# Coating of Mannan on LPD Particles Containing HPV E7 Peptide Significantly Enhances Immunity Against HPV-Positive Tumor

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***Purpose.*** Previously, our laboratory has reported that liposome-protamine-DNA (LPD) nanoparticle is an effective delivery system for tumor-associated antigens. Mannan, which potentially targets antigen-presenting cells, was coated on LPD to further enhance its antitumor activity.

***Methods.*** Cholesterol-conjugated mannan was coated on LPD. The abilities of mannan-coated LPD to target antigen-presenting cells, to activate dendritic cells, and to induce antitumor immunity were investigated and compared to those of LPD alone.

***Results.*** Both *in vitro* and *in vivo* uptake of LPD showed that mannan-coated LPD particles were preferably taken up by dendritic cells and macrophages. In addition, the expression of co-stimulatory molecules CD80/CD86 on DC2.4 cells after co-incubation with mannan-coated LPD was significantly higher than that after co-incubation with LPD. A model major histocompatibility complex class I–restricted peptide antigen from HPV 16 E7 protein was incorporated into LPD to immunize mice against the growth of TC-1 tumor cells expressing E7 protein. Coating with mannan significantly enhanced both preventive and therapeutic activities of LPD/E7. Finally, the release of IFN-γ from isolated splenocytes was significantly enhanced when mice were immunized with mannan-coated LPD/E7 than with LPD/E7 alone.

***Conclusion.*** Targeting of the LPD/E7 to local draining lymph nodes by mannan is partially responsible for the enhanced anti-tumor activity.

**KEY WORDS:** antigen-presenting cells; liposome; mannan; peptide; tumor vaccine.

## INTRODUCTION

Vaccination with synthetic peptide-based vaccines designed to elicit T-cell immunity is an attractive approach to the prevention and treatment of cancers. Tumor cells express antigens that can be recognized by the host immune system. These tumor-associated antigens (TAAs), usually small peptides of 8–10 aa, can be injected into patients to induce immune response that may result in the eradication of cancers. During the past decade, numerous TAAs recognized by tumor-reactive cytotoxic lymphocytes (CTLs) have been identified by various molecular and biochemical methods (1). In animal studies, prophylactic vaccination with synthetic peptide was effective for virally induced tumors. In contrast, for tumors that are not virally induced, prophylactic vaccination was less effective, although clear examples of antitumor efficacy do exist (2).

The use of peptide as vaccine has many advantages, including the fact that the product is chemically defined, stable, safe, and contains only the important epitope (3). However, the potency of peptide-based vaccines is usually poor, if administered alone. For a peptide antigen to induce cell-mediated antitumor immune response effectively, it must make its way into the groove of the major histocompatibility complex (MHC) molecule of the antigen-presenting cells (APCs) and activate them so that the APCs can present the peptide to T cells in the lymphatic tissue. If the peptide is injected alone, it will be mostly degraded before it reaches the APCs. *Ex vivo* loading of peptide to isolated dendritic cells (DCs) followed by re-introducing the DCs to the host has proven to be a feasible alternative (4). However, direct delivery using suitable adjuvant system that can effectively bring the peptide antigen to APCs and activate APCs is still preferred.

Many antigen delivery systems have been designed to deliver peptide antigen. These include the use of peptide associated with adjuvant, encapsulation of peptide into neutral liposomes or biodegradable polymeric particles, and coupling of peptide to synthetic beads, to name a few. Previously, a liposome-based DNA delivery system, called LPD (liposome-protamine-DNA), had been developed in our laboratories (5,6). LPD was engineered by combining cationic liposomes and polycation condensed DNA. Upon mixing, the components rearrange to form a virus-like structure with the condensed DNA located inside the lipid membrane (5,6). When administered systemically, LPD rapidly initiates production of several T-helper type 1 (Th1) cytokines, most notably TNF-α, IL-12, and IFN-γ (7,8). This nonspecific immunostimulation is associated with tumor static effects (7,8). More recently, when a MHC class I–restricted peptide epitope derived from a tumor antigen, the HPV E7 protein, was incorporated into the LPD and then used to immunize mice, a strong antitumor response was observed (9). The LPD/E7 induced E7-specific CTL response that prevented the establishment of E7-expressing TC-1 tumor. Moreover, administration of LPD/E7 to tumor-bearing mice caused tumor regression (9). Further investigation of this LPD as a peptide antigen delivery system is therefore warranted. Besides understanding the mechanisms of immune stimulation from the LPD, optimization (i.e., balancing the efficacy and toxicity) and further improvement of the performance of this LPD-based peptide antigen delivery system are currently sought.

