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J Immunol 2006; 177:2324-2330; doi: 10.4049/jimmunol.177.4.2324
http://www.jimmunol.org/content/177/4/2324

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Antigen Chemically Coupled to the Surface of Liposomes Are Cross-Presented to CD8⁺ T Cells and Induce Potent Antitumor Immunity

Maiko Taneichi,* Hideaki Ishida,† Kiichi Kajino,‡ Kazumasu Ogasawara,† Yuriko Tanaka,* Michiyuki Kasai,* Masahito Mori,‡ Mitsuhiro Nishida,‡ Hiroyuki Yamamura,‡ Junichiro Mizuguchi,§ and Tetsuya Uchida2*

We have previously demonstrated that liposomes with differential lipid components display differential adjuvant effects when Ags are chemically coupled to their surfaces. In the present study, Ag presentation of liposome-coupled OVA was investigated in vitro, and it was found that OVA coupled to liposomes made using unsaturated fatty acid was presented to both CD4⁺ and CD8⁺ T cells, whereas OVA coupled to liposomes made using saturated fatty acid was presented only to CD4⁺ T cells. Confocal laser scanning microscopic analysis demonstrated that a portion of the OVA coupled to liposomes made using unsaturated fatty acid, but not saturated fatty acid, received processing beyond the MHC class II compartment, suggesting that the degradation of OVA might occur in the cytosol, and that the peptides generated in this manner would be presented to CD8⁺ T cells via MHC class I. The ability to induce cross-presentation of an Ag coupled to liposomes consisting of unsaturated fatty acid was further confirmed by in vivo induction of CTL and by the induction of tumor eradication in mice; E.G7 tumors in mice that received combined inoculation with OVA257–264-liposome conjugates, CpG, and anti-IL-10 mAbs were completely eradicated. In those mice, the frequency of CD8⁺ T cells reactive with OVA257–264 peptides in the context of H-2Kb was significantly increased. These results suggested that, by choosing lipid components for liposomes, surface-coupled liposomal Ags might be applicable for the development of tumor vaccines to present tumor Ags to APCs and induce antitumor responses. The Journal of Immunology, 2006, 177: 2324–2330.

Although it has long been a matter of debate whether the human immune system is capable of recognizing and managing spontaneously arising tumors, a number of compelling findings (1–4) have recently indicated that the immune system is clearly capable of recognizing and eliminating tumor cells. Therefore, adoptive immunotherapy could potentially provide a controlled and highly specific stratagem for the treatment of cancer. However, at present, there are some problems to overcome before immunotherapy can be applied to cancer therapies. Successful elimination of experimental tumors in animal models by use of adoptive immunotherapy requires repeated administration of IL-2 to maintain cell survival because, for solid cancers, the capacity of tumor rejection may soon be exhausted unless CTL are rapidly and efficiently recruited to the tumor bed. Also, in humans, adoptive immunotherapy has been (5) plagued by extreme toxicities associated with the simultaneous administration of high doses of IL-2. Second, most of the characterized human tumor Ags have been classified as tumor-associated, because of their demonstrable expression at low levels in some normal cells (6). Thus, the challenge for immunotherapy is to develop strategies that effectively and safely augment antitumor responses (7).

Immunotherapy for the treatment of cancer involves adoptive T cell transfer, tumor-associated-Ag-pulsed dendritic cells (DC; DC-based vaccines), and peptide-based vaccines. In the case of the peptide-based cancer vaccines, the vaccine Ag (e.g., tumor Ag) should be presented via the MHC class I pathway for the induction of Ag-specific CTL. In general, extracellular Ags are presented via MHC class II molecules to CD4⁺ T cells, whereas intracellular Ags are presented via MHC class I molecules to CD8⁺ T cells. To induce Ag-specific CTL, the tumor Ag must be loaded onto the class I MHC processing pathway in the APCs via so-called cross-presentation (8). In cross-presentation, exogenous proteins cross over to the endogenous pathway to gain access to MHC class I. Using this phenomenon, a generation of Ag-specific, primary CD8⁺ CTL responses might be applicable for the development of vaccines for prevention of viral diseases and for the induction of potent protective antitumor immunity.

