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Original Articles

P5 HER2/neu-derived peptide conjugated to liposomes containing MPL adjuvant as an effective prophylactic vaccine formulation for breast cancer

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ABSTRACT

Vaccines containing synthetic peptides derived from tumor-associated antigens (TAA) can elicit potent cytotoxic T lymphocyte (CTL) response if they are formulated in an optimal vaccine delivery system. The aim of this study was to develop a simple and effective lipid-based vaccine delivery system using P5 HER2/neu-derived peptide conjugated to Maleimide-PEG2000-DSPE. The conjugated lipid was then incorporated into liposomes composed of DMPC:DMPG:Chol:DOPE containing Monophosphoryl lipid A (MPL) (Lip-DOPE-P5-MPL). Different liposome formulations were prepared and characterized for their physicochemical properties. To evaluate anti-tumoral efficacy, BALB/c mice were immunized subcutaneously 3 times in two-week intervals and the generated immune response was studied. The results demonstrated that Lip-DOPE-P5-MPL induced a significantly higher IFN-γ production by CD8+ T cells intracellularly which represents higher CTL response in comparison with other control formulations. CTL response induced by this formulation caused the lowest tumor size and the longest survival time in a mice model of TUBO tumor. The encouraging results achieved by Lip-DOPE-P5-MPL formulation could make it a promising candidate in developing effective vaccines against Her2 positive breast cancers.

Introduction

Despite many decades of research on the cancer treatment, cancer is still a major cause of death. Chemotherapy, radiotherapy and surgery are current treatments for cancers, however chemotherapy destroys cells indiscriminately and radiotherapy and surgery are not able to prevent metastases. Due to the disadvantages of current treatments for cancers, tumor immunotherapy has been paid attention during two past decades [1]. Since humoral immunity has a low potential to eliminate solid tumors individually, induction of an effective cell-mediated immunity based on the activation of cytotoxic T lymphocytes (CTLs), namely $CD8^+$ T cells, is aimed in cancer immunotherapy [2,3].

Vaccines containing synthetic peptides derived from tumor-associated antigens (TAA) can elicit potent CTL response if they are formulated optimally. Ag-presenting cells (APCs) mainly dendritic cells (DCs) present peptide antigens to T cells ($CD4^+$ and $CD8^+$) via MHC molecules and initiate immune responses to infectious diseases and tumors [4,5]. Exogenous peptide antigens which are taken up by DCs, pass the endocytic pathway and they are generally presented to $CD4^+$ T cells on MHC class II molecules whereas endogenous antigens enter into the cytosol, load onto MHC class I molecules in the endoplasmic reticulum and are finally presented to $CD8^+$ CTLs [6] .Therefore, efficient delivery of TAAs to DCs, endosomal escape of antigens to the cytosol and activation of CTLs via MHC class I presentation are crucial to induce an effective immune response leading to tumor regression.

Nanoparticle delivery systems carrying antigens have the potential for achieving all the above mentioned goals. Liposomes can offer several advantages over other particulate systems. Basically, liposomes are safe and well-tolerated carriers. They are also completely biodegradable and versatile to be formulated with different lipid constituents, all types of peptide antigens and adjuvants to induce a robust cell-mediated immunity [7,8].

Adjuvants in liposomal vaccine formulations can enhance and prolong immune responses [9]. Among different adjuvants, Monophosphoryl lipid A (MPL) has been used frequently as an efficient adjuvant in liposomal vaccines. MPL has shown adjuvant activity in both cellular and humoral immunity [10]. MPL is a non-toxic derivative from LPS or endotoxin that drives immunity

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responses via TLR4 stimulation [11,12]. FDA approved MPL as a safe adjuvant for human vaccines [13].

Through developing tumor-specific peptide vaccines, various TAAs have been targeted for cancer immunotherapy. As a TAA, HER2/neu protein has provided an opportunity to develop breast cancer vaccines. HER2/neu is a 185 kDa transmembrane glycoprotein and member of the epidermal growth factor receptor family overexpressed in 20–40% of primary breast cancers [14,15].