It has been shown that, as long as antigens remain outside the lymphatic tissues, they will be ignored by the immune systems (10,11). Genetic vaccination has shown that although the majority of the protein antigen expression is located in the peripheral tissues, immune response is initiated by APCs in the draining lymph nodes (12–14). Therefore, for an antigen to effectively induce immunity, it must find its way to the organized lymph organs such as the lymph nodes. This may be achieved by either effective delivery of the antigen to the lymph organ or by delivering a "danger" signal together with the antigen so that lymphocytes, especially APCs, may be recruited to the site of injection and fetch the antigen to local draining lymph nodes for presentation (15). Accordingly, targeting of APCs such as DCs and macrophages using specific ligands is an attractive approach. It has proven that human and murine DCs and macrophages express mannose receptor

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(MR) on their surface (16–18). Several studies have confirmed the feasibility of using mannose or mannan to target protein antigens, liposomes, and other micro(nano)particles to APCs (17,18). For example, Apostolopoulos *et al*. showed that murine MR-bearing macrophages derived from peritoneal exudates and cultured *ex vivo* with oxidized mannan linked to MUC1 antigen can, after adoptive transfer, efficiently present MUC1 to T cells, leading to the generation of CTL and protection from subsequent tumor challenge (19). In another study, Fukasawa *et al*. reported that liposomes that contain an immunodominant peptide (15 aa) of the envelop glycoprotein gp120 of HIV-1 and coated with mannopentaose induced a MHC class I–restricted $CD8^+$ CTL response in mice with a single subcutaneous immunization, whereas uncoated liposomes did not (18). In addition, mannosyl glycoconjugates are present on a range of bacteria, fungi, virus-infected cells, and parasites. Host considers mannan as a "danger" signal and has a mannose-binding-lectin complement pathway as an innate immune response to defend against mannan-bearing objects (20).

In this study, a commercially available mannan conjugate was coated on E7-incorporated LPD to further enhance the resulting immune response. Further enhancement of the immunity will be helpful for us to balance the effectiveness and toxicity of the LPD so that less amount of LPD can be used in future clinical trials. Both prophylactic and therapeutic immunity studies were carried out in a syngeneic mouse tumor model induced by HPV 16 E7 expressing TC-l cells.

## MATERIALS AND METHODS

### Materials

1,2-Dioleoyl-3-trimethylammonium propane (DOTAP) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Con A, protamine sulfate (fraction X from salmon), methyl α-D-glucopyanoside (α-MG), and D(+)-galactose were from Sigma (St. Louis, MO, USA). {*N*-[2-cholesterylcarboxyamino)ethyl]carbamoylmethyl-mannan (Chol-Man) was purchased from Dojindo Molecular Technologies (Gaithersburg, MD, USA). Cy5-labeled oligodeoxylnucleotide (ODN) was from Invitrogen (Carsbad, CA, USA). Phycoerythrin (PE)-, PE-Cy7, or fluorescein (FITC)-labeled antibodies were from BD Pharmingen (San Diego, CA, USA). Plasmid (pNGVL3) containing the CMV promoter and no coding region was obtained from the National Gene Vector Laboratory (Ann Arbor, MI, USA). Plasmid DNA was purified using Qiagen EndoFree Giga-Prep kit (Qiagen, Valencia, CA, USA). The MHC class I–restricted peptide from the HPV 16 E7 protein (aa 49–57, RAHYNIVTF) was synthesized in the University of Pittsburgh Peptide Synthesis Facility by solid phase synthesis using an Advanced ChemTech Model 200 Peptide Synthesizer (Louisville, KY, USA) and purified using high performance liquid chromatography (HPLC).

DC2.4 cells, originally engineered from murine dendritic cells by Dr. Kenneth Rock, were provided by Dr. Louis Falo, Jr., at the University of Pittsburgh Medical Center. DC2.4 cells have proven to be MR positive and a good DC model (21; Han & Huang, unpublished data). TC-1 cells were from Dr. T. C. Wu at the Johns Hopkins University. TC-1 cells are C57BL6 mouse lung endothelial cells transformed with the HPV 16 E6 and E7 oncogenes and activated H-ras. Cells were grown in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin (Sigma), and 100 μg/ml of streptomycin (Sigma).

### Liposome and LPD Preparation

Liposome and LPD preparation were completed as previously described (6,9). Briefly, small unilamellar liposomes composed of DOTAP:cholesterol (1:1 molar ratio) were prepared by thin film hydration followed by membrane extrusion. LPD was comprised of DOTAP/cholesterol liposome, protamine, and plasmid DNA (DNA) in a ratio of 9.0:0.6:1.0 (w/w/w). To prepare LPD, required amounts of liposome and protamine were dispersed in 150 μl of aqueous solution containing 10% of dextrose (Sigma). Then, 150 μl solution containing DNA with or without E7 peptide was added dropwise into the mixture of liposome and protamine while stirring. The complexes were then allowed to incubate at room temperature for at least 20 min prior to further use. The E7 incorporation efficiency was estimated to be ~80% using fluorescein labeled E7 eptide. Free unincorporated E7 was not further removed prior to injection to animals.

Coating of mannan on LPD was completed as previously described with modification (18,22). To coat mannan on LPD, pre-formed LPD suspension (containing 86 μl of liposome in 600 μl of total volume) was mixed with pre-formed Chol-Man dispersion (1 mg/ml) in a ratio of 2:1 (v/v). The mixture was vigorously vortexed for 5 min and then gently shaked at 4°C for 24 h prior to use. The E7 incorporation efficiency was comparable to that in the mannan-free LPD. The particle size and the zeta potential of the LPD were measured following the manufacturer's suggestion using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA, USA) and a Zetasizer 4 (Malven Instruments, Inc. Southborough, MA, USA), respectively.