As for the vaccine adjuvant, the currently approved alum adjuvants are known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity (9–11). Consequently, the development of a novel vaccine adjuvant is essential for the induction of cell-mediated immunity. Among the candidates for adjuvants, liposomes have garnered recent attention (12–18) for their capacity as carriers of vaccines. We previously (19–22) reported that Ags chemically coupled to the surface of liposomes induced Ag-specific IgG but not IgE Ab production.

*Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan; †Department of Pathology, Shiga University of Medical Science, Shiga, Japan; ‡Drug Delivery System Development Division, Nippon Oil and Fat Corporation, Tokyo, Japan; and §Department of Immunology, Tokyo Medical University, Tokyo, Japan

Received for publication December 29, 2005. Accepted for publication May 24, 2006.

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1 This work was supported in part by a grant from the Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation).

2 Address correspondence and reprint requests to Dr. Tetsuya Uchida, Department of Safety Research on Blood and Biological Products, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-city, Tokyo, Japan. E-mail address: tuchida@nih.go.jp

3 Abbreviations used in this paper: DC, dendritic cell; DPPE, dipalmitoyl phosphatidyl ethanolamine; DOPE, dioleoyl phosphatidyl ethanolamine; DSS, disuccinimidyl suberate; SAC, splenic adherent cells; TAA, antitumor-associated Ag.
The inducibility of Ag-specific IgG Ab production by Ag-liposome conjugates varied among the liposome preparations used for the production of Ag-liposome conjugates; the greater the membrane mobility in liposomes, the more Ab production induced by Ag-liposome conjugates (23). In fact, alteration of lipid composition has been reported to modulate immune responses (24–27).

In the present study, OVA was coupled to liposomes consisting either of saturated or unsaturated fatty acid, and the inducibility of cross-presentation by these OVA-liposome conjugates was investigated by monitoring in vitro cytokine production by OVA-specific CD4+ or CD8+ T cells cocultured with Ag-liposome conjugates in the presence of APCs. OVA coupled to liposomes consisting of unsaturated fatty acid was presented to both CD4+ and CD8+ T cells. We then made conjugates of OVA257–264 with liposomes consisting of unsaturated fatty acid, and investigated in vivo CTL induction and the effect of administrating these conjugates to E.G7-bearing mice.

Materials and Methods

Mice

BALB/c mice (8 wk of age, female) were purchased from Charles River Laboratories. C57BL/6 mice (6–8 wk of age, female) were purchased from SLC (Shizuoka, Japan). All mice were maintained under specific pathogen-free conditions.

Chemicals

All phospholipids were obtained from Nippon Oil and Fat. Reagent grades of cholesterol were purchased from Wako Pure Chemical.

Reagents

Synthetic CpG ODN (5002: TCCATGACGTTCTTGATGTT) was purchased from Hokkaido System Science and was phosphorothiate protected to avoid nucleoside-dependent degradation. Mouse MHC class-I (Kb)-binding peptide OVA323–339 (SIINFEKL) was also obtained from Hokkaido System Science. FITC-conjugated anti-mouse CD8 mAb or PE-conjugated H-2Kb/OVA257–264 tetramer was purchased from BD Biosciences or MBL, respectively.

Antigens

OVA (grade VII) was purchased from Sigma-Aldrich. For the analysis of the processing of liposome-coupled OVA by macrophages, DQ-OVA, which exhibits green fluorescence upon proteolytic degradation, was purchased from Molecular Probes.

Fluorescence labeling of OVA

OVA was labeled with fluorescence using an AlexaFluor 488 protein labeling kit (Molecular Probes) according to the manufacturer’s protocol.

Liposomes

Liposomes with two different lipid components were used in the present study. Saturated liposomes consisted of dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoyl phosphatidyl glycerol, and cholesterol in a 4:3:2:7 molar ratio, and unsaturated liposomes consisted of dioleoyl phosphatidylcholine, dioleoyl phosphatidyl ethanolamine (DOPE), dioleoyl phosphatidyl glycerol, and cholesterol in a 4:3:2:7 molar ratio. The crude liposome solution was passed through a membrane filter (Nucleopore polycarbonate filter; Costar) with a pore size of 0.2 μm.

