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ORIGINAL ARTICLE

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Liposome-polycation-DNA (LPD) particle as a carrier and adjuvant for protein-based vaccines: Therapeutic effect against cervical cancer

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Abstract With the successful identification of many tumor-specific antigens, tumor-associated antigens, and the potential of using unfractioned tumor cell derivatives as tumor antigens, a system and/or adjuvant that can deliver these antigens and help them to induce strong and effective anti-tumor immune responses is greatly needed. Previously, we reported that a MHC class I-restricted peptide epitope derived from human papillomavirus (HPV) 16 E7 protein, when incorporated into a clinically proven safe LPD (liposome-polycation-DNA) particle, was able to effectively eradicate tumors established in mice. Cervical cancer is the second most common cancer among women worldwide. HPV infection is clearly linked to this cancer. Vaccines based on the early (E) gene products of HPV could be effective in controlling it. However, besides the fact that epitope vaccines have many limitations particularly, concerning the diverse HLAs in humans, the use of the epitope as an antigen prevented us from fully characterizing the immune responses induced by the LPD as a vaccine carrier and/or adjuvant in previous studies. In the present study, by using the HPV 16 E7 protein as an antigen, we first showed that LPD, as a vaccine carrier and adjuvant induced strong and robust immune responses, both cellular and antibody. We then showed that immunization with LPD particles incorporated with either the wild type HPV 16 E7 protein or a potentially safer mutant induced strong immune responses that caused complete regressions of a model cervical cancer tumor established in murines. LPD could be a potent vaccine carrier and/or adjuvant for many antigens.

Keywords Point mutation · Antibody · CTL · Tumor treatment · Adjuvant · Vaccine delivery

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Introduction

After decades of debate over whether the immune system can actually fight tumors, growing and compelling evidence now suggests that the immune cells play an important role in the control of malignancy [1–3]. However, there still are many major hurdles in developing cancer vaccines, including the identification of antigens that induce immune responses specifically targeting tumor cells without harming normal cells and the development of methods that induce immune responses sufficient to eradicate tumors [4]. Over the last decade, numerous tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) recognized by tumor-reactive CTLs have been identified [5, 6]. Unfractionated tumor derivatives, such as total tumor lysates, peptides, or proteins, may also be used as tumor antigens. Studies have shown that immunization by reintroducing dendritic cells (DCs) pulsed with tumor antigens or total tumor derivatives into patients led to strong anti-tumor activities [7–18]. However, ex vivo vaccination with autologous patient DCs is not preferred because it is costly, time-consuming, and very inconvenient to patients. Novel delivery systems and/or adjuvants that allow direct injection of tumor antigens to induce anti-tumor immune responses are needed.

Cervical cancer is the second most common cancer among women worldwide. About 45% of cervical cancer patients die as a result of distant metastasis [19]. Over the last 20 years, the relationship between HPV infection and cervical cancer has been extensively investigated.

More than 95% of cervical cancer cells contain HPV DNA, with HPV 16 being the most common type, accounting for about 50% of all cases [20]. HPV contains a double stranded DNA genome, which can be separated into three regions: the early genes (E), the late genes (L), and the non-coding upstream regulatory region (URR). L genes encode structural proteins such as the major capsid protein L1 and the minor capsid protein L2. The major transforming proteins are coded by the E genes such as E6 and E7; their expression induces cell immortalization and transformation in many cell types [21, 22]. E6 and E7 of the "high risk" viruses bind to p53 and pRB, respectively, and inhibit their tumor suppressor functions [23, 24]. For example, E7 targets pRB family proteins for ubiquitin-mediated proteolysis that leads to decreased pRB level [24]. Also, E7 protein has a strong affinity to pRB protein. Binding of E7 to pRB releases E2F factor. This eventually causes the cell growth to lose control [24, 25].

Vaccination has been suggested as an effective approach to prevent and treat cervical cancers caused by HPV. The recently developed HPV virus-like particles (VLP) by over expression of L1 alone or co expression of L1 and L2 have demonstrated a great potential as a prophylactic vaccine [26–29]. However, for therapeutic purpose, a HPV capsid protein-based vaccine is unlikely to have any significant benefit because these proteins are not detectable in the basal epithelial cells that developed into tumors. E6 and E7 proteins are expressed in almost all the cervical cancer cells and their precursors [30, 31]. They are excellent cervical cancer-specific antigens that may be developed into therapeutic vaccines. In fact, many animal studies have shown that immunization with either E7 protein, peptide epitopes derived from E7 protein, or E7 gene carried on a plasmid or a viral vector caused regression of experimentally grafted E7-expressing tumors [30, 32–47], if an appropriate adjuvant was used. Several clinical trials have also been completed [24, 48–52]. For example, in a phase I trial, 18 women with high grade cervical or vulvar intraepithelial neoplasia (CIN) and positive HPV-16 were given a HLA-A2-restricted peptide ($E7_{12-20}$) in Montanide ISA-51 (Seppic, Inc), which has been used many in clinical trials [52–64]. DC infiltrate was observed in 6 out of 6 patients. CTL response was observed in 10 out of 16 patients. Also, 3 out of 18 patients cleared their dysplasia after vaccination [50].