### Con A Agglutination Assay

Con A agglutination assay was completed as previously described with modification (23). Briefly, 100 μl of samples were added into 1 ml of Con A (1 mg/ml) in phosphate-buffered saline (PBS) (10 mM, pH 7.4) with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. The turbidity (OD360 nm) at room temperature was monitored for 100 s using a DU-640 Spectrophotometer (Beckman Coulter). For specificity studies, α-MG (0.5 M) or D-(+)-galactose (0.25 M) were incubated with Con A (1 mg/ml) for 5 min at room temperature prior to the addition of mannan-coated LPD.

### *In vitro* Uptake of LPD by DC2.4 Cells

For this study, LPD was prepared with DNA composed of 5% (w/w) FITC-labeled ODN. Cell binding and uptake studies were preformed with DC2.4 cells that were approximately 80% confluent. Cells were seeded in 48-well plates at a density of $1.0 \times 10^6$ cells/well and allowed to grow for 16 h. The cells were then incubated with LPD (50 μl), coated or uncoated with mannan, for 0, 0.25, 0.5, 1.25, and 4 h at 4°C or 37°C under 5% $CO_2$. The incubation was terminated by centrifuging the plates at 4°C (2000 rpm, 5 min). Cells were washed three times with cold PBS (10 mM, pH 7.4). Fluorescence intensity associated with the cells was measured using a Perkin Elmer Luminoscence Spectrometer C550B (Selton, CT, USA) (Ex, 496 nm; Em, 512 nm). For the uptake of

mannan-coated LPD at 37°C, one group of cells (n = 3) were also pre-treated with 50 μl of free Chol-Man for 30 min prior to the addition of mannan-coated LPD, and the incubation was stopped 30 min later. Fluorescence data were corrected for light scattering by using unlabeled LPD as a control. Incubation at 4°C was included to measure the binding of LPD to the cells.

### Uptake of LPD by Lymphocytes in Popliteal Lymph Nodes After Footpad Injection

Briefly, 50 μl of LPD in aqueous suspension containing Cy5-labeled ODN (5%, w/w), coated or uncoated with mannan, was subcutaneously (s.c.) injected into the footpads of the hind legs of C56BL/6 mice (n = 4). Sixteen hours after the injection, the popliteal lymph nodes were removed, pooled, and suspended in 5 ml of serum-free RPMI medium. Collagen was digested and lymphocytes were prepared. One million cells were stained with FITC-labeled anti-mouse CD11b and PE-labeled anti-CD11c antibodies at 4°C for 20 min. The cells were then washed twice with BD Pharmingen washing buffer and re-suspended into 200 μl of buffer for flow cytometry analysis (CyAn XL, DakoCytomation Colorado, Inc., Fort Collins, CO, USA). Percent of lymphocytes that are Cy5 positive and percent of Cy5 positive cells that are CD11b positive or CD11c positive were measured.

### Expression of Co-stimulatory Molecules (CD80/86) on DC2.4 Cells After *in vitro* Stimulation

DC2.4 cells ($1.5 \times 10^6$/well) were seeded into 6-well plates and then incubated with 75 μl of LPD or mannan-coated LPD (liposome content was adjusted to the same concentration) at 37°C, 5% $CO_2$. As controls, cells were also treated with equivalent amount of Chol-Man alone or 5% dextrose. Sixteen hours later, the cells were washed twice with BD Pharmingen buffer. One million cells were then stained with FITC-labeled CD80 antibody and PE-labeled CD86 antibody for 20 min at 4°C. After washing twice, the cells were analyzed with a flow cytometer as mentioned above. Data were reported as the percentage of DC2.4 cells that were CD86 or CD80 positive.

### Immunization and Treatment

Six-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were used in all animal studies. National Institutes of Health guidelines for the care and use of laboratory animals were observed. For vaccinations, mice were injected s.c. with 150 μl of the formulations on day 0 and 9. The formulations used are 5% dextrose only, Chol-Man mixed with E7 peptide (10 μg), LPD with E7 peptide (10 μg), and mannan-coated LPD with E7 peptide (10 μg). The corresponding cationic liposome volume injected was about 14 μl. On day 13, mice were challenged by s.c. injection of $5 \times 10^5$ TC-1 cells and were observed for the formation of tumors by palpation. The size of the tumor was measured using a caliper 2–3 times a week. Tumor size was determined by multiplying the two largest dimensions of the tumor.

For treatments, s.c. tumors were established by injecting $5 \times 10^5$ TC-1 cells on day 0. On day 6, mice were then s.c. injected with the formulations mentioned above. Tumor size was monitored as aforementioned. For both prevention and treatment studies, two separate experiments (n = 4 in each, n = 8 total) were carried out due to the difficulty in maintaining the TC-1 cells at the same condition in the entire injection period if too many mice were to be injected with the tumor cells.