Coupling of OVA to liposomes

Liposomal conjugates with plain OVA, Alexa-labeled OVA, or DQ-OVA were prepared essentially in the same way as described previously (20) via disuccinimidyl suberate (DSS). Briefly, a mixture of 10 ml of anhydrous chloroform solution containing 0.136 mM DPPe (saturated liposomes) or DPPE (unsaturated liposomes) and 24 μl of triethylamine was added to drops of 26.6 ml of anhydrous chloroform solution containing 0.681 mM DSS and stirred for 5 h at 40°C. The solvent was evaporated under reduced pressure, and 18 ml of a 2:1 mixture of ethyl acetate and tetrahydrofuran was added to dissolve the residue. Thirty six milliliters of 100 mM sodium phosphate (pH 5.5) and 90 ml of saturated NaCl aqueous solution were added to the solution, shaken for 1 min, and allowed to separate. To remove undesirable materials, the upper layer was washed with the same buffer and, after evaporation of the solvent, 3 ml of acetone was added to dissolve the residue. Ice-cold acetone (100 ml) was added in drops and kept on ice for 30 min to precipitate. Crystals were collected and dissolved in 5 ml of chloroform. After evaporation, 34.4 mg of DPPE-DSS was obtained. 0.18 mM dipalmitoyl phosphatidylcholine, 0.03 mM DPPE-DSS, 0.21 mM cholesterol, and 0.06 mM dimyristoyl phosphatidyl glycerol were dissolved in 10 ml of chloroform/methanol (saturated liposomes). For unsaturated liposomes, DOPE-DSS, dioleoyl phosphatidylcholine, dioleoyl phosphatidyl glycerol, and cholesterol were used and the preparation was done in the same manner as above. The solvent was then removed under reduced pressure and 5.8 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. The vesicle dispersion was extruded through a 0.2-μm polycarbonate filter to adjust the dosage size. A 2-ml suspension of DSS-introduced liposome and 0.5 ml of 5 mg/ml OVA257–264 solution were mixed and stirred for 3 days at 4°C. The liposome-coupled and -uncoupled peptides were separated as described above using CL-4B column chromatography.

Quantification of OVA coupled to liposome

For the measurement of OVA coupled to liposome, radiolabeled OVA (methyl-14C; purchased from New England Nuclear) was mixed with cold OVA and used for coupling with liposome and for determining the calibration curve. The radioactivity of the resulting OVA-liposome solution was counted using a calibration curve. The amounts of OVA coupled to saturated and unsaturated liposomes were 48.1 and 47.8 μg/ml, respectively.

Cell culture

All incubations were performed in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies).

Preparation of splenic adherent cells (SAC) and CD4+ and CD8+ T cells

Spleen cell suspensions were prepared in RPMI 1640 containing 10% FCS. Cells (5 × 10^6) in 5 ml of medium containing 10% FCS were plated into 50-mm plastic tissue culture dishes (no. 3002; BD Biosciences) and were incubated at 37°C in a humidified 5% CO2 atmosphere for 2 h. After culture, nonadherent cells were removed by vigorous washing in warm medium, and adherent cells were then harvested with a cell scraper. CD4+ and CD8+ T cell purification from spleen cells of mice immunized with OVA-alum was performed with the magnetic cell sorter system MACS according to the manufacturer’s protocol using anti-CD4 and anti-CD8 Ab-coated microbeads (Miltenyi Biotec). T cells were suspended in RPMI 1640 containing 10% FCS at a cell density of 2 × 10^6/ml.

Culture of CD4+ and CD8+ T cells with SAC pulsed with OVA

OVA-liposome conjugates made using saturated or unsaturated liposomes were added to the culture of SAC and incubated for 2 h. The final concentration of OVA-liposome added to the macrophage culture was 500 μg/ml, which included 24 μg of OVA/ml. For controls, OVA was added to the culture at final concentrations of 24 μg/ml. SAC were then washed three times in ice-cold medium and 2 × 10^6 cells were cocultured with 5 × 10^4 CD4+ or CD8+ T cells in a 48-well plate (no. 3047; BD Biosciences). A preliminary experiment showed that the optimal culture period in the above culture condition was 2 days for IFN-γ production by CD4+ T cells and 5 days for IL-5 production by CD4+ and CD8+ T cells.
and IFN-γ production by CD8+ T cells. After incubation in a CO2 incubator for 2 or 5 days, the culture supernatants were collected and assayed for cytokines.

