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Research Paper

Immunogenicity of Liposomes Containing Lipid Core Peptides and the Adjuvant Quil A

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Purpose. The purpose of this study was to investigate the immunogenicity of liposomes containing mannosylated lipid core peptide (manLCP) constructs, both *in vitro* and *in vivo*, with or without the addition of the immune stimulating adjuvant Quil A.
Methods. Mouse bone marrow dendritic cells (BMDC) were cultured with liposome formulations for 48 h, and the resulting level of BMDC activation was determined by flow cytometry. BMDC pulsed with liposome formulations were incubated with 5,6-carboxyfluoroscein diacetate succinimidyl ester-labeled T cells for 72 h and the resulting T cell proliferation was determined by flow cytometry. To investigate the immunogenicity of formulations *in vivo*, groups of C57Bl/6J mice were immunized by subcutaneous injection, and the resulting antigen-specific cytotoxic and protective immune responses toward tumor challenge evaluated.
Results. Despite being unable to demonstrate the activation of BMDC, BMDC pulsed with liposomes containing manLCP constructs were able to stimulate the proliferation of naïve T cells *in vitro*. However, *in vivo* only liposomes containing both manLCP and Quil A were able to stimulate a strong antigen-specific cytotoxic immune response. Liposomes containing manLCP and Quil A within the same particle were able to protect against the growth of tumor cells to a similar level as if the antigen was administered in alum with CD4 help.
Conclusion. ManLCPs administered in liposomes are able to stimulate strong cytotoxic and protective immune responses if Quil A is also incorporated as an adjuvant.

KEY WORDS: CD8 T cell; lipid core peptide; liposome; Quil A; vaccine.

INTRODUCTION

Synthetic lipid core peptide (LCP) constructs containing minimal $CD8^+$ T cell epitopes of model proteins conjugated to a polylysine backbone have previously been investigated for their ability to stimulate strong MHC class II, $CD4^+$ T-cell-mediated immune responses (1–3). We have recently shown that these LCP constructs are also capable of stimulating MHC class I restricted, $CD8^+$ T-cell-mediated immune responses, but only if the constructs are administered in alum and with ovalbumin protein to provide CD4 help (4). The requirement for CD4 help in priming $CD8^+$ T-cell immune responses is controversial with both CD4-dependent and CD4-independent $CD8^+$ T cell activation being described (5–9). In general, it seems that weakly immunogenic antigens stimulate stronger CD8 cytotoxic immune responses if CD4 help is provided.

Previous experiments investigating the immunogenicity of peptide and lipopeptide antigens have found that delivering the antigen in a particulate delivery system is one means of increasing the resulting immune response (10,11). In general, particulate delivery systems, such as immune stimulating complexes (ISCOMs) and liposomes, more closely resemble the size of the pathogens phagocytic cells have evolved to recognize, thereby enhancing antigen phagocytosis and presentation by specialized antigen presenting cells. In addition, ISCOMs contain the immune stimulating adjuvant Quil A or its derivatives, which have been demonstrated to be potent stimulators of both antibody- and cell-mediated immune responses (12,13). Liposomes can also be modified to include additional immune stimulating molecules such as cytokines and targeting molecules that are capable of increasing the immune response toward entrapped protein and peptide antigens (14–18).

Although ISCOMs have been shown to be capable of stimulating effective immune responses and indeed have been licensed for use in several veterinary vaccines, their potential toxicity and lipid matrix structure, which means only antigens with hydrophobic domains can be incorporated,

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ABBREVIATIONS: BMDC, bone marrow dendritic cell; DC, dendritic cell; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; ISCOMs, immune stimulating complexes; LCP, lipid core peptide; manLCP, mannose-conjugated LCP; PBS, phosphate-buffered saline; PC, L-α-phosphatidylcholine; PRR, pattern recognition receptor; TEM, transmission electron microscopy.

has limited their use in more vaccine formulations. Liposomes, on the other hand, have a demonstrated safety profile in both vaccine and drug formulations (19). They also possess the ability to entrap a wide variety of potential antigens, thereby increasing their generic utility (20).

LCPs, due to their amphipathic nature, lend themselves toward being easily incorporated into the bilayer of liposomes. The aim of this study was therefore to determine if incorporating LCP constructs into liposomes could obviate the need for alum and CD4 help in stimulating strong CD8 cytotoxic immune responses both *in vitro* and *in vivo*. In an attempt to boost the immune response further, LCP constructs were made with a mannose residue attached to each peptide (see Fig. 1), as it has been postulated that interactions between mannose residues and C-type lectins such as the mannose receptor and DEC-205 may increase antigen uptake and subsequent immune activation (21–24). In addition, the effect on immunogenicity of incorporating a small amount of Quil A into the LCP liposomes was also investigated.

MATERIALS AND METHODS

Mice

C57Bl/6J and OT-I mice were bred and maintained in micro-isolators under specific-pathogen-free conditions at the Hercus Tairei Resource Unit, Dunedin, New Zealand. All experiments were approved by the University of Otago Animal Ethics Committee.

Reagents

L-α-Phosphatidylcholine (from egg yolk) and ovalbumin protein (Grade V, 98% purity) were purchased from Sigma (St. Louis, MO, USA). Antimouse 2.4G2 (Fcγ III/II receptor), CD11c, CD86, CD40, CD8, Vα2, and propidium iodide staining solution were purchased from BD Pharmingen (San Diego, CA, USA) and were used according to the manufacturer's instructions. 5,6-Carboxyfluoroscein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR, USA) and was used in accordance with the manufacturer's instructions. Alum (Alu-Gel-S) was purchased from Serva (Heidelberg, Germany). Lyophilized purified Quil A (Superfos) was purchased from Biosector (Frederikssund, Denmark).

LCP Constructs

LCPs containing four copies of the minimal CD8 T cell epitope SIINFEKL attached to a polylysine backbone were synthesized according to a method described by White *et al.* (4). In this study, the SIINFEKL epitopes were also conjugated to mannose residues resulting in the formation of manLCP to better target C-type lectins on the surface of dendritic cells.