Previously, a liposome-based DNA delivery system named LPD, was developed by us [65, 66]. 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 inside the lipid membrane [65, 66]. When administered systemically, the LPD rapidly initiates the production of several T helper type 1 (Th1) cytokines, most notably TNF-α, IL-12, and IFN-γ [67, 68]. This non-specific immunostimulation is associated with tumoristatic effects [67, 68]. More recently, we have found that when a MHC class I-restricted peptide epitope ($E7_{49-57}$) derived from the HPV 16 E7 protein was incorporated into the LPD and then used to immunize mice, strong anti-tumor responses, including CTL responses specific to $E7_{49-57}$, were observed [69]. Administration of LPD/$E7_{49-57}$ to tumor-bearing mice caused tumor regressions [69].

Although immunization with the $E7_{49-57}$ peptide epitope resulted in a potent anti-tumor activity, immunization with the whole E7 protein might be more effective because there are multiple MHC class I and II restricted epitopes on the E7 protein. The existence of highly diverse MHC I and II molecules among human populations also makes the whole E7 protein more attractive [70]. In addition, the E7 protein as an antigen will allow us to further characterize the immune responses induced by the LPD as a vaccine carrier and/or adjuvant. In the present study, using the E7 protein as an antigen, we have shown that the LPD as an antigen carrier induced very strong and robust CTL and antibody responses. The immune responses are sufficient to treat tumors pre-established in mice.

Materials and methods

Materials

Plasmid pET-E7 was a gift from Dr. Jeong-Im Sin at the Catholic University of Korea (Seoul, Korea). Dioleoyl trimethylammonium propane (DOTAP) and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Protamine sulfate (fraction X from salmon) was purchased from Sigma-Aldrich (St. Louis, MO). Plasmid (pNGVL3) containing the CMV promoter without coding insert was obtained from the National Gene Vector Laboratory (Ann Arbor, MI). It was purified using Qiagen EndoFree Giga-Prep kit (Qiagen, Valencia, CA). TC-1 cells were obtained from Dr. T. C. Wu of the Johns Hopkins University. The cells were C57BL/6 mouse lung endothelial cells transformed with HPV 16 E6 and E7 oncogenes and activated H-ras gene. Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin (Sigma-Aldrich, St. Louis, MO), and 100 μg/ml of streptomycin (Sigma).

Construction of mutants on the E7 gene

It has been shown that change of amino acid D21 into G21 in HPV 16 E7 protein dramatically decreased its tumor transformation ability, and that replacement of G22 of a 'low risk' HPV type 6 E7 protein by D22 increased its tumor transformation ability to a level comparable to that of "high risk" HPV type 16 E7 protein [71, 72]. Furthermore, in the C terminal of E7, there are two CXXC motifs, which form a zinc finger structure. Disruption of this structure by replacing one of the C residues with a G has been shown to severely

reduce the cell immortalization ability of E7 [73, 74]. Overlapping PCR was used to introduce mutations in the E7 protein. Amino acids D21 and C94 on HPV 16 E7 protein were changed to G21 and G94 by changing their codons from GAT to GGT and TGT to GGT, respectively. The four primers used were P1, 5-TTGGGATCCACCATGCATGGAGATACACCTAC-3; P2, 5-CGGAATTCATTCTTATGGTTTCTGAGAACCGATGGGGCACACA-3; P3, 5-GAGACAACTGGTCTCTACTGTTAT-3; and P4, 5-ACAGTAGAGACCAGTTGTCTCTGG-3. Using pET-E7 as the template, two separate PCRs were completed using primer pairs P1/P4 and P2/P3. The PCR products were purified using QiaQuick PCR purification kit (Valencia, CA). Another PCR was completed using P1 and P2 as primers and molar equivalents of the products from the previous two PCRs as templates. The PCR conditions were 94°C for 5 min followed by 35 cycles of 94°C, 0.5 min, 56°C, 1 min, and 72°C, 0.5 min. Another 5 min of incubation at 72°C was included prior to the end of the PCR reaction. Taq DNA polymerase and dNTP were obtained from Promega (Madison, WI). After purification, the PCR product was then ligated to the pGEM-T vector from Promega. The ligation reaction was then transferred into *E. coli* DH5α strain. Positive colonies were selected using LB/ampicillin/IPTG/X-Gal plates. After confirmation of mutations on the E7 gene of the pGEM-T-E7m by DNA sequencing, the plasmid was digested with *Bam*HI and *Eco*RI. The resulting DNA fragment was gel purified and then cloned into the *Bam*HI and *Eco*RI site of pCDNA3.1(+) vector (Invitrogen) and pET vector (Novagen, Madison, WI), respectively. The plasmid constructs were transferred into *E. coli* DH5α and selected against ampicillin and kanamycin, respectively .

Purification of recombinant E7 and E7m proteins

His-tagged recombinant E7 and E7m proteins were purified as previously described [75]. To purify E7 and E7m proteins, the pET-E7 and pET-E7m plasmids were purified and transformed into BL21 (DE3) cells. A single colony was seeded into LB broth supplemented with kanamycin at a final concentration of 50 μg/ml. The cells were incubated at 37°C shaker until the absorbance at 600 nm was between 0.6 and 0.8. Protein production was induced using 0.5 mM of isopropy-1-thio-β-D-galactopyranoside for 4 h. The cell pellets were collected by centrifugation at 8,000 g for 20 min, frozen-thawed once at −20°C, and then re-suspended in 8 M urea buffer (pH 8.0) (5 ml per 1 mg of wet cell pellet). The cells were lysed by stirring at 37°C for 1 h. The lysate was centrifuged at 10,000 g for 30 min at room temperature. The supernatant was collected and mixed with 50% Ni-NTA slurry at a ratio of 4:1. The mixture was gently shaken for 1 h at room temperature. The lysate-resin mixture was then loaded into an empty column provided by Qiagen, and washed with 5 vol of Buffer B (8 M urea, pH 8.0), followed by 10 vol of Buffer C (8 M urea, pH 6.3). His-tagged proteins were eluted with 10 ml of Buffer C containing 200 mM imidazole. The protein solution was then dialyzed in 6 M urea buffer for 2 h followed by two more hours in 4 M urea buffer. After overnight dialysis in phosphate buffered saline (pH 7.0), the protein solution was collected and passed through a Detoxi-Gel endotoxin removing gel column from Pierce (Rockford, IL). The protein concentration was quantified using Coomassie Plus protein assay reagent (Pierce). The protein purity was confirmed by electrophoresis on a BioRad Ready Gel (4–20% SDS polyacrylamide gel) (Hercules, CA). Endotoxin was determined to be <100 EU/mg protein using the Limulus Amebocyte Lysate (LAL) pyrochrome kit from the Associates of Cape Cod Incorporated (Falmouth, MA). Similar to previous reports, the recombinant E7 protein migrated as a 23 KDa protein on SDS-PAGE gel [28]. The yield was roughly 1 mg/l of bacterial culture.