In our previous study, four peptides containing MHC class I restricted multi-epitope from rat HER2/neu protein were designed by *in silico* analysis and the effectiveness of these peptides was evaluated by administration to BALB/c mice. As results showed that two of these peptides (p5 and p435) were effective in inducing CTL responses, it was hypothesized that encapsulating P5 or P435 in lipid carriers may enhance CTL immune responses more than peptides alone. Encapsulating peptides in LPD (liposome-polycation-DNA) nanoparticles included DOTAP as a cationic lipid and CpG ODN as an immune-stimulatory adjuvant confirmed the hypothesis [16]. However, LPD is a complex carrier and PS-type CpG ODN at high dose may elicit systemic toxicity [17].

For these reasons, in the present study, we utilized liposomes composed of DMPC:DMPG:Chol:DOPE containing MPL for efficiently introducing P5 peptide to cytosol of APCs and generating a strong CTL response. In our earlier challenging study, we developed an optimized procedure for encapsulating P5 peptide in the inner cavity of liposomes by passive loading [18]. As encapsulation efficiency was low, in this study, P5 peptide (ELAAWCRWGFLLALLPPGIAGGGC) was covalently conjugated to Maleimide-PEG_{2000}-DSPE to improve peptide incorporation into liposomes. The effectiveness of liposomal formulation of P5 peptide in the induction of CTL response was evaluated in BALB/c mice and in TUBO *in vivo* tumor mice model, which overexpresses the HER2/neu oncogene.

Materials and methods

Materials

Peptide P5 (ELAAWCRWGFLLALLPPGIAGGGC, purity > 95%) was synthesized by ChinaPeptides Co. (Shanghai, China). Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphoglycerol (DMPG), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (Maleimide-PEG2000-DSPE) were purchased from Avanti Polar Lipid (Alabaster, USA). Cholesterol and Monophosphoryl lipid A from *Salmonella enterica* (MPL) were purchased from Sigma-Aldrich (Steinheim, Germany). Cytofix/Cytoperm™ Plus, PMA/ionomycin cocktail, anti-CD8a-PE-cy5, anti CD4-PE-cy5, anti-IFN-γ- FITC and anti-IL-4-PE antibodies were purchased from BD Biosciences (San Diego, USA). All other solvents and reagents were used as chemical grade.

Animal and cell lines

Four to six week old female BALB/c mice were purchased from Pasteur Institute (Tehran, Iran). The experimental protocols were approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences in accordance with animal welfare guidelines.

TUBO, a cloned cell line that overexpresses the rHER2/neu protein, was kindly provided by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy) and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 20% fetal bovine serum (FBS). A murine colon carcinoma cell line, CT26, was purchased from Pasteur Institute (Tehran, Iran) and cultured in RPMI-1640 medium supplemented with 10% FBS.

Conjugation of P5 peptide to PEG_{2000}-DSPE

P5 peptide was conjugated to Maleimide-PEG_{2000}-DSPE through covalent binding between the thiol group of cysteine residue of peptide and the pyrrole group of maleimide. Peptide was reacted with Maleimide-PEG_{2000}-DSPE in a molar ratio of 1.2:1 (peptide:maleimide) in DMSO:chloroform (1:1) solution at room temperature for 24 h. Thin layer chromatography (TLC) was used to confirm the formation of P5-PEG_{2000}-DSPE. A TLC plate (silica gel 60 F254, Merck, USA) was placed in a TLC chamber containing mobile phase composed of chloroform, methanol, and water at 90:18:2 (v/v). The chamber was saturated with iodine vapor to stain the TLC plate. The conjugation of peptide with PEG_{2000}-DSPE was also ascertained indirectly by determining unconjugated peptide fraction using HPLC. KNAUER smart line HPLC (Berlin, Germany) was equipped with a Nucleosil C18, 5 μm, 150 × 4.6 mm, 100A° column (KENAUER) and an UV detector (KENAUER S2600) set at 220 nm. The mobile phases employed were A (water + 0.1% TFA) and B (acetonitrile + 0.1% TFA). Elution program was a gradient starting with 100% A and increasing to 30% B in 2 min, 60% B in 10 min and 90% B in 2 min. The flow rate was set to 1 ml/min.