Liposome Preparation and Characterization

Liposome formulations were prepared by hydration of thin lipid films. Briefly, the required quantity of L-α-phospha-

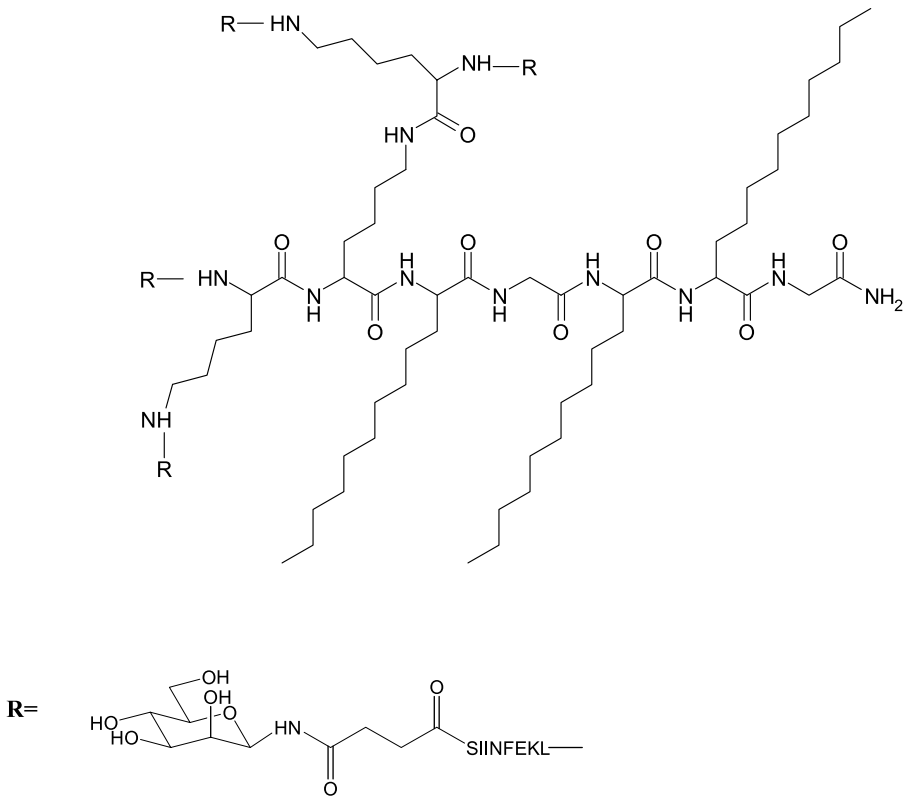

Fig. 1. Schematic representation of the lipid core peptide construct. "R" represents the SIINFEKL peptide linked to a mannose residue.

tidylcholine (PC) was dissolved in approximately 10 mL chloroform in a round-bottom flask, and then the solvent was evaporated to dryness at 45°C under reduced pressure on a rotary evaporator. The lipid film was flushed with nitrogen gas to ensure removal of all residual solvent. A 2-mL vol of 10 mM HEPES buffer (pH 7.4) was added to the lipid film and hydration aided by handshaking. For liposomes containing manLCP constructs, 95 mg PC was dried onto the wall of a round-bottom flask and hydrated with 2 mL HEPES buffer. Following hydration, the liposome dispersions were centrifuged (100,000 *g*, 30 min, 15°C) and the supernatant discarded. manLCP (5 mg) dissolved in DMSO at a concentration of 50 mg/mL was added to the liposome pellet and vortexed to mix. The pellet was then redispersed in HEPES buffer to a final volume of 2 mL. For liposomes containing Quil A, 100 mg PC was dried onto the wall of a round-bottom flask and hydrated with 2 mL HEPES buffer containing an appropriate amount of Quil A. For liposomes containing both manLCP and Quil A, Quil A-containing liposomes were initially prepared as described above. Following hydration, the liposome dispersion was centrifuged and the supernatant discarded as before. The manLCP constructs were added to the pellet prior to redispersion in HEPES buffer as described.

Following liposome preparation, all formulations were subjected to repeated extrusion through polycarbonate membranes of 800 nm pore size to reduce vesicle size and lamellarity. The size distribution of the liposome dispersions in 10 mM HEPES buffer (pH 7.4) was measured by photon correlation spectroscopy (Zetasizer 3000; Malvern Instruments, Malvern, UK). The liposomal nature of the particles formed was confirmed by transmission electron microscopy using methods previously described (25).

Incorporation of manLCP constructs into liposomes was determined by using an ATTO-TAG™ CBQCA Amine Derivatization Kit obtained from Molecular Probes (Eugene, OR, USA), and used in accordance with the manufacturer's instructions.

Generation and Activation of Bone Marrow Dendritic Cells

Bone marrow dendritic cells (BMDC) were generated by culturing bone marrow stem cells from C57Bl/6J mice in complete Iscove's Modified Dulbecco's Medium (cIMDM; IMDM supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamax, and 0.01% 2-mercaptoethanol; all from Invitrogen, Carlsbad, CA, USA) with 20 ng/mL recombinant granulocyte/macrophage colony stimulating factor (clone kindly supplied by Dr G. Buchan, University of Otago) for 6 days at 37°C, 5% CO_2. Cells were seeded at 5×10^5 cells/mL in cIMDM for pulsing with liposome formulations. Cell viability after incubation for 48 h was determined by staining with propidium iodide solution and determining the proportion of propidium iodide^{-ve} cells by flow cytometry.

Activation of BMDC was measured by flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA), and data were analyzed by using CellQuest Pro (Becton Dickinson). Cells were stained with the BMDC marker CD11c, and then the level of costimulatory markers CD86 and MHC class II on CD11c^{+ve} propidium iodide^{-ve} cells determined 48 h after the addition of liposome formulations. The fold increase in the mean fluorescence intensity (MFI) of activation marker expression was determined by dividing the MFI of each activation marker expressed on formulation pulsed BMDC by the MFI of each activation marker on CD11c^{+ve} propidium iodide^{-ve} cells in the negative control (no formulation).