### IFN-γ Release from Splenocytes

Mice (n = 2) were immunized as mentioned above on days 0 and 9. On day 13, mice were sacrificed and the splenocytes ($5 \times 10^5$ cells in 250 μl, n = 3) were stimulated with 1 μg/ml of E7 peptide for 24 h. The cells were then spun down, and the IFN-γ level in the supernatant was assayed using a mouse IFN-γ ELISA kit from Pierce (Rockford, IL, USA). Similar experiments were carried out twice.

### Statistical Analyses

Except where mentioned, statistical analyses were completed by performing one-way analysis of variance (ANOVA) followed by pair-wise comparisons with Fisher's protected least significant difference procedure (PLSD). The tumor growth and regression curves were analyzed using GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA, USA). A p value of less than or equal to 0.05 was considered to be significant.

## RESULTS AND DISCUSSION

Previously, we have reported the application of LPD as a delivery system for peptide epitope (9). When a MHC class I–restricted E7 peptide was incorporated in the LPD, immunization of mice (C57BL/6) with the LPD led to promising prophylactic and therapeutic effects against a mouse tumor model induced by TC-1 cells. As part of our efforts to further optimize and improve this LPD-based peptide vaccine delivery system, mannan was coated on the LPD to enhance the uptake of LPD by APCs, and thereby to further enhance the resulting immunity. Enhanced immunity will help us to balance the effectiveness and toxicity of the LPD in future studies.

### Verification of the Specific Protein Binding Ability of Mannan in the LPD Formulation

Mannan was coated on the LPD simply by taking advantage of the cholesterol group conjugated to the mannan (18,22). Therefore, after prolonged co-incubation, mannan could be deposited on the surface of the LPD by inserting itself via the hydrophobic cholesterol group. Con A is a tetrameric protein that binds specifically to mannose and glucose in their reduced form. Methyl α-D-mannopyranoside (α-MM) and methyl α-D-glucopyranoside (α-MG) are two competitive inhibitors. Because of its polymeric nature, binding of mannan with Con A can cause the formation of aggregates, which can be quantified by the turbidity increase (24). Figure 1A clearly showed that Con A bound to the mannan-coated LPD, but not mannan-free LPD. The fact that preincubation of Con A with methyl α-D-glucopyranoside (α-MG), but not D-galactose, inhibited the turbidity increase showed that the binding was specific. The slight increase in the OD 360 from the mannan-free LPD is thought to be due to some nonspe-

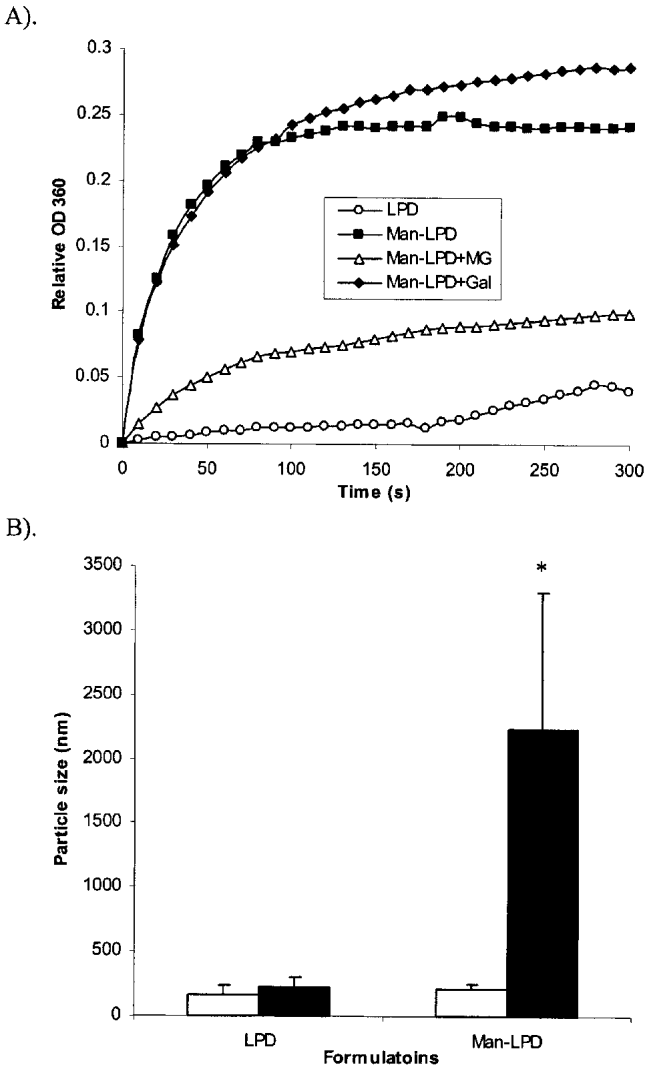


**Fig. 1.** Con A agglutination assay. (A) Relative turbidity (OD360 nm) increase as a function of time when LPD, coated or uncoated with mannan, were mixed with Con A. For the mannan-coated LPD (Man-LPD), Con A was also preincubated with either methyl α-glycopyranoside (Man-LPD+MG) or galactose (Man-LPD+Gal) prior to being mixed with Man-LPD. (B) Particle size of LPD before (white bars) and after (black bars) being mixed with Con A for 300 s. Data reported are mean ± SD (n = 3). *Indicates that the size of Man-LPD increased significantly after co-incubation with Con A.