Liposome preparation

Liposomes (Lip-DOPE) composed of DMPC:DMPG:Chol:DOPE at a molar ratio of 30:4:6:10 were prepared using lipid film hydration method. Control liposomes (Lip) were also prepared in the same molar ratio as above without using DOPE. Lipids were first dissolved in chloroform and then they were combined in sterile glass tubes. The required amount of MPL and P5-PEG_{2000}-DSPE conjugate was added to the lipid solutions to prepare liposomes containing P5 peptide and MPL (Lip-P5-MPL). The lipid solutions were dried to a thin film by rotary evaporation (Heidolph, Germany) under reduced pressure. Films were freeze-dried (VD-800F, Taitech, Japan) overnight to remove the solvents completely. Lipids were then hydrated in HEPES buffer (10 mM, pH 7.2) containing 5% dextrose, vortexed and bath-sonicated to disperse completely the lipids into the buffer. The resulting multilamellar vesicles (MLVs) were extruded using a mini extruder (Avestin, Canada) to form 100 nm small unilamellar vesicles (SUVs) with a uniform size. The final formulations contained 0.1 mg/ml P5 peptide and 0.25 mg/ml monophosphoryl lipid A in liposome with a lipid concentration of 50 mM.

Liposome characterization

The P5 peptide content in liposomal formulations was determined by the same HPLC method as described in "Conjugation of P5 Peptide to PEG_{2000}-DSPE." Liposome preparations were disrupted with 1.5% (v/v) $C_{12}E_{10}$ detergent and then assayed to determine MPL content by an LAL chromogenic endpoint assay (QCL-1000, Lonza, Walkersville, MD) [19]. The amount of total lipids was determined based on phospholipids by using a phosphorus assay method [20]. Vesicle size, polydispersity index and zeta potential of liposomes were determined by dynamic light scattering (Malvern Instruments, Malvern, UK). Liposomes were stored at 4 °C under argon.

Animal immunization and splenocyte collection

BALB/c mice (10 per group) were immunized with different liposomal formulations three times at two-week intervals subcutaneously. The liposome dose of 5 μmol per mouse was used for each injection. Free P5 peptide (10 μg/mouse) and HEPES-dextrose buffer were used as control groups.

Two weeks after the last booster, the mice (four per group) were sacrificed and their splenocytes aseptically collected to evaluate cellular immune responses.

Enzyme-linked immunospot (ELISpot) assays

ELISpot assays were carried out using mouse ELISpot kits from U-cytech (Utrecht, The Netherlands) according to the manufacturer's instruction. Briefly, one day before mice sacrifice, ELISpot 96-well plates were coated with anti-IL-4 and anti-IFN-γ antibodies and incubated overnight at 4 °C. Splenocytes were cultured in triplicate wells in a final volume of 200 μl with medium containing P5 peptide (10 μg/ml) in precoated plates. Splenocytes were incubated for 24 h at 37 °C in tissue culture incubator. When spots appeared, counting was done with Kodak 1D image analysis software (Version 3.5, Eastman Kodak, Rochester, New York).