T Cell Proliferation *in Vitro*

To examine the ability of the liposome formulations to stimulate CD8$^+$ T cell proliferation *in vitro*, BMDC pulsed with formulations for 48 h were washed to remove excess formulation and then incubated with OT-I splenocytes stained with CFSE for 72 h, at a DC/T ratio of 1:25. CFSE staining of Vα2$^+$ CD8$^+$ OT-I transgenic splenocytes was determined by flow cytometry and analyzed by using CellQuest Pro.

Cytotoxic T Cell Stimulation *in Vivo*

Groups of C57Bl/6J mice (n = 3) were immunized twice (days 0 and 14) by subcutaneous injection of manLCP formulations into the back of the neck. Groups of mice received either 10 μg manLCP in 200 μL alum or 10 μg manLCP incorporated into or admixed with liposomes. The liposome groups were immunized with liposomes containing either (a) manLCP alone, (b) liposomes containing both manLCP and Quil A, (c) manLCP and Quil A incorporated into separate liposomes and mixed prior to administration, or (d) manLCP admixed with Quil A liposomes prior to administration. All liposome groups received 10 μg manLCP in 1 mg total lipid administered in a total volume of 200 μL adjusted with PBS. Nine days after the second immunization, mice were injected intravenously with fluorescently labeled C57Bl/6J splenocytes pulsed with either 10 μg/mL SIINFEKL peptide or no peptide as a control. A group of naïve mice were also injected with pulsed and labeled splenocytes as a control. The following day, the mice were euthanased and the brachial and axillary lymph nodes removed. The number of fluorescently labeled target cells remaining in the lymphoid organs was analyzed by flow cytometry. Data from each animal were normalized to the number of nonpeptide pulsed targets recovered. The amount of antigen-specific killing of the peptide pulsed target cells was calculated by using the following formula:

$$\%\ cytotoxicity = 100 - (a/b \times 100)$$

where a is the number of peptide pulsed target cells recovered from experimental animals and b is the number of peptide pulsed target cells recovered from the naïve controls.

Tumor Challenge

Groups of C57Bl/6J mice (n = 5) were immunized twice (days 0 and 14) by subcutaneous injection either with 10 μg manLCP in 200 μL alum with the addition of 10 μg of ovalbumin protein to provide CD4$^+$ T-helper cell stimulation, or with 10 μg manLCP in liposome formulations. The liposome formulations included liposomes containing manLCP-alone, liposomes containing both manLCP and Quil A, and manLCP admixed with Quil A liposomes prior to administration. All

liposome groups received 10 μg manLCP in 1 mg total lipid administered in a final volume of 200 μL adjusted with PBS. Immunized mice and nonimmunized controls were challenged 30 days after the second immunization with 1×10^6 EG.7-OVA tumor cells (26) administered via subcutaneous injection into the flank. Mice were monitored for tumor growth every 2–3 days. Tumor measurements for each group of mice were stopped, and all animals were euthanased when any one animal in the group developed a tumor in excess of 200 mm^2.

Statistical Analysis

Statistical analysis was performed by using a one-way analysis of variance at a 95% confidence interval to test for significant differences.

RESULTS

Liposome Characterization

Four different liposome formulations were prepared for these studies containing either no additions (unmodified PC liposomes), 5% w/w manLCP, 2% w/w Quil A, or both 5% w/w manLCP and 2% w/w Quil A. A schematic representation of the manLCP constructs used in these studies is presented in Fig. 1. Each construct contained four copies of the minimal $CD8^+$ T cell epitope SIINFEKL conjugated to a polylysine backbone linked to three C_{10} lipid tails. In each case, the SIINFEKL peptide was also linked to a mannose residue in an attempt to enhance recognition of the particles by targeting C-type lectins on the surface of the BMDC. The mean size and polydispersity of the resulting liposome dispersions was determined by photon correlation spectroscopy. All liposome formulations were of a similar mean size distribution; however, even after repeated extrusions through 800 nm polycarbonate membranes the polydispersity of the dispersions remained high (≥0.70), indicating that the size distributions were heterogeneous in nature (Table I).

Using a protein derivatization assay, the incorporation of manLCP constructs into liposomes was found to be at least 60%, with the remainder being detected in the washes.

Previous experiments have shown that in formulations containing <10% w/w Quil A, the predominate particulate structures formed have characteristics typical of liposomes, that is, concentric bilayers separated by and surrounding aqueous spaces (25). Transmission electron micrographs (TEMs) were taken of representative liposome formulations used here to confirm that the addition of 2% w/w Quil A to PC liposomes did not affect the nature of the particulate structures formed. In all cases, the particles had structural characteristics typical of liposomes with no evidence of ISCOM formation as observed in formulations containing higher amounts of Quil A.

Table I. Size Distribution of Liposome Formulations

Formulation	Size (nm)[a]	Polydispersity index
Unmodified PC liposomes	952 ± 40	0.80
ManLCP liposomes	993 ± 18	0.79
Quil A liposomes	990 ± 46	0.70
ManLCP-Quil A liposomes	989 ± 50	0.88

[a] Values represent the mean ± SD of ten measurements on three independent formulations.

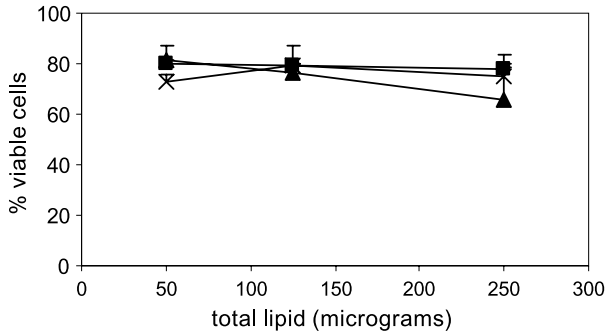

Fig. 2. Viability of BMDC after incubation with liposomes containing Quil A. BMDC were incubated for 48 h at 37°C with liposomes containing 0% (×), 1% (■), or 2% (▲) w/w/Quil A. Cells were stained with propidium iodide staining solution and then the proportion of propidium $iodide^{-ve}$ (% viable) cells determined by flow cytometry. Data represent the mean ± SD of three independent replicates.