Verification of E7m protein's inability to activate E2F driven genes

An indirect method was used to verify that the E7m is unable to activate E2F driven genes. E7 and E7m genes were inserted into the *Bam*HI and *Eco*RI sites of pCDNA3.1(+) vector. The resulting plasmids were amplified in *E. coli* DH5α strain and purified. Plasmid cdc25A *Sac* I-luc, in which luciferase gene is driven by a 1173 (−755 to + 418) bp *Sac* I fragment of the cdc25A promoter, is a gift from Dr. D. DiMaio of Yale University [46]. The *Sac* I fragment has the E2F binding site. Plasmid cdc25A *Sac* I-luc, pCDNA3.1(+) with or without E7 or E7m insert, and a CMV driven β-galactosidase gene containing plasmid were co-transfected into confluent 293 cells (5×10^5 /well, incubated at 37°C and 5% CO_2 overnight, DMEM medium with 10% FBS) with Lipofectamine (Invitrogen). The plasmid ratio was 2:1:10 (w/w/w) with pCDNA3.1(+) plasmid at 1 μg/well. Four hours after the addition of the plasmids, the medium was replaced with a fresh medium. After another 44 h, cells were washed with cold PBS (10 mM, pH 7.4) twice and lysed. Luciferase activity, β-galactosidase expression, and total protein amount were then determined. The same experiment was repeated three times.

Preparation of liposome and LPD

Liposomes and LPD were prepared as previously described [69]. Briefly, small unilamellar liposomes composed of DOTAP: cholesterol (molar ratio 1:1) were prepared by thin film hydration followed by membrane extrusion. DOTAP concentration was fixed at 10 mg/ml. The LPD was comprised of DOTAP/cholesterol liposomes, protamine, and plasmid DNA in a ratio of 9.0:0.6:1.0 (w/w/w). To prepare LPD, required amounts

of liposome (43 μl) and protamine (15 μl, 2 mg/ml) were dispersed in 150 μl of aqueous solution containing 10% of dextrose (Sigma). Then, 150 μl aqueous solution containing pDNA (50 μg) with E7 protein or E7m protein (40 μg) was added drop wise into the mixture of liposome and protamine with gentle shaking. The complex was then allowed to stay at room temperature for at least 20 min prior to use. The incorporation efficiency of the protein was estimated by SDS-PAGE gel electrophoresis to be around 60%.

The particle size of the protein incorporated LPD particles were measured using a Coulter N4 Plus particle sizer (Beckman Coulter, San Francisco, CA). The size of the E7 incorporated LPD was 191 ± 7 (polydispersity index, 0.001) nm; the size of the E7m incorporated LPD was 210 ± 32 (PI, 0.026) nm. They were not significantly different from each other.

Mouse tumor treatment study

Six-week-old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were used in all animal studies. National Institutes of Health guidelines for the care and use of laboratory animals were observed. Subcutaneous tumors were established by injecting 5×10^5 TC-1 cells in the flank on day 0. On specific day(s), mice were then subcutaneously injected with the E7 protein alone (20 μg/mouse), LPD/E7, or E7 adjuvanted with Alum (15 μg/mouse). One group of mice was left untreated. All formulations were in 150 μl of 5% dextrose to maintain isotonicity. The corresponding cationic liposome amount injected was about 140 μg. When Montanide ISA-51 was used, 50 μl (20 μg) E7m in 15% dextrose solution was mixed with 100 μl of Montanide ISA-51 (Seppic, Inc., France). The mixture is a very viscous white suspension. Tumor size was measured using a caliper and reported by multiplying the two largest dimensions of the tumor. Same experiment was repeated two or three times.

ELISA

Specific serum IgG levels against E7 and E7m proteins were determined using ELISA. Briefly, Costar high binding 96-well assay plates were coated with 100 μl (10 mM sodium phosphate buffer, pH 9.6) of E7 or E7m protein (1 μg/well) overnight at 4°C. The plates were then washed once with phosphate buffered saline (10 mM, pH 7.4) and blocked for 1 h at 37°C with 5% bovine serum albumin (BSA) (Sigma) solution (100 μl/well) made in sodium phosphate buffer as above. The plates were then washed five times with PBS/Tween 20 buffer. Mouse serum was diluted 10, 100, and 1000 times with 4% BSA, and 50 μl was added into the well and incubated for 2 h at 37°C. After washing five times with PBS/Tween 20 buffer, anti-mouse IgG HRP $F(ab')_2$ fragment from sheep (Southern Biotechnology Associates, Inc., Birmingham, AL) (diluted 1:5,000 in 1% BSA) was added (50 μl/well) and incubated for 1 h at 37°C. The plates were washed five times with PBS/Tween 20 buffer. Finally, the samples were developed with 100 μl TMB substrate (BioRad) for 30 min at room temperature and then stopped with 100 μl of 2% (w/v) oxalic acid. The OD of each well was measured using an Ultramark BioRad Microplate Imaging System at 405 nm.