cific binding between Con A and the highly positively charged LPD. To further confirm the binding of Con A to the mannan-coated LPD, the size of LPD after mixing with Con A for 300 s was measured. Although the size of the mannan-free LPD (166 ± 67 nm) was not significantly changed, large aggregates (2–4 μm) were formed in the mannan-coated LPD samples, strongly indicating the binding of mannan-LPD with Con A (Fig. 1B). In fact, after about 2 h of co-incubation, large visible precipitates were observed in the Con A/mannan-coated LPD mixture. Finally, the zeta potentials of mannan-free LPD and mannan-coated LPD were +28.9 ± 0.8 mV and +21.0 ± 0.4 mV, respectively. This is understandable because for the mannan-coated LPD, the cationic charge of LPD should be partially shielded by mannan (25).

### *In vitro* and *in vivo* Uptake of LPD by Antigen-Presenting Cells

Human and murine DCs and macrophages have mannose receptors on their surface (26). Several previous reports have used mannose as a ligand to target proteins, liposomes, or other particles to DCs and macrophages (18,22). As shown in Fig. 2A, the DC2.4 cells took up significantly more mannan-coated LPD than mannan-free LPD. The uptake of LPD

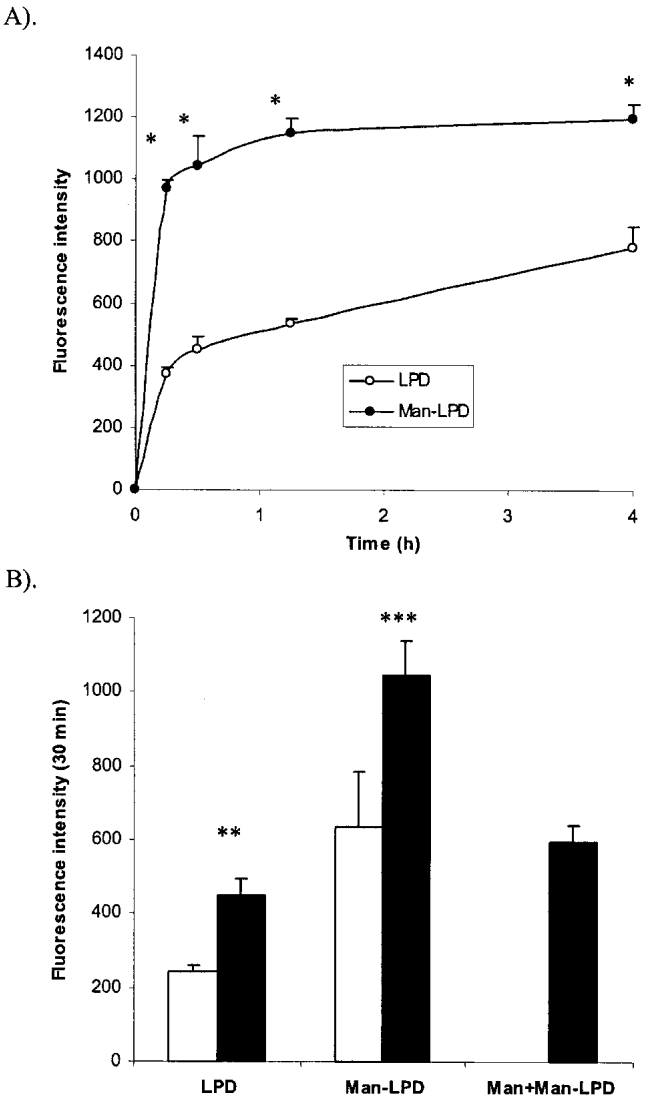


**Fig. 2.** Binding and uptake of LPD by DC2.4 cells. (A) Uptake kinetics of FITC-labeled LPD by DC2.4 cells. Cells ($1.0 \times 10^6$) were incubated with LPD, coated or uncoated with mannan. At various time points, the incubation was stopped, and fluorescence intensities associated with the cells were measured. *Indicates the fluorescence intensities of Man-LPD are significantly different from that of the LPD at 0.25, 0.5, 1.25, and 4 h. (B) Binding and uptake of LPD and mannan-coated LPD by DC2.4 cells after 30 min incubation at either 4°C (white bars) or 37°C (black bars). For Man+Man-LPD, the cells were preincubated with 50 μg of free Chol-Man 30 min prior to the addition of mannan-coated LPD. Data reported are mean ± SD (n = 3). **Indicates the result of LPD incubated at 37°C is significantly different from that incubated at 4°C. ***Indicates the result of Man-LPD incubated at 37°C is significantly different from that incubated at 4°C and significantly different from that of Man+Man-LPD.

by DC2.4 cells is via endocytosis (Han and Huang, unpublished data). Indeed, the fluorescence intensity associated with the DC2.4 cells was significantly higher at 37°C than at 4°C (Fig. 2B). Moreover, the fact that preincubating the DC2.4 cells with free Chol-Man in aqueous dispersion significantly blocked the uptake of the mannan-coated LPD by the DC2.4 cells indicates that the uptake was a receptor-mediated process (Fig. 2B).