Viability of BMDC After Incubation with Liposomes Containing Quil A

Viability of BMDC after incubating with liposomes containing up to 2% w/w Quil A for 48 h was determined by staining with propidium iodide solution. Propidium iodide is excluded from viable cells and therefore the proportion of propidium $iodide^{-ve}$ cells in each population can be measured by flow cytometry. As shown in Fig. 2, the inclusion of up to 2% w/w Quil A into PC liposomes had little effect on cell viability even at relatively high lipid concentrations.

Activation of BMDC *in Vitro*

Activation of BMDC by various liposome formulations *in vitro* was examined after incubating the liposome formulations with mouse BMDC for 48 h. Activation of BMDC was determined by staining the cells with fluorescent antibodies against the cell surface costimulatory molecules CD86 and MHC class II, and by comparing the levels of expression with that found on control BMDC not incubated with any formulation. As shown in Fig. 3, incubating BMDC with the different liposome formulations resulted in a similar level of activation marker expression, both CD86 (Fig. 3A) and MHC class II (Fig. 3B) for all formulations studied, with only marginally increased marker expression over levels found in control BMDC. The increase in CD86 MFI was slightly higher for formulations containing manLCP constructs compared with formulations not containing the constructs (Fig. 3A); however, these differences were not statistically significant.

T-Cell Proliferation *in Vitro*

Although the levels of BMDC activation after incubation with liposome formulations were not significantly increased over control levels, previous experience in our laboratory has shown that due to the relative insensitivity of

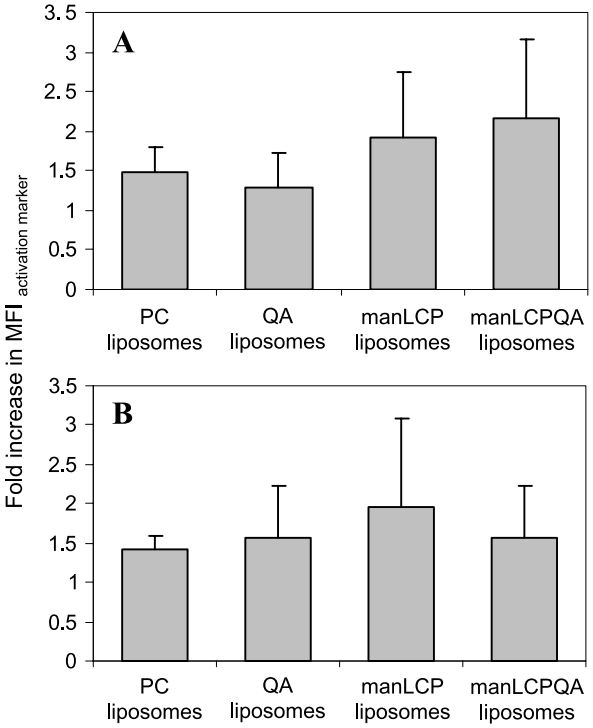

Fig. 3. Activation of BMDC after incubation with unmodified liposomes or liposomes containing 2% Quil A, 5% manLCP, or both 5% manLCP and 2% Quil A. Expression of activation markers CD86 (A) and MHC class II (B) on the surface of BMDC was determined after incubating cells with 250 μg liposome formulations for 48 h at 37°C. Activation marker expression was determined by comparison of the mean fluorescence intensity (MFI) of fluorescent antibodies against each activation marker on $CD11c^+$ BMDC pulsed with formulations ($MFI_{activation\ marker}$) with the MFI of activation markers expressed on $CD11c^+$ BMDC not pulsed with liposomes and calculated as a fold increase in MFI over background. Data represent the mean ± SD of three independent replicates.

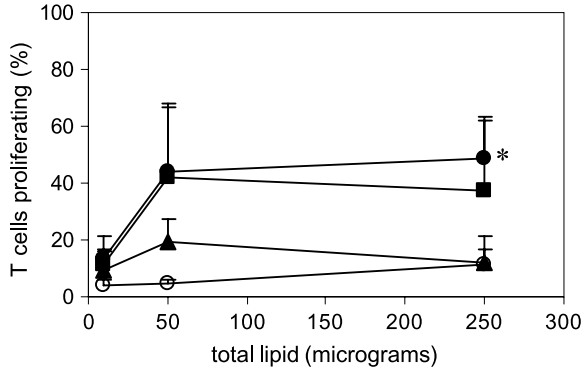

Fig. 4. Proliferation of $V\alpha2^+$ $CD8^+$ T cells after incubation with BMDC pulsed with liposome formulations. BMDC were pulsed for 48 h at 37°C with varying amounts of unmodified PC liposomes (○), Quil A-containing liposomes (▲), manLCP-containing liposomes (■), and manLCP- and Quil A-containing liposomes (●). DC were then harvested and cocultured with CFSE-labeled splenocytes from an OT-I transgenic mouse at a DC/T ratio of 1:25. After 3 days the percentage of proliferating $V\alpha2^+$ $CD8^+$ T cells was determined by flow cytometry. $^*P < 0.05$ compared with both unmodified PC liposomes and Quil A-containing liposomes. Data represent the mean ± SD of three independent replicates.

this assay, poor BMDC activation *in vitro* does not necessarily preclude the ability of formulations to stimulate $CD8^+$ T cells *in vitro* and *in vivo*. Therefore we next determined the ability of BMDC pulsed with the various liposome formulations for 48 h to stimulate proliferation of naïve $CD8^+$ T cells *in vitro*. The percentage of the $CD8^+$ T cell population proliferating after 72 h was determined by flow cytometry. As shown in Fig. 4, BMDC pulsed with unmodified PC liposomes were unable to stimulate proliferation of $CD8^+$ T cells. Similarly, BMDC pulsed with liposomes containing Quil A alone but lacking incorporated CD8 epitopes were unable to stimulate significant levels of $CD8^+$ T cell proliferation. BMDC pulsed with liposomes containing manLCP constructs either with or without the addition of Quil A were able to stimulate a much greater level of $CD8^+$ T cell proliferation; however, only in formulations containing both manLCP and Quil A was proliferation significantly greater than in cultures incubated with BMDC pulsed with unmodified liposomes.