CTL assay and cytokine release from splenocytes

CTL activity was measured using CytoTox 96 NonRadioactive Cytotoxicity Assay Kit (Promega, Madison, WI). Mice were immunized subcutaneously on days 0 and 14 as mentioned above. On day 28, they were sacrificed, and splenocytes were prepared and cultured in RPMI medium with 10% FBS, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, 40 U/ml IL-2, and 1 μg/ml a nine amino acid E7 peptide (aa 49–57, RAHYNIVTF) for 4 days. Effector cells were plated into 96-well plates at various effector/target (E:T) ratios. Targets used were EL4 cells pulsed with E7 peptide. Before being mixed with effectors, the targets were washed two times with the medium and re-suspended at 2×10^5 cells/ml. The lysis reaction was carried out for 4 h at 37°C, after which the plates were centrifuged, and 100 μl of medium was carefully removed from each well and assayed for lactate dehydrogenase activity. Specific lysis was calculated with the absorbance at 490 nm as suggested by the manufacturer.

Also, splenocytes (1×10^6 cells in 300 μl, $n=6$) were stimulated with 10 μg/mL of E7 protein for 48 h. The cells were spun down, and the IL-4 and IFN-γ level in the supernatant was measured using ELISA kits from Pierce (Rockford, IL).

Statistical analysis

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

Results

LPD/E7 induced strong cellular and antibody immune responses in mice

The use of a single MHC class I-restricted peptide epitope, $E7_{49\text{-}57}$, did not allow us to fully characterize the immune responses induced by the LPD as a vaccine

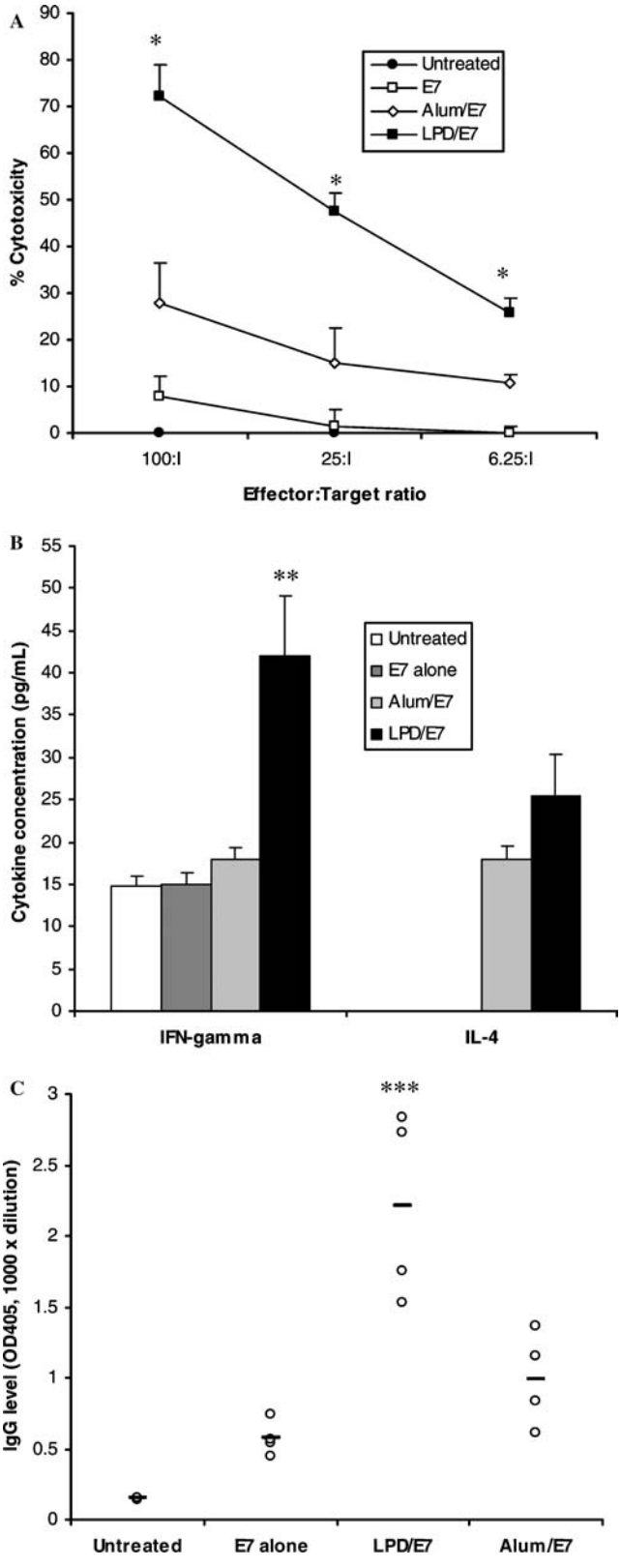

Fig. 1 LPD/E7 protein induced anti-E7 immune responses. Mice ($n=4$–5) were immunized with E7 protein alone (20 μg/mouse), LPD/E7 protein, or E7 protein adjuvanted with Alum (15 μg/mouse) (Alum/E7) on days 0 and 14. On day 28, they were bled via tail vein. Also, Spleens from mice in the same group were pooled, and splenocytes were prepared. All formulations were in 5% sterile dextrose. As a control, one group of mice was left untreated. **a** CTL response. *Asterisk* indicate that the values from every treatment are different from each other. Shown are mean ± S.D ($n=3$). The CTL result is a representative from three independent experiments. **b** Cytokine released from splenocytes after stimulation with E7 protein (10 μg/ml). Shown are concentrations averaged from six different readings (±S.D.). *Double asterisk* indicates the INF-γ from LPD/E7 immunized mice is significantly higher than that from the others. **c** Specific total IgG level in serum after diluted by 1,000-fold. A ANOVA analysis on the three treatments showed a P value of 0.001. *Triple asterisk* indicates that the value from LPD/E7 is significantly different from that of the others