Cytokine assays
IL-5 and IFN-γ in the culture supernatant were measured using the Biotrak mouse ELISA system (Amersham International). All tests were assayed in duplicate, and the SE in each test was always <5% of the mean value.

Cloned macrophage hybridoma

Macrophage hybridoma clone 39, obtained from the fusion of SAC from CKB mice and P388D1 (28), was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 75-cm² flask (no. 3111; BD Biosciences).

Construction and expression of a fusion protein, DM-DSRed, in macrophage clone 39

The DNA fragment coding the full-length H2-DMb2 (29) was amplified by PCR with two primers (5′-ATGGCTGTCACCTGTGGTCTGCTGTCGTG GTG-3′ and 5′-GATGCCCCTCTTGGGTAGTTGATCC-3′). The PCR product was cloned into the CMV promoter-driven expression plasmid pDsRedN1 (BD Clontech). This construct omitted the stop codon of H2-DMb2 and encoded the H2-DMb2 fused with DsRed. The cloned plasmid DNA was transfected to macrophage hybridoma clone 39 with Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. During the transfection to clone 39, the medium containing cDNA and the transfection reagent was replaced with fresh medium after an 8-h transfection, and then clone 39 was cultured for 40 h. To obtain stable cell lines, clone 39 was passaged at 1:5 into RPMI 1640 containing 10% FCS with 50 μg/ml G418 (G-418; Sigma-Aldrich). Cells showing the best fluorescence were selected by using a FACSVantage cell sorter. After cell sorting, clone 39 that expressed DM-DSRed was cultured in RPMI 1640 containing 10% FCS with 200 μg/ml G418.

Confocal laser scanning microscopy

To investigate the internalization of OVA-liposome conjugates by macrophages, the DM-DSRed-expressing cloned macrophages 39 were cultured for 18 h at 37°C on 8-hole heavy Teflon-coated slides (Bokusui Brown) and then incubated with Alexa-ova-liposome conjugates or with DQ-ova-liposome conjugates, prepared using saturated or unsaturated liposomes, for 2 h at 37°C. The slides were then washed with MEM and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, they were incubated for 10 min in 0.1 M glycine-HCl (pH 7.0) to block the remaining aldehyde residue. They were then washed twice in PBS. After washing, the slides were scaled with PBS, and the average diameter of the tumor mass in each group was calculated.

Table 1. Cytokine production by splenic CD4/CD8 T cells of mice immunized with OVA after coculture with OVA-pulsed SAC

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>In Vitro Ag</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-5</td>
<td>IFN-γ</td>
<td>IL-5</td>
</tr>
<tr>
<td>None</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>OVA solution</td>
<td>96.2 ± 12.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>OVA-liposome</td>
<td>910.2 ± 23.0</td>
<td>88.7 ± 45.0</td>
<td>115.1 ± 28.6</td>
</tr>
<tr>
<td>OVA-liposome</td>
<td>1065.5 ± 31.9</td>
<td>149.9 ± 83.8</td>
<td></td>
</tr>
</tbody>
</table>

* Splenic CD4/CD8 T cells were taken from mice immunized with OVA and were cultured with OVA-pulsed SAC as described in Materials and Methods. Data represent the mean cytokine concentration (picograms per milliliter) in the culture supernatants and SE of triplicate culture. ND, Not detected.
that expressed DM-DsRed. After incubation for 2 h, the recovered macrophages were analyzed using confocal laser scanning microscopy. Macrophages expressed DM-DsRed (Mφ alone in Figs. 1 and 2). The yellow spots in the panel labeled saturated in Fig. 1 illustrate that Alexa-OVA coupled to liposomes were colocalized with DM. In contrast, in the panel labeled unsaturated in Fig. 1, both green and yellow spots were observed, suggesting that a portion of the Alexa-OVA coupled to unsaturated liposomes was not colocalized with DM. In the next experiment, DQ-OVA, which exhibits green fluorescence upon proteolytic degradation, was coupled to liposomes instead of Alexa-OVA, and similar investigations were performed. Interestingly, the results shown in Fig. 2 demonstrate that a portion of the DQ-OVA coupled to unsaturated liposome received processing beyond the class II compartment.