Cytotoxic T-Cell Stimulation *in Vivo*

We next compared the ability of formulations containing manLCP or manLCP and Quil A to stimulate cytotoxic $CD8^+$ T cells *in vivo*. Groups of C57Bl/6J mice were immunized twice with liposomes containing manLCP or manLCP and Quil A. To further investigate whether manLCP needed to be physically incorporated into the same liposome particle as the Quil A, two further vaccines were prepared consisting of manLCP-containing liposomes admixed with Quil A-containing liposomes, and manLCP admixed with Quil A-containing liposomes. Immunization with manLCP in alum was used as a control. Nine days after the second immu-

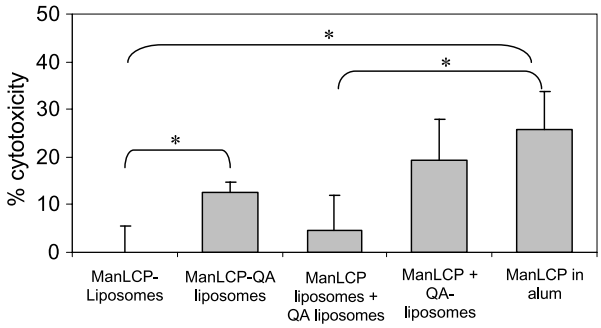

Fig. 5. Stimulation of cytotoxic $CD8^+$ T cells *in vivo* by liposome formulations. Groups of C57Bl/6J mice ($n = 3$) were immunized twice with manLCP-containing liposomes (manLCP liposomes), manLCP- and Quil A-containing liposomes (manLCP-QA liposomes), manLCP liposomes admixed with Quil A-containing liposomes (manLCP liposomes + QA liposomes), manLCP admixed with QA liposomes (manLCP + QA liposomes), or manLCP in alum. Nine days after the second immunization, mice were injected intravenously with CFSE-labeled target cells pulsed with 10 μg SIINFEKL peptide or no peptide. The following day, mice were euthanased. The remaining antigen-pulsed target cells in draining lymph nodes was determined by flow cytometry and the percentage cytotoxicity of peptide pulsed target cells calculated. Data represent the mean ± SD of three animals in each group and is representative of three independent experiments. $^*P < 0.05$ as indicated.

nization, mice were injected intravenously with CFSE-labeled target cells pulsed with 10 μg SIINFEKL peptide or no peptide as a control. The following day mice were euthanased and the remaining antigen-pulsed target cells in draining lymph nodes were determined by flow cytometry.

Mice immunized with liposomes containing manLCP alone were not able to mount a CD8 cytotoxic immune response comparable to manLCP in alum (Fig. 5). Adding 2% Quil A to manLCP liposomes, however, significantly enhanced the resulting cytotoxic immune response to a level similar to that stimulated by manLCP in alum. A similar level of antigen-specific cytotoxicity was achieved if manLCP was simply admixed with Quil A-containing liposomes prior to immunization. However, if manLCP was incorporated into separate liposomes from the Quil A-containing liposomes, then the resulting *in vivo* immune response was lower and not significantly different from that achieved following immunization with manLCP liposomes alone.

Tumor Challenge

Finally, to determine the ability of liposomes containing manLCP and Quil A to act as prophylactic cancer vaccines and protect against tumor challenge, groups of C57Bl/6J mice were immunized subcutaneously twice with liposomes containing both manLCP and Quil A. Groups of mice were also immunized with manLCP-containing liposomes, manLCP admixed with Quil A-containing liposomes, and manLCP in alum with ovalbumin protein to stimulate CD4 help. Thirty days after the second immunization, 1×10^6 EG.7-OVA tumor cells were administered into the flank of immunized mice or nonimmunized controls, and tumor growth was monitored every 2–3 days. As shown in Fig. 6, all naïve mice challenged with EG.7-OVA tumor cells quickly developed measurable tumors. In contrast, mice immunized with manLCP plus ovalbumin protein in alum were well protected, with four out of five animals remaining tumor-free 4 weeks after tumor challenge. Mice immunized with manLCP admixed with Quil A-containing liposomes were similarly well protected from tumor growth. In groups of mice immunized with liposomes containing manLCP or both manLCP and Quil A, two out of five, and three out of five animals, respectively, remained tumor-free at the end of the study—indicating an intermediate level of immune protection.

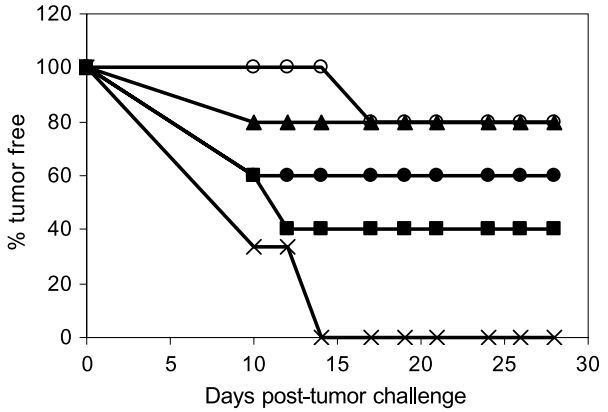

Fig. 6. Tumor development in mice immunized with liposome formulations. Groups of C57Bl/6J mice (n = 5) were immunized twice with liposomes containing manLCP (■) or both manLCP and Quil A (●), manLCP admixed with Quil A liposomes (▲), or manLCP in alum with ovalbumin protein (○). Thirty days after the second immunization 1×10^6 EG.7-OVA tumor cells were administered into the flank of immunized mice or nonimmunized controls (×, n = 3). Tumor growth was monitored every 2–3 days, and the percentage of tumor-free animals in each group was calculated. Data represent two independent replicates.