Intracellular cytokine assay via flow cytometric analysis

Splenocytes (10^6 cells/ml) in medium containing GolgiPlug™ (1 μl/ml) was stimulated with PMA/ionomycin cocktail (2 μl/ml) for 4 h at 37 °C. After stimulation, 10^5 splenocytes were transferred into flow cytometry tubes and washed two times with stain buffer (2% FCS in PBS). Splenocytes were stained with 1 μl anti-CD8a-PE-cy5 antibody and 1 μl anti CD4-PE-cy5 antibody in separate tubes for 30 min at 4 °C. The cells were washed with stain buffer and fixed using Cytofix/Cytoperm™ solution. Fixed cells were washed two times with Perm/Wash™ buffer and then stained with 1 μl anti-IFN-γ- FITC antibody for 30 min at 4 °C. CD4 cells were also stained with 1 μl anti-IL-4-PE antibody. The cells were washed with Perm/Wash™ buffer and suspended in 300 μl stain buffer for flow cytometric analysis (BD FACSCalibur™, BD Biosciences, San Jose, USA).

In vitro CTL assay

Two weeks after the last booster, splenocytes were isolated from four mice per group and re-stimulated in vitro with P5 peptide (10 μg/ml) and recombinant IL-2 (20 U/ml) for 5 days. After stimulation, Splenocytes, as effector cells, were transferred to U-bottomed plates in triplicate wells. TUBO tumor cells in DMEM-20% were incubated with 12.5 μM Calceine AM at 37 °C for one hour in the dark [21]. After removing the excess dye, TUBO cells (2×10^4), as target cells, were added to splenocytes and incubated at 37 °C for 4 hours in the dark. Culture medium only and medium

containing 2% Triton X-100 were added to the wells to determine the minimum and maximum release by target cells, respectively. Fluorescence in supernatants was read on a fluorimeter (FLx800, BioTek Instruments Inc. USA) with excitation at 485 nm and emission at 538 nm. The specific lysis was calculated as follows: percentage of specific lysis = (release by CTLs – minimum release by targets)/(maximum release by targets – minimum release by targets). CT26 cells which labeled similarly to the TUBO cells were used as negative control to prove that cytotoxic activity is specific.

In vivo *tumor protection assay*

The immunized mice (six per group) were challenged on day 14 post last vaccination via subcutaneous injection in the right flank with 5×10^5 TUBO cells in 50 µl PBS buffer. The tumor volume ([length × width × height] × 0.5), the time to reach end point (TTE) (from the equation of the line obtained by exponential regression of the tumor growth curve) and the percent of tumor growth delay (%TGD) (based on the difference between the median TTE of treatment group (T) and the median TTE of the control group (C) (%TGD = [(T – C)/C] × 100]) were calculated for each mouse [22,23]. For ethical consideration, mice were sacrificed when (a) the tumor volume was greater than 1000 mm^3, (b) the body weight loss was over 15% of initial weight or (c) the mice became lethargic or sick and unable to feed.

Statistical analysis

The results were analyzed by one-way ANOVA and Tukey test to assess the significance of the differences among various formulations. Mouse survival was analyzed by log-rank test (GraphPad Prism, version 5, San Diego, California). Results with $P < 0.05$ were considered to be statistically significant.

Results

Synthesis of P5-PEG-DSPE

Formation of P5-PEG-DSPE was identified by thin layer chromatography. Disappearance of PEG-DSPE spot from the reaction mixture was confirmed by iodine vapor (Fig. 1A). The extent of unconjugated (free) peptide was determined using HPLC post reaction with PEG-DSPE and the results indicated almost complete reaction between maleamide group in PEG-DSPE and thiol on P5 (Fig. 1B).

Table 1
Vesicle size, pdI and zeta potential of liposomal formulations (n = 3; mean ± SD).

Formulation	Vesicle size (nm)	pdI	Zeta potential (mV)
Lip-DOPE-MPL (DMPC/DMPG/Chol/DOPE/MPL)	126.3 ± 3.5	0.138 ± 0.019	–44.6 ± 1.28
Lip-P5 (DMPC/DMPG/Chol/P5)	135.7 ± 6.1	0.173 ± 0.014	–41.7 ± 1.65
Lip-P5-MPL (DMPC/DMPG/Chol/P5/MPL)	128.9 ± 6.3	0.147 ± 0.011	–47.4 ± 1.46
Lip-DOPE-P5 (DMPC/DMPG/Chol/DOPE/P5)	142.3 ± 5.6	0.184 ± 0.008	–42.4 ± 2.21
Lip-DOPE-P5-MPL (DMPC/DMPG/Chol/DOPE/P5/MPL)	132.7 ± 9.3	0.176 ± 0.026	–44.7 ± 2.55

Physical properties of liposomal formulations

P5-PEG-DSPE was incorporated into different liposomal formulations to induce an effective CTL response. For each formulation, physical property including liposome size, polydispersity index (pdI) and zeta potential was determined as shown in Table 1.