carrier and/or adjuvant in previous studies. In the present study, purified HPV 16 E7 protein was incorporated into the LPD particles and used to immunize mice. E7 protein should allow us to characterize both CTL and antibody responses. As expected, a strong CTL response was induced when mice were immunized with the LPD/E7 protein (Fig. 1a). The significantly enhanced IFN-γ release,but not that of IL-4, from splenocytes isolated from the LPD/E7 protein immunized mice indicates that the immune response is biased toward Th1 (Fig 1b). Immunization with the LPD/E7 protein induced a strong E7-specific antibody response too (Fig. 1c). The E7 specific IgG level in the serum of mice immunized with LPD/E7 protein was significantly higher than that from mice immunized with E7 protein admixed with Alum (Fig. 1c, $P=0.017$). In all these studies, very weak responses were induced when mice were immunized with the E7 protein alone.

Treatment with LPD/E7 led to complete regressions of tumors in mice

Because the LPD/E7 protein induced strong CTL and T helper cell responses (Fig. 1a, b), we expected the immune responses have anti-tumor activity. To test this, TC-1 tumor cells were subcutaneously seeded into mice, 4 days later, the mice were treated with the LPD/E7 protein. As shown in Fig. 2, treatment of tumor-bearing mice with the LPD/E7 caused complete regression of the tumors. In contrast, E7 protein alone or E7 protein admixed with Alum did not induce inhibitory effects on tumor growth. On day 25, the size of tumors in E7/Alum or mice treated with E7 alone were similar to that of the untreated mice ($P=0.26$).

Construction of E7 mutant

Point mutations D21 to G21 and C94 to G94 were introduced into the E7 protein using overlapping PCR. DNA sequencing confirmed the mutations. It has been shown that replacing amino acid D21 on HPV 16 E7 gene protein by G21 deletes the cell transformation ability of the E7 protein [25, 72]. Also, disruption of the zinc finger structure in the C terminal of E7 protein by

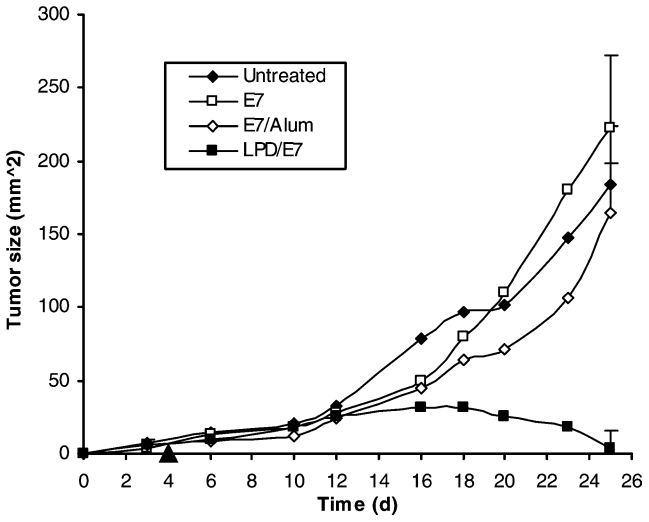

Fig. 2 Treatment with the LPD/E7 protein caused complete tumor regressions. Mice ($n=5$) were subcutaneously injected with TC-1 cells (5×10^5 /mouse) on day 0. On day 4, they were treated with E7 protein alone (20 μg/mouse), LPD/E7 protein, and E7 protein adjuvanted with Alum (15 μg/mouse) (Alum/E7). Shown is the tumor growth kinetics. On day 25, the tumor sizes in the LPD/E7 treated mice are significantly smaller than that from other treatments ($P<<0.05$). Standard deviations are only showed for day 25 for easy viewing. Only one representative of three independent experiments is shown. *Filled triangle* indicates the treatment time point

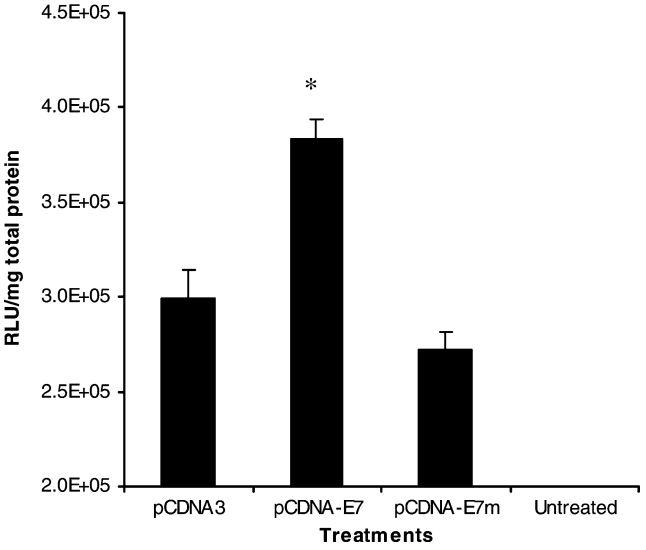

Fig. 3 Relative luciferase unit (RLU) in 293 cells transfected with a CMV driven β-galactosidase gene containing plasmid, cdc25A *Sac* I-luc, and pCDNA3(+) with or without E7/E7m gene (see Materials and Methods for details). *Asterisk* indicates that the value for pCDNA-E7 is different from that of the others ($P=0.002$). Also, the value for pCDNA3 is not different from that for pCDNA-E7m ($P=0.19$). Data reported are mean ± S.D. ($n=6$). Experiment was repeated three times. Shown is one representative