DISCUSSION

We have previously shown that LCP constructs containing multiple copies of minimal $CD8^+$ T cell epitopes are capable of stimulating $CD8^+$ T cell immune responses *in vivo*. The responses generated, however, are modest unless the constructs are administered in alum to provide a depot and with ovalbumin protein to stimulate $CD4^+$ T helper cells (4). Under these more optimal vaccine conditions, LCPs are able to stimulate strong cytotoxic immune responses that completely protect mice from subsequent tumor challenge. In an attempt to obviate the need for using alum and providing CD4 help in LCP vaccines, we wished to investigate the immunogenicity of LCPs incorporated into liposomal formulations. Although in previous studies liposomal incorporation has been found to increase the immune response generated toward weakly immunogenic peptides and lipopeptides (10,11,27), we have found the inherent immunogenicity of unmodified liposomes to be relatively low (18). We therefore included a small amount of the immune stimulating adjuvant Quil A into additional liposome formulations, and investigated the effects of this inclusion on the resulting immune response.

Liposomes were prepared containing 5% w/w manLCP with or without the addition of 2% w/w Quil A by modification of the lipid film hydration method. Hydration of thin lipid films tends to produce liposomes that are relatively large and multilamellar in nature with a heterogeneous size distribution (28). As particle size is important in phagocytosis, the mean size distribution of the resulting liposome dispersions was reduced by high-pressure extrusion through polycarbonate membranes. Particle size analysis of the liposome formulations containing manLCP, Quil A, or both manLCP and Quil A revealed the size distributions to be similar to that of the unmodified PC liposomes, indicating that the size of the resulting liposomes was not affected by the inclusion of 5% w/w manLCP and/or 2% w/w Quil A moieties. In addition, TEM analysis indicated that the inclusion of this low amount of Quil A did not disrupt the integrity of the bilayer, and the particles formed were still liposomal in nature.

Before investigating the ability of the liposome formulations to stimulate *in vivo* immune responses, we first determined whether the formulations could activate mouse BMDC and subsequently stimulate proliferation of $CD8^+$ T cells *in vitro*. Dendritic cells (DCs) are important phagocytic cells involved in pathogen recognition and presentation of processed antigens to naïve T and B cells. To activate effector cells efficiently, the DCs themselves must first become activated, for example, via ligation of certain pathogen associated molecular patterns by Toll-like receptors and other pattern recognition receptors (PRRs) on the surface of the DC. DC

activation results in the up-regulation of costimulatory molecules on the DC surface, which provide additional signals that are required to fully activate the naïve effector cell. Without DC activation and costimulation, a productive immune response will not develop and, in fact, a tolerogenic immune response may even result (29).

Previously, we showed that both LCP and manLCP (data not shown; (4)) are able to induce significant DC activation *in vitro*, which may be attributable to interaction between the LCP constructs and PRRs on DCs. To investigate DC activation after incubation with manLCP when the constructs are incorporated into liposomes, mouse BMDC were incubated with liposome formulations containing either manLCP, Quil A, or both manLCP and Quil A. After 48 h, the level of CD86 and MHC class II activation marker expression on the surface of the BMDC was determined by flow cytometry. It was found that the liposomes containing manLCP and both manLCP and Quil A were unable to stimulate a significantly greater increase in the expression of CD86 and MHC class II on the surface of BMDC compared with unmodified liposomes or liposomes containing Quil A alone. The low level of BMDC activation after incubation with unmodified PC liposomes is in line with previous experiments that have found unmodified liposomes possess only low inherent immunogenicity (18). The lack of increased BMDC activation after incubation with liposomes containing Quil A and/or manLCP constructs, however, was disappointing, although this may reflect the low amount of these moieties being delivered to the cells in these formulations. The amount of LCP (5% w/w) used in the current study was chosen after consideration of the literature investigating the immunogenicity of peptides and lipopetides when incorporated into liposomes (10,11,27). It had been hoped that even though the amount of manLCP used in these experiments was low, the fact that it was being delivered to BMDC in a particulate form may compensate for the decrease in dosage. It is also possible that by incorporating the constructs into liposomes, the lipid tails were no longer available for interaction with DC PRRs, and this may have contributed to the low level of activation observed in these *in vitro* studies.

The ability of Quil A to stimulate immune responses is thought to rely in part on its ability to associate with cholesterol in cell membranes forming transmembrane pores that allow for enhanced cytosolic entry of antigens into DCs (30). This pore-forming ability is also thought to be responsible for the high cytolytic activity and hence toxicity associated with the use of Quil A as an immune stimulating adjuvant (31). We have previously shown that the incorporation of increasing amounts of Quil A into liposomes results in a change in the structure of the particles formed from liposomes at a Quil A content below approximately 10% w/w, through to ISCOMs and small ring-like micellar structures at progressively higher Quil A concentrations (25). Associated with the higher Quil A content, ISCOMs and ring-like micelles show high cell toxicity *in vitro*. We therefore chose to use a Quil A content of 2% w/w in this current study to avoid the changes in particle structure and associated toxicity associated with higher Quil A content. It is likely, therefore, that this low level of Quil A was not sufficient to stimulate the significant up-regulation of BMDC activation markers *in vitro*. However, the fact that no increase in BMDC activation was seen may also reflect a lack of sensitivity in the assay to discriminate between small differences in BMDC activation, as we have previously shown that a lack of BMDC activation *in vitro* does not preclude the ability of formulation pulsed BMDC to stimulate T cells *in vitro* (18).

We therefore investigated the ability of BMDC pulsed with various liposome formulations to stimulate *in vitro* proliferation of naïve $CD8^+$ T cells isolated from OT-I transgenic mice. As expected, BMDC pulsed with unmodified liposomes and liposomes containing Quil A alone were unable to stimulate proliferation of $CD8^+$ T cells as no $CD8^+$ T cell antigens were present in these formulations. In comparison, BMDC pulsed with liposomes containing manLCP constructs were able to stimulate strong proliferation of $CD8^+$ T cell populations. The only formulations showing a statistically significant enhancement in proliferation compared with proliferation in T cell cultures incubated with unpulsed BMDC, however, were the liposomes containing both manLCP and Quil A. This confirms previous findings that measuring BMDC activation *in vitro* may not be a sufficiently sensitive method for determining the immunogenicity of formulations as T cell activation can still occur in the absence of significant DC activation. It also suggests that this T cell activation is antigen-specific as no proliferation occurred in the absence of manLCP constructs. These results indicate that the addition of a small quantity of Quil A to the liposome formulations may enhance the ability of BMDC to induce T cell proliferation over that induced in its absence.