Table I shows the uptake of Cy5-labeled LPD, coated or uncoated with mannan, by lymphocytes in the popliteal lymph nodes after the LPD was subcutaneously injected into the footpads. About 20% of the lymphocytes from the mannan-free LPD injected mice were LPD positive, comparing to about 15% from the mannan-coated LPD injected mice. Besides the integrity of the lymphatic tissue, it is reported that both particle size and the surface characteristics of particles influence the rate of particle drainage from the subcutaneous injection site into local draining lymph nodes (27). Therefore, the differences in the physical characteristics, both particle size and zeta potential, between the two different LPDs might be accountable for the difference observed in the present study. The sizes of the mannan-free LPD and mannan-coated LPD were 166 ± 67 nm (polydispersity index, 0.105) and 210 ± 45 nm (PI, 0.057), respectively. Also, as shown above, the zeta potential of the mannan-free LPD (+28.9 ± 0.8 mV) was more positive than that of the mannan-coated LPD (+21.0 ± 0.4 mV). More importantly, the percentage of $Cy5^+$ lymphocytes that were macrophages and DCs was 1.5- and 2-fold higher in mice injected with mannan-coated LPD than in those injected with mannnan-free LPD (Table I), clearly demonstrating the feasibility of using mannan as a ligand to target the LPD to APCs.

### Expression of Co-stimulatory Molecules on DC2.4 Cells After Stimulation by LPD

The above study on lymphocyte uptake of LPD clearly demonstrated that mannan-coated LPD were preferentially taken up by APCs. However, uptake of peptides by APCs alone is not enough to initiate a CTL response. In fact, some co-stimulatory molecules such as CD80 and CD86 as secondary signal are also required for successful presentation of the peptide to T cells by APCs (28). Therefore, the ability of the LPD to stimulate the expression of the co-stimulatory molecules on the surface of DC2.4 cells was studied. As shown in Table II, incubation of Chol-Man alone with DC2.4 cells resulted in CD80 and CD86 levels comparable to that of the background levels. However, incubation with LPD led to significantly enhanced CD80 and CD86 levels on the surface of DC2.4 cells. The same results were observed on isolated mouse primary DC cells (Han *et al.*, unpublished data). Moreover, this enhancement was further enhanced by coating mannan on the LPD. This observation, in combination with the known fact that LPD can successfully promote peptide antigen presentation by the MHC class-1 molecule (9), suggests that mannan-coated LPD should be superior to mannan-free LPD in inducing anti-tumor activity.

**Table I.** Uptake of LPD by Cells in Popliteal Lymph Nodes After Subcutaneous Injection Into Footpad

| | Marker | LPD | Man-LPD |
|---|---|---|---|
| % Cells that are $Cy5^+$ | $Cy5^+$ | 20.1 | 15.3 |
| % $Cy5^+$ cells that are macrophage | $Cy5^+$, $CD11b^+$ | 6.8 | 10.2 |
| % $Cy5^+$ cells that are dendritic cell | $Cy5^+$, $CD11c^+$ | 2.5 | 5.1 |

Data were from flow cytometry analyses. Man-LPD, mannan-coated LPD. LPD, liposome-protamine-DNA.

**Table II.** Expression of CD80 and CD86 on the Surface of DC2.4 Cells After Stimulation

| | CD80 (%) | CD86 (%) |
|---|---|---|
| Negative control | 21 ± 3 | 19 ± 4 |
| Chol-Man | 24 ± 4 | 25 ± 5 |
| LPD | 62 ± 1[a] | 39 ± 1[a] |
| Man-LPD | 76 ± 5[a,b] | 48 ± 2[a,b] |

Values are shown as the percent of CD80/CD86 positive cells. Data reported are mean ± SD. LPD, liposome-protamine-DNA; Man-LPD, mannan-coated LPD.

[a] The values from LPD and Man-LPD are significantly different from that of the negative control.

[b] The values from LPD are different from that from Man-LPD.

### Prophylactic and Therapeutic Applications of Mannan-Coated LPD

The kinetics of the formation of tumors in mice immunized with mannan-free or mannan-coated LPD/E7 is shown in Fig. 3. Apparently, the mannan-coated LPD was superior to the mannan-free LPD in terms of preventing tumor formation. Of the mice immunized with mannan-coated LPD/E7, 1 out of 8 mice developed tumors when challenged with