deleting one of the CXXC motifs significantly damages the oncogenic activity of E7 [73, 74]. Therefore, only an indirect method was used to confirm that the mutated E7 protein is unable to activate E2F-driven genes [24]. The wild type E7 protein can bind to pRB, causing the activation of E2F; whereas the mutant E7 protein created should not be able to do the same. When 293 cells were co-transfected with a luciferase gene driven by an E2F responsive element (cdc25A *Sac* I-luc) and an E7 protein encoding plasmid (pCDNA-E7), significantly higher luciferase expression was observed as compared with cdc25A *Sac* I-luc co-transfected with an empty plasmid (pCDNA3) ($P=0.02$) (Fig. 3). However, when pCDNA-E7m was co-transfected with cdc25A *Sac* I-luc, the resulting luciferase level was comparable to that of cdc25A *Sac* I-luc co-transfected with pCDNA3 ($P=0.19$). Taken together, the data were consistent with the notion that the mutations on E7 have removed its ability to bind to pRB and thus its oncogenic activity.

LPD incorporated with the mutant E7 protein induced immune responses comparable to that induced by LPD incorporated with the wild-type E7 protein

To investigate whether the mutations introduced into the E7 protein affect its ability to induce anti-tumor and antibody responses, both recombinant E7 and E7m proteins were purified, incorporated into LPD, and used to immunize mice. The resulting specific antibody level

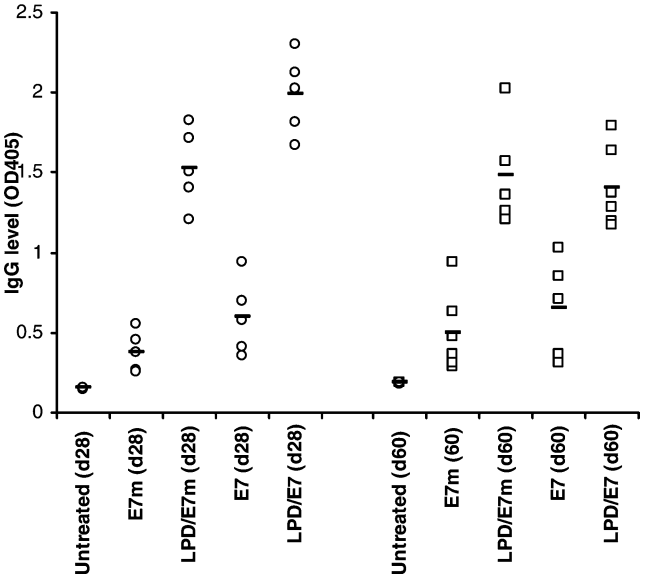

Fig. 4 Specific total IgG levels in mice immunized with E7m, LPD/E7m, E7, and LPD/E7. Mice ($n=5$–6) were immunized on days 0 and 14. Shown are IgG levels in serum on day 28 (*open circle*) and day 60 (*open square*). The IgG levels from E7 or E7m alone immunized mice were significantly lower than those from LPD/E7 or LPD/E7m immunized mice, respectively. On day 28, the value for LPD/E7m was lower than that for LPD/E7 ($P = 0.02$); on day 60, the values from these two treatments were similar ($P=0.53$). For LPD/E7m, the value from day 28 was similar to that for day 60 ($P=0.81$); for LPD/E7, these two values are different ($P=0.004$). The *dark lines* are the means ($n=5$–6)

and tumor therapy activity were compared. Shown in Fig. 4 are the specific IgG levels in serum on day 28 and day 60. Both LPD/E7 and LPD/E7m induced significantly higher IgG levels than E7 and E7m alone without LPD. Interestingly, on day 28, the IgG level from LPD/E7 was higher than that from LPD/E7m ($P=0.019$); whereas on day 60, the IgG level from LPD/E7 was comparable to that from LPD/E7m ($P=0.53$). This is due to the significantly decreased IgG level in LPD/E7 immunized mice on day 60 ($P=0.004$). It is possible that the antibody response from the mutated E7 protein lasted longer than that from the wild type E7 protein. The above data are the antibody levels when measured against the E7 protein. Similar results were obtained when they were measured against the E7m protein (data not shown).

Treatment of tumor bearing mice with LPD/E7m is as effective as with LPD/E7 in causing tumor regressions (Fig. 5). In addition, the LPD particles as an antigen carrier or adjuvant are more effective than the Montanide ISA-51 in treating tumors (Fig. 6). LPD/E7m treatment (once, 6 days after tumor inoculation) caused complete tumor regressions, whereas Montanide ISA-51/E7m did not induce significant anti-tumor activity (Fig. 6).