Finally, we investigated the ability of liposome formulations containing manLCP and/or Quil A to stimulate cytotoxic immune responses *in vivo* and protect against subsequent tumor challenge. Groups of C57Bl/6J mice were immunized with liposomal vaccines containing manLCP, or manLCP and Quil A, either in the same or in different liposome particles. All vaccines were well tolerated after subcutaneous injection with no evidence of irritation, redness, or swelling at the site of injection. The liposomes containing manLCP alone were unable to stimulate an antigen-specific cytotoxic response *in vivo* after subcutaneous administration, suggesting that formulating the manLCP in liposomes on their own is not sufficient to increase the immunogenicity of the manLCP constructs. Inclusion of 2% Quil A into manLCP-containing liposomes, however, significantly enhanced the cytotoxic immune response as measured by killing of peptide-pulsed target cells. ManLCP incorporated in Quil A-containing liposomes during liposome formulation and manLCP admixed with Quil A-containing liposomes prior to administration elicited similar levels of antigen-specific cytotoxicity *in vivo*, not significantly different from manLCP administered in alum. However, if manLCP and Quil A were incorporated into separate liposomes and admixed before administration, the resulting level of cytotoxicity was reduced to near-background levels. This finding suggests that the antigen and adjuvant need to be delivered in the same liposome delivery system. It is conceivable that because of the amphipathic nature of the manLCP constructs, free manLCP admixed with Quil A-containing liposomes becomes associated with the liposomal membrane during vaccine preparation, and thus effectively becomes the same as the manLCP-Quil A-containing liposome formulations, explaining the similar results seen with these two formulations. If manLCP is already

incorporated into liposomes, this interaction would not occur and manLCP and Quil A would remain in separate particles.

The ability of manLCP- and Quil A-containing liposomes to act as prophylactic vaccines and protect against later tumor challenge was also investigated and found to confirm the above *in vivo* cytotoxicity findings. Immunization with manLCP admixed with Quil A-containing liposomes achieved the same level of tumor protection in mice as manLCP administered under more optimal vaccine conditions (four out of five animals protected at 4 weeks posttumor challenge). The level of protection after immunization with manLCP-Quil A-containing liposomes was less than that after immunization with the admixed formulation group (three out of five animals protected), but higher than that achieved using manLCP liposomes alone.

The findings presented here are consistent with those of Lipford *et al.* (32) using peptide antigen incorporated into Quil A-containing liposomes. Lipford *et al.* demonstrated that approximately 30% specific lysis of peptide-pulsed target cells could be achieved *in vitro* by incubating with cytotoxic T cells removed from mice immunized with peptide-containing Quil A liposomes. Our results demonstrate that a similar level of cytotoxicity can be achieved *in vivo* by using manLCP-containing Quil A liposomes.

The ability of Quil A-containing liposomes to replace the requirement for ovalbumin protein to stimulate CD4 help in *in vivo* immune responses is also consistent with previous findings regarding the mechanism of action behind the immunogenicity of Quil A as an adjuvant. ISCOMs containing Quil A are known to stimulate the production of IL-1 (33), a potent inducer of $CD4^+$ T helper cells, which in turn stimulates activation of $CD8^+$ immune responses toward otherwise weak immunogens (5). Quil A can therefore potentially replace other stimulators of CD4 help in vaccine formulations. In the absence of Quil A, manLCP-containing liposomes seem unable to stimulate such strong immune responses.

CONCLUSION

The findings presented here suggest that liposomal incorporation of manLCP constructs is not sufficient on its own to replace the need for alum in stimulating cytotoxic immune responses *in vivo*. However, liposomes containing just 2% w/w Quil A are capable of replacing the need for alum in initiating *in vivo* $CD8^+$ cytotoxic immune responses toward manLCP constructs. In addition, vaccines containing manLCP and Quil A are able to protect against subsequent tumor challenge as effectively as if the constructs are administered in alum and with ovalbumin protein to stimulate CD4 help. To stimulate this protective immune response, it seems that the adjuvant needs to be delivered within the same particle as the antigen, as the immune response generated when the manLCP and Quil A were administered in separate liposomes was reduced to near-background levels. Importantly, the low level of Quil A incorporated into these liposome formulations seemed to be well tolerated after subcutaneous injection. Interestingly, the ability of various liposome formulations to stimulate *in vivo* immune responses did not reflect their ability to stimulate mouse BMDC *in vitro.* Therefore, *in vitro* testing alone should not be relied upon to determine the immunogenicity of potential vaccine formulations.