The particle size of all liposomal formulations ranged from 110 to 150 nm in diameter which was desirable for vaccine formulations [24]. Negatively charged liposomes were also homogenous and had a uniform size with monomodal distribution (pdI < 0.2).

Content of P5 peptide and MPL in liposomal formulations

Since the dose of peptide antigen and adjuvant can significantly influence the efficacy of formulations, P5 peptide and MPL content were accurately determined in different liposomal formulations (Table 2). Based on the lipid dose of 5 µmol, a similar peptide and MPL doses per mouse were administered for each formulation.

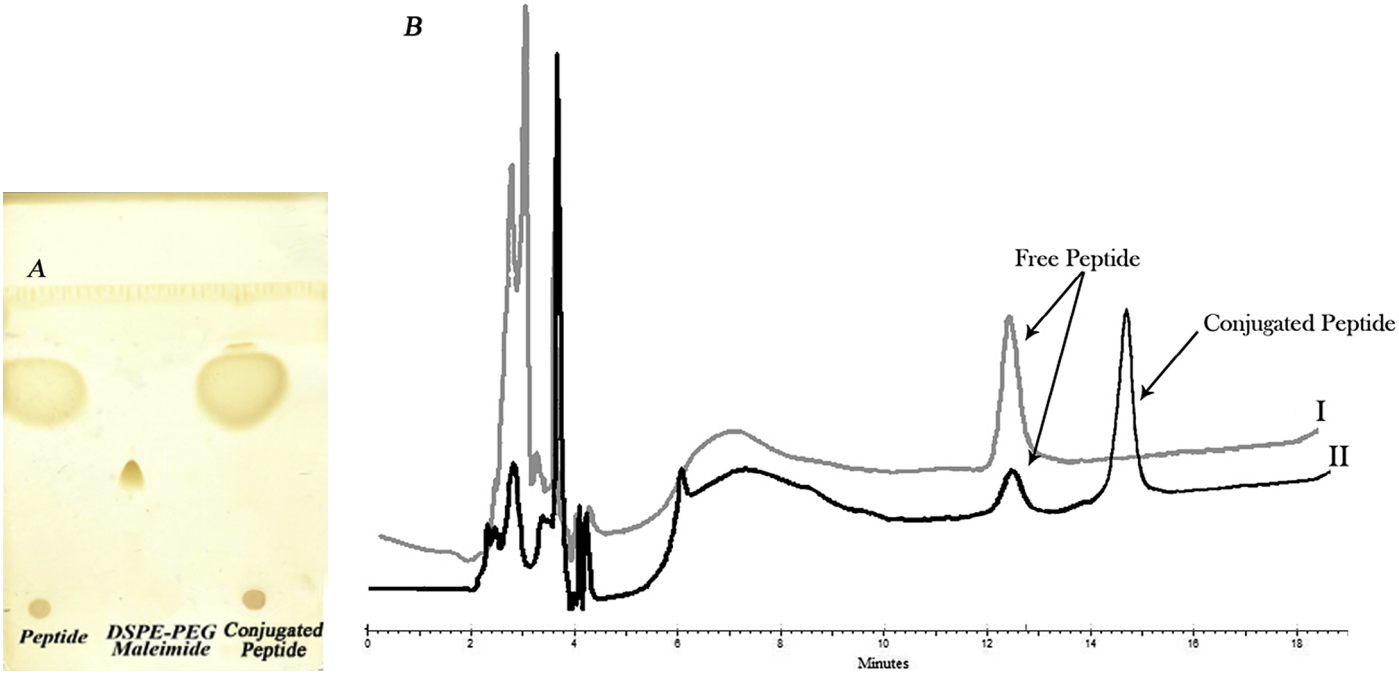

Fig. 1. Confirmation of P5 peptide conjugation to Maleimide-PEG-DSPE using TLC (A) and HPLC (B). (A) A TLC plate was placed in TLC chamber containing mobile phase composed of chloroform, methanol, and water at 90:18:2 (v/v). Disappearance of PEG-DSPE spot from the reaction mixture was confirmed by iodine vapor. (B. I) Standard free peptide eluted with a retention time of ~12 minutes. (B. II) The extent of unconjugated (free) peptide was determined post reaction with PEG-DSPE (see method).

Table 2
Content of P5 peptide and MPL in liposomal formulations.

Formulation	P5 content (μg/μmol lipid)	MPL content (μg/μmol lipid)	P5 dose* (μg per mouse)	MPL dose* (μg per mouse)
Lip-DOPE-MPL	–	4.91	–	24.55
Lip-P5	1.85	–	9.25	–
Lip-P5-MPL	1.89	4.82	9.45	24.10
Lip-DOPE-P5	1.96	–	9.80	–
Lip-DOPE-P5-MPL	1.92	5.08	9.60	25.40

* P5 peptide dose and MPL dose were determined based on a lipid dose of 5 μmol given per mouse.

