4 and CD8 T-cell proliferation, while unmodified liposomes tend to induce B cells at the popliteal lymph node of mouse during a DTH response (data not shown). Although we did not confirm the
localization of MGlu-HPG-conjugated liposomes in BMDCs, the above mechanisms explain very well why MGlu-HPG–modified liposome initiates antigen-specific humoral and cellular immunity. These results indicated that MGlu-lyposome–conjugated liposome is an effective antigen carrier for the development of novel vaccines.

Since prophylactic vaccination is the most cost effective strategy for preventing infectious disease, vaccines to bacterial toxins and many common viral infections have been developed and made widely available. This study showed for the first time that antigen-specific cellular and humoral immunity can be enhanced in vivo by the encapsulation of antigen within MGlu-HPG–modified liposomes. Thus, this study demonstrates that MGlu-HPG–modified liposomes are a promising antigen carrier for the development of novel vaccines. Further studies, including the optimization of MGlu-HPG–modified liposomes and immunization with various peptide- or antigen-carrying MGlu-HPG–modified liposomes in other animals, are in progress for the clinical development of this novel vaccine candidate.

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Figure legends

Fig. 1. Uptake by dendritic cells. Bone marrow-derived dendritic cells (BMDCs) were incubated with 1 μg/ml of FITC-labeled free OVA or OVA-containing liposomes with or without the polymer modification for 1 h at 4°C or 37°C. After incubation, the uptake of FITC-labeled OVA was detected by flow cytometry. The percentage of FITC-positive cells is shown. The results are expressed as the mean ± SEM of five independent experiments. Significant differences between the groups are indicated by *, P < 0.05.

Fig. 2. Stimulation of OVA-immunized splenocytes in vitro. Splenocytes (2 × 10^6/ml in 2 ml) isolated from OVA-immunized C57BL/6 mice were incubated with 5 μg/ml of free OVA or OVA-containing liposomes with or without the polymer modification for 72 h (A) or 96 h (B). Interferon (IFN)-γ secretion was detected by ELISA (A) and cell proliferation was detected by analysis of 5-bromo-2-deoxyuridine (BrdU) uptake (B). The results are expressed as the mean ± SEM of at least three independent experiments. Significant differences between the groups are indicated by *, P < 0.05, and **, P < 0.01.
Fig. 3. **OVA stimulation of immunized splenocytes in vitro.** Splenocytes (2 × 10^6/ml in 2 ml) isolated from C57BL/6 mice immunized with free OVA or with liposomes with or without the polymer modification were incubated with 1–25 μg/mL of OVA for 72 h (A) or 96 h (B). Interferon (IFN)-γ secretion was detected by ELISA (A) and cell proliferation was detected by analysis of BrdU uptake (B). The results are expressed as the mean ± SEM of at least three independent experiments. Significant differences between the groups are indicated by *, P < 0.05, and **, P < 0.01.

Fig. 4. **MHC restriction of OVA-specific responses.** Splenocytes (2 × 10^6/ml in 2 ml) isolated from C57BL/6 mice immunized with OVA-encapsulating MGlu-HPG liposomes were incubated with 5 μg/ml of OVA and either 1 or 10 μg/ml of anti-mouse H-2D^b (MHC class I), anti-mouse I-A/I-E (MHC class II), or isotype control antibodies for 96 h. Cell proliferation was detected by BrdU uptake. The results are expressed as the mean ± SEM of at least three independent experiments. Significant differences between the groups are indicated by *, P < 0.05.

Fig. 5. **OVA-specific antibody production.** OVA-specific whole IgG antibody titers in the serum of C57BL/6 mice immunized with free OVA or with OVA-containing
liposomes with or without the polymer modification were measured using an endpoint ELISA on day 28 (A) and throughout the entire experimental period (B). The results are expressed as the mean ± SEM of at least four mice. Significant differences between the groups are indicated by *, $P < 0.05$, and **, $P < 0.01$.

Fig. 6. DTH response in mice. For sensitization, 10 μg of OVA was applied with PBS, unmodified liposome, or MGl-HPG–modified liposome to the dorsal skin of C57BL/6 mice by subcutaneous injection. Control mice received PBS only. Seven days after sensitization, 30 μg of OVA, applied using the same compounds as at sensitization, was injected into the left footpad of mice, and PBS was injected into the right footpad. Footpad thickness was measured 24 h after challenge. The data are the mean ± SEM of at least three mice per group. Significant differences between the groups are indicated by *, $P < 0.05$, and **, $P < 0.01$. 
Fig. 1.
Fig. 2.

A

Stimulation
(in vitro)

- PBS
- OVA
- Lipo-OVA
- MGLu-HPG-lipo-OVA

B

Stimulation
(in vitro)

- PBS
- OVA
- Lipo-OVA
- MGLu-HPG-lipo-OVA

IFN-γ (ng/ml)

FITC-positive cells (%)

Immunization

PBS
OVA
Fig. 3.

A

Immunization
- PBS
- OVA
- Lipo-OVA
- MGlu-HPG-lipo-OVA

Stimulation (in vitro)
- PBS
- OVA (μg/ml)

B

Immunization
- PBS
- OVA
- Lipo-OVA
- MGlu-HPG-lipo-OVA

Stimulation (in vitro)
- PBS
- OVA (μg/ml)
Fig. 5.

A

B

Specific-antibody titer (whole IgG, log10)

PBS OVA Lipo-OVA MGlu-HPG-lipo-OVA

Specific-antibody titer (whole IgG, log10)

PBS OVA Lipo-OVA MGlu-HPG-lipo-OVA

0 7 14 21 28

(Day)
Fig. 6.

PBS
OVA

Lipo-OVA
MGlu-HPG-
lipo-OVA

-200 0 200 400 600 800 1000 1200

Swelling (µm)

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