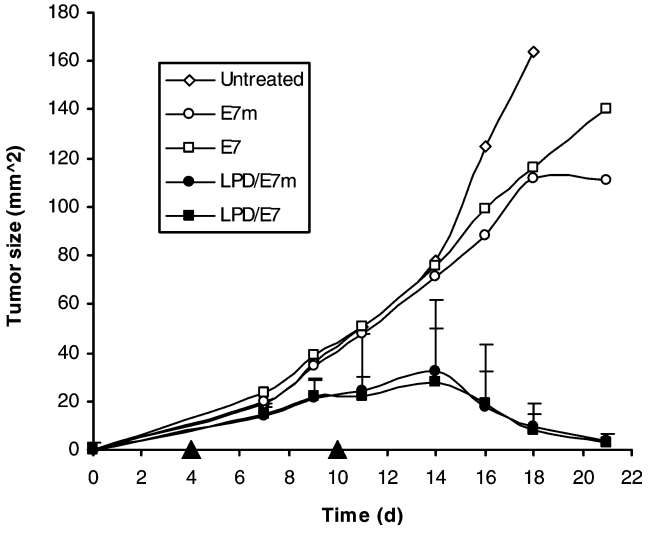

Fig. 5 LPD/E7m and LPD/E7 are equally effective in treating tumor. Mice ($n=5$ or 10, 10 for LPD/E7 and LPD/E7m) were injected with TC-1 cells (5×10^5 /mouse) on day 0. On days 4 and 10, they were treated with E7 or E7m alone, LPD/E7 or LPD/E7m. The protein dose was 20 μg/mouse. Tumor sizes were reported as a function of time. Statistical analysis showed that the values for LPD/E7m and LPD/E7 are not different (Prism). *Error bars* are standard deviation. One representative of two independent experiments is shown. *Filled triangles* indicate the treatment time points

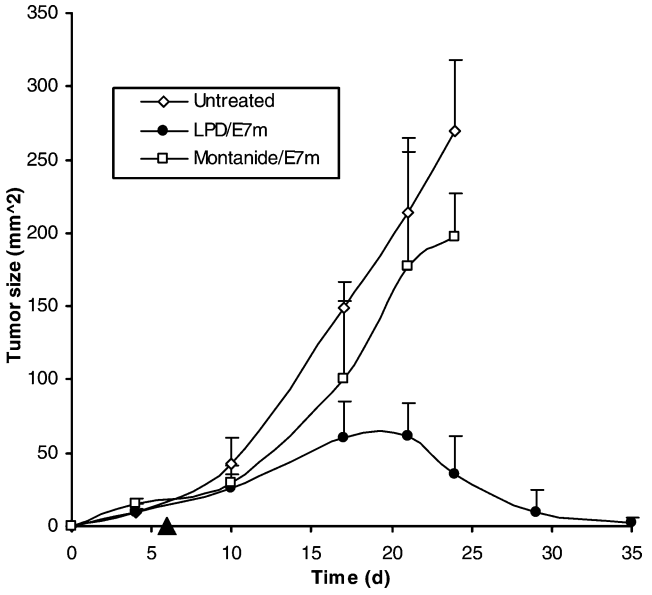

Fig. 6 LPD/E7m induced stronger anti-tumor response than E7m incorporated into Montanide ISA-51. Mice ($n=7$) were injected with TC-1 cells (5×10^5 /mouse) on day 0. On day 6, they were treated with LPD/E7m or E7m incorporated into Montanide ISA-51. The protein dose was 20 μg/mouse. Tumor sizes were reported as a function of time. *Error bars* are standard deviation. Shown is one representative of two independent experiments. The *filled triangle* indicates the treatment time point

Discussion

We have shown that LPD as a protein antigen carrier and adjuvant induced strong and robust immune responses to eradicate established tumors in a mouse model. In fact, the anti-tumor activity from LPD as an antigen carrier and adjuvant is even stronger than that from the Montanide ISA-51, one of the Montanide series that have been used in many previous clinical trials as a vaccine adjuvant [52–64]. Considering the good toxicity profile of LPD in previous clinical trials [76], LPD has a great potential as a vaccine carrier and adjuvant. The HPV 16 E7 protein was used as an antigen to test the adjuvanticity of LPD. Although antibody response and Th2 cytokine release, such as IL-4, may not be required for the killing of cervical cancer cells, we still measured these aspects of immune responses to thoroughly understand the resulting immune response. To our surprise, both strong CTL and potent antibody responses have been induced. Thus, LPD may be readily used to prepare vaccines against HIV or malaria, for which both neutralizing antibody and CTL responses are required.

Another objective of the present study is to test the feasibility of using the HPV 16 E7 protein-incorporated LPD (LPD/E7 protein) as a therapeutic vaccine to treat cervical cancer. In three different dosing schedules [day 4 only (Fig. 2), days 4 and 10 (Fig. 5), and day 6 only (Fig. 6)], treatment with LPDs incorporated with either the wild-type or the mutant E7 protein always led to complete tumor regression in the end. Thus, we believe that our LPD particles incorporated with cervical cancer specific antigens (E6 and E7 from the "high risk" HPV types) could be a potent vaccine for cervical cancer therapy.