Induction of IFN-γ response by Lip-DOPE-P5-MPL formulation

The results showed that splenocytes isolated from the mice immunized with liposomes composed of DMPC:DMPG:Chol:DOPE containing P5 peptide and MPL (Lip-DOPE-P5-MPL) secreted a significantly higher amount of IFN-γ than the other liposomal formulations, P5 peptide alone and HEPES buffer (Fig. 2A). None of the liposomal formulations induced considerable IL-4 response in mice (Fig. 2B).

Induction of CD8+ response by Lip-DOPE-P5-MPL formulation

Flow cytometric analysis using CD8, CD4 and IFN-γ markers demonstrated CD8+ T lymphocytes mostly contributed to IFN-γ production (Fig. 3A and B). Moreover, the results showed Lip-DOPE-P5-MPL formulation induced a significantly higher level production of IFN-γ in CD8+ lymphocytes (higher MFI level) which represented a higher number of IFN-γ producing cells in CD8+ population or higher CTL population in comparison with other groups (Fig. 3A). Flow cytometric results also showed IL-4 production in $CD^{+}4$ cells that implies T cell-dependent humoral immunity was not induced significantly in all groups. (Fig. 3C).

Induction of antigen-specific CTL response by Lip-DOPE-P5-MPL formulation

In vitro CTL activity assay using rHER2/neu-expressing TUBO tumor cells indicated immunization with Lip-DOPE-P5-MPL formulation generated significantly higher CTL response to P5 antigen than other formulations (Fig. 4). The CTL activity was established significantly at both various effector-to-target ratios. The