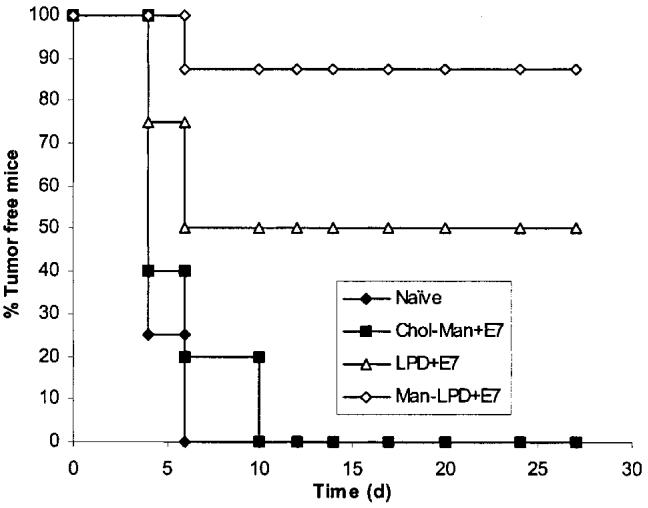


**Fig. 3.** Tumor formation kinetics in immunized mice subcutaneously challenged with TC-1 cells. Mice (n = 8) were subcutaneously injected with LPD+E7 (Δ), mannan-coated LPD+E7 (Man-LPD+E7) (◇), Chol-Man+E7 (■), or 5% dextrose alone (Naive) (◆) on days 0 and 9. The E7 dose was 10 μg/mouse. The corresponding liposome dose injected was about 14 μl/mouse. On day 13, the mice were s.c. challenged with TC-1 cells ($0.5 \times 10^6$). Tumor formation was monitored 2 or 3 times a week. Twenty-seven days after the injection of TC-1 cells, the size of the tumor on the single mouse (1 out 8) treated with mannan-coated LPD/E7 was 124 $mm^2$, whereas the tumor size on mice treated with mannan-free LPD/E7 was 307 ± 64 $mm^2$.

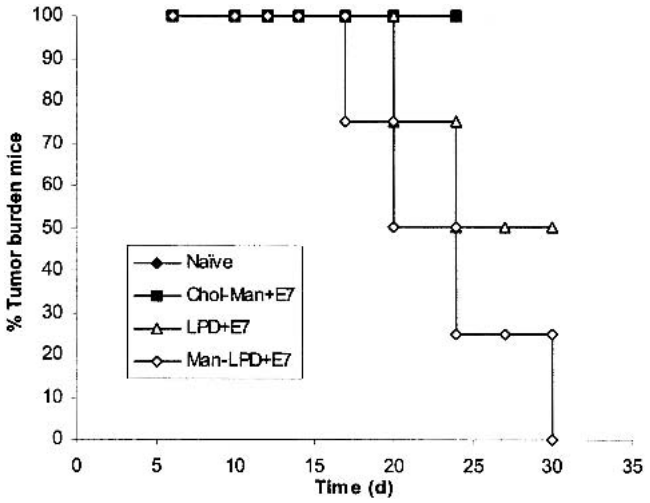


**Fig. 4.** Eradication of established tumors by immunization with LPD/E7, coated or uncoated with mannan. Subcutaneous tumors were established by injecting $0.5 \times 10^6$ TC-1 cells on day 0 to mice (n = 8). On day 6, mice were then subcutaneously injected with LPD+E7 (Δ), mannan-coated LPD+E7 (Man-LPD+E7) (◇), Chol-Man+E7 (■), or 5% dextrose alone (Naive) (◆). The E7 dose was 10 μg/mouse. Naïve mice and Chol-Man+E7 treated mice were sacrificed on day 25 due to the large tumor they developed. Statistic analysis showed that Man-LPD+E7 is different from LPD+E7 ($p = 0.0425$).

TC-1 cells 13 days after the initial immunization. However, for the mannan-free LPD/E7 immunized mice, 50% developed tumors during the same period. Moreover, 27 days after the injection of TC-1 cells, of those mice that developed tumors, the tumor in mice treated with mannan-coated LPD/E7 (~124 $mm^2$) was smaller than those in mice treated with mannan-free LPD/E7 (307 ± 64 $mm^2$).

Figure 4 shows the results from a tumor treatment study. As expected, tumors on the naïve mice and Chol-Man/E7 treated mice grew rapidly. In contrast, in the LPD/E7 (coated or uncoated with mannan) immunized mice, some of the tumors started to regress at various time points. Thirty days after the injection of tumor cells (24 days after the single immunization), all of the mannan-coated LPD/E7 immunized mice were free of tumor, and only 50% of the mannan-free LPD/E7 immunized mice were free of tumor. Statistic analysis showed that the tumor regression curves for mice immunized with mannan-coated LPD/E7 and mannan-free LPD/E7 are different ($p = 0.0425$). In conclusion, these animal studies demonstrated that coating of mannan on the surface of LPD/E7 helped to improve the antitumor activity of LPD/E7.

In our previous report, the E7 dose injected per mouse was 20 μg for tumor prevention and 10 μg for tumor treatment. The corresponding liposome and DNA doses were 21.5 μl and 25 μg per mouse, respectively (9). In addition, in previous tumor treatment study, mice were injected with LPD/E7 twice 3 and 6 days after the inoculation of tumor cells (9). In the current studies, the dose of E7 peptide was 10 μg in both prevention and treatment studies. The dose of liposome and DNA were two thirds of that used by Dileo *et al.* Also, in the current treatment study, mice were injected only once with the reduced dose 6 days after tumor cell inoculation. The dosage and dosage schedule were adjusted to clearly show the advantage of coating with mannan. Also, the adjustment is in line with our effort to balance the effectiveness and the potential toxicity of the LPD particles.