Only second to breast cancer, cervical cancer is prevalent among women worldwide. Evidence clearly points to 'high risk' HPV viruses as the causal agent of cervical cancer. In the U.S., the widespread use of the 'Pap' smear screening has resulted in a 70% decline in mortality from cervical cancers in the last five decades [77]. Unfortunately, it was estimated that 50% of cervical cancer in the US occurred in previously screened patients. Also, women in developing countries do not have easy access to this screening program [78]. Therefore, vaccination seems to be the most promising approach. Due to the almost universal presence of E6 and E7 proteins in cervical cancer cells, successful immune responses raised against E6 and/or E7 protein should theoretically kill the cervical cancer cells. In fact, many vaccines have been constructed based on partial or full length E6 and/or E7 in the form of protein, peptide, or DNA [79]. Our LPD particles, incorporated with the E7 protein, induced very strong CTL and anti-tumor responses. The Th1 response as indicated by the high IFN-γproduction may also be helpful. It is believed that the $CD4^+$ T cell response is not required for tumor killing, but still beneficial [80]. In fact, our previous tumor prevention study showed that, after immunization with the LPD/E7 protein, about 40% of $CD4^+$ cell-depleted mice developed tumors when challenged with TC-1 cells, in comparison to the fact that all the LPD/E7 protein immunized normal mice were tumor free (unpublished data). Montanide series are a group of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two [81]. The various Montanide ISA adjuvants are water-in-oil emulsions, oil-in-water emulsions, or water-in-oil-in-water emulsions. Different adjuvants accommodate different aqueous phase/oil phase ratios because of the variety of surfactant and oil combinations. The manufacturer mentioned that the performance of these adjuvants is similar to the incomplete Freund's adjuvant (IFA) for antibody production; however the inflammatory response is usually less. ISA-51 is a W/O emulsion based on mineral oil and designed to enhance immune responses. It has already been assessed in various clinical trials (I/II/III) [81]. The data from the current study (Fig. 6) clearly demonstrated that our LPD particles induced a much stronger anti-tumor response than the ISA-51 did. In another study using Montanide ISA-51, we found that treatment of tumor-bearing mice with antigen-incorporated ISA-51 failed to induce a detectable CTL response (unpublished data). This may explain why treatment with the ISA-51/E7 protein failed to cause tumor regression in the present study. In our study, when the E7 solution and ISA-51 were mixed in a 1:2 ratio (v/v), a very viscous whitish suspension was formed, making it extremely difficult to inject. The suspension formed a solid mass in the injection site, which took many days to diffuse. We are not sure whether an alternative and improved E7 protein and ISA-51 combination will lead to improved performance.

In general, a protein alone as an antigen does not induce (or only weakly induces) an immune response. Therefore, an effective adjuvant is necessary. Unfortunately, upto the present, Alum still represents the only approved vaccine adjuvant for humans in the U.S. Alum is a weak adjuvant for inducing antibody response, and is even worse as a cell-mediated immune (CMI) response adjuvant. The strong antibody and CMI including CTL responses against E7 protein, when the E7 was dosed as a LPD/E7 protein, demonstrated the potential of LPD as a vaccine carrier and adjuvant. Previous data showed that as high as 17% of DCs in the popliteal lymph nodes picked up LPD particles when the LPDs were subcutaneously injected into mouse footpad [69]. LPD nanoparticles enter DCs via endocytosis (unpublished data). A protein as an antigen usually does not induce CTL response because it is taken up via the endosome/lysosome path, degraded inside them, and then presented to the MHC II molecules. Recent studies have shown that carrying a protein in certain particulates allows it to enter the cytoplasma directly. The protein will be processed by the proteosome, presented to the MHC I molecules, then induce a CTL response [82, 83]. The fact that the E7 protein incorporated into LPD was able to induce a CTL response might be explained similarly. For those E7 proteins that were delivered to DCs directly, some may be delivered into the cytoplasm, presented 'endogenously', and induce a CTL response; some may stay in the endosome and lysosome and induce antibody response [84]. As to the mechanism of immune induction by LPD as an adjuvant, our previous studies have shown that the empty plasmid DNA incorporated inside the liposomes and the cationic lipids (or liposomes) are both responsible and required for its full activity [85]. It is known that the bacterial plasmid DNA is a potent vaccine adjuvant because of its unmethylated CpG motifs [86]. Studies by us and others have also shown that certain cationic lipids, such as the DOTAP used to prepare liposomes in our studies, are immunostimulatory [87]. Treatment of DCs with DOTAP induced both CD80/CD86 expression on DCs surface and the release of proinflammatory cytokines such as TNF-α by DCs [85, 87]. LPD as a vaccine carrier and adjuvant has many advantages. (1) Incorporation of antigen in the aqueous core of liposomes prevents the antigen from enzymatic degradation; (2) Two components of the LPD, the plasmid DNA and the cationic liposomes, are both immunostimulatory; (3) The immune response from LPD is balanced, both humoral and cellular responses are induced; (4) The LPD is not complicated to prepare because the incorporation of protamine-condensed DNA into liposomes is a spontaneous process; (5) The safety profile of LPD has been established in a recent Phase I clinical trial [76].

It is known that the HPV 16 E7 protein is tumorigenic. However, the risk from E7 in protein form as a vaccine is much lower than that when the E7 is coded in plasmid DNA for immunization. More toxicity studies are needed to determine whether these mutations are

needed for an E7 protein-based vaccine. If a mutated version of the E7 protein is proven to be necessary in the future, our LPD particles should also be effective in delivering the mutated proteins as long as the MHC I-restricted epitope(s) are not altered or deleted. As shown in present studies, when incorporated with the mutated E7 protein, LPD particles are still effective in inducing anti-TC-1 immune responses.

In summary, we report that LPD as a protein-based antigen carrier and adjuvant induces a very potent and robust immune response, including CTL and antibody responses. It may be used to treat diseases such as cervical cancer, when specific antigens such as E7 protein is incorporated inside. We are currently confirming the anti-tumor efficacy of the LPD/E7m in humanized mice.

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