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REFERENCES

1. C. Olive, M. Batzloff, A. Horvath, T. Clair, P. Yarwood, I. Toth, and M. F. Good. Potential of lipid core peptide technology as a novel self-adjuvanting vaccine delivery system for multiple different synthetic peptide immunogens. *Infect. Immun.* **71**: 2373–2383 (2003).
2. C. Olive, M. Batzloff, A. Horvath, A. Wong, T. Clair, P. Yarwood, I. Toth, and M. Good. A lipid core peptide construct containing a conserved region determinant of the group a streptococcal M protein elicits heterologous opsonic antibodies. *Infect. Immun.* **70**:2734–2738 (2002).
3. W. Hayman, I. Toth, N. Flinn, M. Scanlon, and M. Good. Enhancing the immunogenicity and modulating the fine epitope recognition of antisera to a helical group A streptococcal peptide vaccine candidate from the M protein using lipid-core peptide technology. *Immunol. Cell Biol.* **80**:178–187 (2002).
4. K. White, P. Kearns, I. Toth, and S. Hook. Increased adjuvant activity of minimal CD8 T cell peptides incorporated into lipid-core-peptides. *Immunol. Cell Biol.* **82**:517–522 (2004).
5. Y. Zhan, A. Corbett, J. Brady, R. Sutherland, and A. Lew. CD4 help-independent induction of cytotoxic CD8 cells to allogeneic P815 tumor cells is absolutely dependent on co-stimulation. *J. Immunol.* **165**:3612–3619 (2000).
6. B. Wang, C. Norbury, R. Greenwood, J. Bennink, J. Yewdell, and J. Frelinger. Multiple paths for activation of naive CD8(+) T cells: CD4-independent help. *J. Immunol.* **167**:1283–1289 (2001).
7. J. Wang and A. Livingstone. Cutting edge: CD4(+) T cell help can be essential for primary CD8(+) T cell responses *in vivo*. *J. Immunol.***171**:6339–6343 (2003).
8. A. Kaiser, N. Bercovici, J. Abastado, and A. Nardin. Naive CD8(+) T cell recruitment and proliferation are dependent on stage of dendritic cell maturation. *Eur. J. Immunol.* **33**:162–171 (2003).
9. H. Hu, H. Winter, J. Ma, M. Croft, W. Urba, and B. Gox. CD28, TNF receptor, and IL-12 are critical for CD4-independent cross-priming of therapeutic antitumor CD8(+) T cells. *J. Immunol.* **169**:4897–4904 (2002).
10. H. Guan, W. Budzynski, R. Koganty, M. Krantz, M. Reddish, J. Rogers, B. Longenecker, and J. Samuel. Liposomal formulations of synthetic MUC1 peptides: effects of encapsulation *versus* surface display of peptides on immune responses. *Bioconjug. Chem.* **9**:451–458 (1998).
11. I. Fernandes, B. Frisch, S. Muller, and F. Schuber. Synthetic lipopeptides incorporated in liposomes: *in vitro* stimulation of the proliferation of murine splenocytes and *in vivo* induction of an immune response against a peptide antigen. *Mol. Immunol.* **34**:569–576 (1997).
12. L. Flebbe and H. Braley-Mullen. Immunopotentiating effects of the adjuvants SGP and Quil A: I. Antibody responses to T-dependent and T-independent antigens. *Cell. Immunol.* **99**: 119–127 (1986).
13. S. Kamstrup, R. San Martin, A. Doberti, H. Grande, and K. Dalsgaard. Preparation and characterisation of Quillaja saponin with less heterogeneity than Quil-A. *Vaccine* **18**:2244–2249 (2000).
14. M. Baca-Estrada, M. Foldvari, M. Snider, S. van Drunen Littel-vanden Hurk, and L. Babiuk. Effect of IL-4 and IL-12 liposomal formulations on the induction of immune response to bovine herpes virus type-1 glycoprotein D. *Vaccine* **15**:1753–1760 (1997).
15. A. Ninomiya, K. Ogasawara, K. Kajino, A. Takada, and H. Kida. Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice. *Vaccine* **20**:3123–3129 (2002).
16. M. van Slooten, G. Storm, A. Zoephel, Z. Kupcu, O. Boerman, D. Crommelin, E. Wagner, and R. Kircheis. Liposomes contain-

ing interferon-gamma as adjuvant in tumor cell vaccines. *Pharm. Res.* **17**:42–48 (2000).
17. M. Jaafari and M. Foldvari. Targeting of liposomes to melanoma cells with high levels of ICAM-1 expression through adhesive peptides from immunoglobulin domains. *J. Pharm. Sci.* **91**: 396–404 (2002).
18. M. Copland, M. Baird, T. Rades, J. McKenzie, B. Becker, F. Reck, P. Tyler, and N. Davies. Liposomal delivery of antigen to human dendritic cells. *Vaccine* **21**:883–890 (2003).
19. G. Kersten and D. Crommelin. Liposomes and ISCOMs as vaccine formulations. *Biochim. Biophys. Acta* **1241**:117–138 (1995).
20. P. Demana, N. Davies, B. Berger, and T. Rades. Incorporation of ovalbumin into ISCOMs and related colloidal particles prepared by the lipid film hydration method. *Int. J. Pharm.* **278**:263–274 (2004).
21. L. East and C. Isacke. The mannose receptor family. *Biochim. Biophys. Acta* **1572**:364–386 (2002).
22. F. Sallusto and A. Lanzavecchia. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and down-regulated by tumour necrosis factor α. *J. Exp. Med.* **179**:1109–1118 (1994).
23. K. Mahnke, M. Guo, S. Lee, H. Sepulveda, S. Swain, M. Nussenzweg, and R. Steinman. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J. Cell Biol.* **151**:673–683 (2000).
24. L. Bonifaz, D. Bonnyay, K. Mahnke, M. Rivera, M. Nussenzweig, and R. Steinman. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* **196**:1627–38 (2002).
25. P. Demana, C. Fehske, K. White, T. Rades, and S. Hook. Effect of incorporation of the adjuvant Quil A on structure and immune stimulatory capacity of liposomes. *Immunol. Cell Biol.* **82**:547–554 (2004).
26. M. Moore, F. Carbone, and M. Bevan. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* **54**:777–785 (1988).
27. A. Agrawal, A. Puri, W. Haq, and C. Gupta. Enhanced immunostimulant activity and protective effect of a synthetic lipopeptide after liposomization against *Plasmodium berghei* infection in mice. *J. Drug Target.* **9**:231–239 (2001).
28. R. New. *Liposomes: A Practical Approach*, Oxford University Press, New York, 1990.
29. M. Steinman, D. Hawiger, and M. C. Nussenzweig. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* **21**:685–711 (2003).
30. C. Kensil. Saponins as vaccine adjuvants. *Crit. Rev. Ther. Drug Carrier Syst.* **13**:1–55 (1996).
31. B. Rönnberg, M. Fekadu, and B. Morein. Adjuvant activity of non-toxic *Quillaja saponaria* Molina components for use in ISCOM matrix. *Vaccine* **13**:1375–1382 (1995).
32. G. Lipford, H. Wagner, and K. Heeg. Vaccination with immunodominant peptides encapsulated in Quil A-containing liposomes induces peptide-specific primary CD8+ cytotoxic T-cells. *Vaccine* **12**:73–80 (1994).
33. S. Behboudi, B. Morein, and M. Villacres-Eriksson. *In vitro* activation of antigen-presenting cells (APC) by defined composition of *Quillaja saponaria* Molina triterpenoids. *Clin. Exp. Immunol.* **105**:26–30 (1996).