Perhaps the enhanced cellular immunity, as indicated by the significantly enhanced Th1 type cytokine (IFN-γ) release, was somehow responsible for the enhanced antitumor activity seen in mice treated with mannan-coated LPD/E7 (Fig. 5). The enhanced IFN-γ release from splenocytes isolated from mannan-coated LPD immunized mice agrees well with other reports. Toda *et al.* showed that mannan-coated liposome mediated DNA vaccination also enhanced the Th1-mediated immunity (22). More importantly, they found that *in vivo* injection of anti–IFN-γ antibody greatly inhibited the CTL response from a mannan-coated liposome mediated HIV vaccine (22). The authors concluded that the activation of macrophages and other APCs through the Th1 cytokine system is an important feature of the mannan-mediated immune response.

Nevertheless, results from the tumor prevention and treatment studies clearly demonstrate the benefit of coating the LPD with mannan. Besides the possible role played by the enhanced Th1 cytokine such as IFN-γ, we speculate that the enhanced uptake of mannan-coated LPD by the lymphocytes, especially by APCs such as DCs and macrophages, in local draining lymph nodes contributed greatly to the enhanced activity. As mentioned above, LPD can successfully present peptide antigen to the MHC class I molecule in APCs and can stimulate the expression of co-stimulatory molecules. Therefore, targeting of more LPD particles to the lymphatic DCs

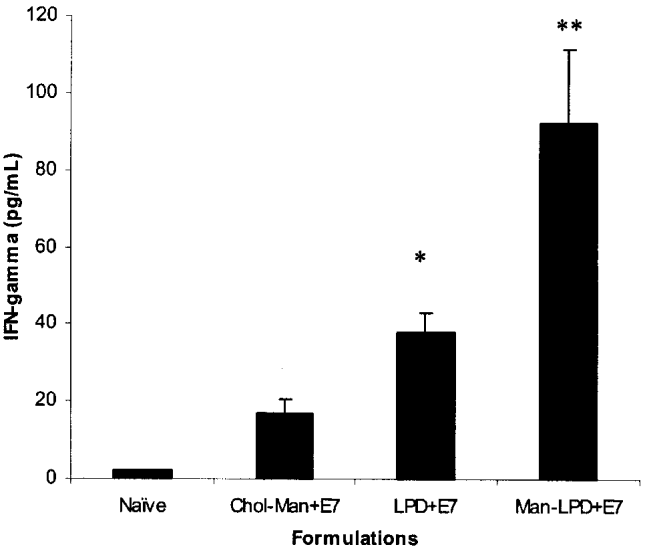


**Fig. 5.** IFN-γ release from isolated splenocytes after *in vitro* stimulation with E7 peptide. Mice (n = 2) were immunized with LPD+E7, mannan-coated LPD+E7 (Man-LPD+E7), Chol-Man+E7, or 5% dextrose alone (Naïve) on days 0 and 9 (10 μg of E7 per mouse). On day 13, spleens were removed and pooled. Splenocytes were prepared and stimulated ($5 \times 10^5$ splenocytes) with E7 peptide for 24 h. IFN-γ released into the medium was measured using ELISA. One representative of two experiments showing similar results is shown here. Data reported are mean ± SD (n = 3). *Indicates that the IFN-γ level from splenocytes isolated from mice immunized with LPD+E7 is significantly different from that of the control. **Indicates that the IFN-γ level from splenocytes isolated from mice immunized with Man-LPD+E7 is significantly different from that from splenocytes isolated from mice immunized with LPD+E7.

and macrophages by coating with mannan is expected to enhance the resulting immune responses. It also could be possible that the mouse immune system considered mannan as a "danger" signal and started a stronger innate immune response against the mannan-coated than the mannan-free LPD/E7.

Mechanistically, more work still needs to be carried out to elucidate how the E7-carrying LPD initiated the strong antitumor immunity. Both the cationic liposomes and the bacterial plasmid DNA may be responsible. It has been shown that cationic vesicles were taken up efficiently by macrophages and DCs and that potent *in vivo* CTL and humoral immune responses were elicited by cationic vesicles, compared to anionic vesicles (29). Bacterial DNA has proven to be a potent immunostimulatory molecule (30). A combination of bacterial DNA and cationic liposomes is therefore expected to significantly boost the resulting immune response against the antigen. Lipoplex, a complex formed by mixing cationic liposomes with plasmid DNA, is one of the systems that combined cationic liposomes and DNA. LPD, however, is different from lipoplex. Studies have shown that LPD is a virus-like structure with the protamine condensed DNA located inside the lipid membrane (6). LPD can be lyophilized, stored for extended periods, re-hydrated, and used without any loss of efficacy (31). The fact that coating of mannan on the LPD led to enhanced antitumor activity demonstrates the potential to further improve the immunostimulation activity of LPD and to balance its immunostimulation activity with its toxicity. More investigations are warranted.

Finally, E7 peptide, a MHC class I–restricted epitope from HPV 16 E7 protein, was used as antigen in the current study. It should be mentioned that this delivery system should be applicable for other tumor-associated antigens such as prostate cancer and various her2/neu-expressing cancers. In addition, LPD may also be used as antigen delivery system for other intracellular pathogens, such as HIV and the causative agents for tuberculosis and malaria, for which effective vaccines are greatly needed.

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