In vivo CTL induction by OVA-liposome conjugates

Cross-presentation of OVA coupled to unsaturated liposomes was further confirmed using experiments of in vivo CTL induction. As shown in Fig. 3, both OVA\textsubscript{257–264} (D) and whole OVA (E) coupled to unsaturated liposomes successfully induced CTL against target cells pulsed with OVA\textsubscript{257–264} but not with control NP\textsubscript{366–374}. In contrast, a mixture of OVA\textsubscript{257–264} and unsaturated liposomes (Fig. 3B), and OVA\textsubscript{257–264} coupled to saturated liposome (Fig. 3C) failed to induce CTL against target cells pulsed with OVA\textsubscript{257–264}.

To examine the effectiveness of liposome-coupled peptides in vivo, we performed tumor-rejecting experiments. B6 mice were injected s.c. with E.G7 cells transfected with OVA DNA, and solid tumors with a diameter of $\geq 5$ mm were established 7–10 days after the injection. Liposome-coupled peptides, OVA\textsubscript{257–264} with CpG and anti-IL-10 Ab, were injected twice around the tumor mass as described in Materials and Methods. As shown in Fig. 4, a significant ($p < 0.001$) decrease of mean tumor diameter was observed as early as 7 days after inoculation of liposome-coupled OVA\textsubscript{257–264} with CpG and anti-IL-10 Ab, and the tumors were completely eradicated in 12 days. In contrast, injection of CpG and anti-IL-10 Ab with peptide solution containing the same amount of OVA\textsubscript{257–264} as liposome-coupled OVA\textsubscript{257–264} did not eradicate the established tumors. These results suggested that the liposome-coupled OVA\textsubscript{257–264} might effectively present OVA \textsubscript{257–264} to CTL, resulting in tumor rejection.

To determine whether liposome-coupled OVA\textsubscript{257–264} contributes to CTL activation, we analyzed splenic T cells in tumor-bearing mice with or without the treatment. CD8\textsuperscript{+} gated cells were analyzed with a tetramer-detecting OVA\textsubscript{257–264} plus H-2K\textsuperscript{b}-specific T cells. As shown in Fig. 5, spleen cells in normal mice were slightly stained with the tetramer at a background level. The tetramer-positive cells accounted for 5.2% of the total CD8\textsuperscript{+} cells of tumor-eradicated mice, whereas they made up 1.8 and 2.3% in normal mice and nontreated, tumor-bearing mice, respectively.
were affected by contamination of the liposome preparation with endotoxin, since the liposomes used in the present study were produced using endotoxin-free materials in the GMP-verified production facilities of Nippon O.I. & Fats, which produces injection-grade liposomes for clinical use. In fact, SAC incubated for 2 h with OVA-liposome and subsequently cultured for 24 h did not produce any detectable cytokines, and the addition of polymyxin B in the experiments shown in Table I did not affect the results (data not shown).

We next investigated the ability of Ag-liposome conjugates to induce antitumor immunity. The aim of cancer vaccination is to generate an immune-mediated antitumor-associated Ag (TAA) response resulting in the elimination of the tumor. The Ag of choice may be the whole protein alone or with immune stimulatory components, or defined epitopes (e.g., peptides) of the target Ag (7). Recent preclinical studies (37) have demonstrated that combined therapies involving the use of vaccines with cytokines, activators of DC such as TLR ligands or mAb to CD40, or recombinant vectors that provide a stimulus to the innate immune system resulted in enhanced antitumor responses. In the present study, antigenic peptides were chemically coupled to the surface of liposomes and inoculated into tumor-bearing mice in combination with CpG and anti-IL-10 mAbs. This treatment successfully induced eradication of the tumor mass, whereas inoculation of mice with CpG and anti-IL-10 mAbs with peptide solution containing the same amount of OVA257–264 did not affect the results of E.G7 tumor growth (Fig. 4). It has been reported that CpG and anti-IL-10 receptor Ab reverse tumor-induced DC paralysis, resulting in tumor rejection by CTL activated by the DC (38). However, under the conditions used in the present study, a combined inoculation of CpG and anti-IL-10 Abs in tumor-bearing mice did not inhibit the growth of the E.G7 tumor. In addition, the same Ag dose of plain peptide solution did not affect tumor growth even when inoculated in combination with CpG and anti-IL-10. Thus, liposome-coupled OVA peptide might be critical for the tumor eradication observed in the present study, suggesting that the administration of tumor Ag is indispensable for the induction of Ag-specific CTL, and peptide-liposome conjugates might effectively induce cross-presentation in APCs and induce a CTL response. In fact, tetramer staining (Fig. 4) demonstrated that Ag-specific CTL were significantly generated in mice that received
The experiment was repeated three times with similar results. We have investigated (25, 39) the potential ability of surface-linked liposomal Ags for the application to vaccine development, whereas most of the investigations regarding liposomes as a drug-delivery system have been done by encapsulating Ags into liposomes. During the course of this investigation, several advantages of the liposome-coupled Ags over the liposome-encapsulated Ags became apparent. First, a predominant coupling efficiency of Ags to liposomes: following our previously reported procedure (20) for coupling Ags to liposomes, ~50% of the Ags bound to the surface of liposomes, whereas in the Ag encapsulation, a 60-fold higher volume of Ags was required to obtain the same amount of conjugates (our unpublished observation). Second, Ag-specific and IgE-selective unresponsiveness induced by surface-linked liposomal Ags: Ags chemically coupled to the surface of liposomes induced Ag-specific IgG but not IgE Ab production in mice (19) and also in monkeys (40), suggesting the potential ability of surface-linked liposomal Ags for application to the development of vaccines with minimal allergic side effects. In addition, during the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-linked liposomal Ag, we found the existence of an alternative mechanism, not involving T cells, in the regulation of IgE synthesis (41). Third, an enhanced recognition of liposomal Ags by APCs: because liposomes basically consist of immunologically inert fatty acid, they are hardly recognized by APCs. Therefore, some contrivance, such as the introduction of mannose on the surface of liposomes (42), is required in Ag-encapsulated liposomes to enhance the recognition of liposomes by APCs. In contrast, in surface-linked liposomal Ags, Ags expressed on the surface of liposomes might be recognized more efficiently by APCs, which might result in an enhanced presentation to T cells. In fact, surface-linked liposomal Ags induced a significantly higher level of Ag-specific IgG production than that by liposome-encapsulated Ags in mice (our unpublished observation). In addition, a significant difference, which correlated closely with the adjuvant activity of liposomes, was observed in the recognition of liposomal Ags by APCs between liposomes with different lipid components; more Ags coupled to the unsaturated liposomes were engulfed by macrophages in vitro and a higher level of Ag-specific Ab production was induced in vivo than when saturated liposomes were used, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, i.e., recognition of Ag by APCs (43). In addition to this quantitative difference between liposomes with differential lipid components, in the present study, a qualitative difference (i.e., the differential ability to induce cross-presentation) was observed between saturated and unsaturated liposomes. Although the precise mechanism underlying this difference is currently unclear, the significant difference in membrane mobility observed between these liposomes (23) might affect their ability to induce cross-presentation.

Because a detailed characterization of many tumor cell surface molecules that act as TAAs is now available (44), immunotherapy has become an increasingly essential component of cancer therapies (7). Emphasis to date has been placed on the development of cancer vaccines to enhance the immunogenicity of weak TAAs. In this context, surface-linked liposomal Ag might potentially serve as a candidate protocol for tumor vaccine preparation to present tumor Ags to APCs and induce effective antitumor responses.

Disclosures
The authors have no financial conflict of interest.

References
LIPOSOME-COUPLED PEPTIDES INDUCE ANTITUMOR IMMUNITY