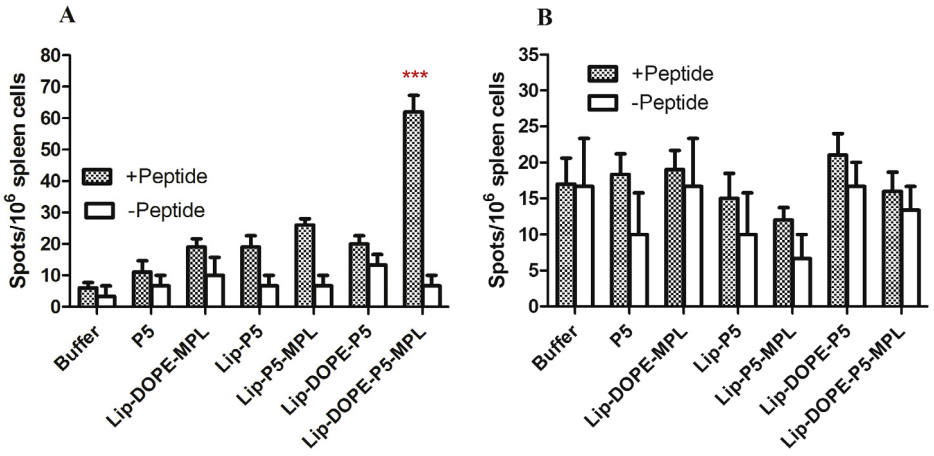

Fig. 2. The efficacy of different liposomal formulations in inducing IFN-γ (A) and IL-4 (B) production. BALB/c mice (10 per group) were immunized three times at two-week intervals with different liposomal formulations, P5 peptide alone or HEPES buffer. On day 14 post last booster, four mice from each group were sacrificed and their splenocytes were restimulated with P5 peptide. IFN-γ and IL-4 release from splenocytes induced by different liposomal formulations was determined using ELISpot assay. The data indicate the mean ± S.E.M. (n = 4). *** denotes significant difference from all other formulations.

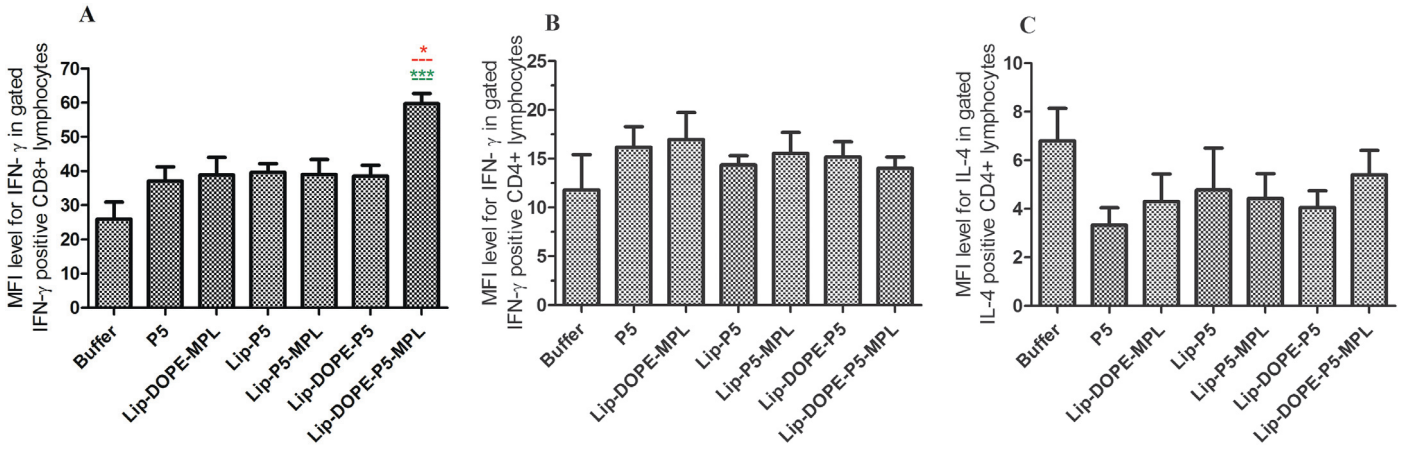

Fig. 3. Geometric mean fluorescence intensity (MFI) level for IFN-γ in gated CD8 (A) and CD4 (B) lymphocyte populations and MFI level for IL-4 in gated CD4s (C). Isolated splenocytes of immunized mice were re-stimulated in vitro with PMA/ionomycin and stained with CD4, CD8, IFN-γ and IL-4 markers. MFI level for IFN-γ and IL-4 in gated populations were determined by flow cytometric analysis. The data indicate the mean ± S.E.M. (n = 4). *** and * denote significant difference from buffer and all other formulations, respectively.

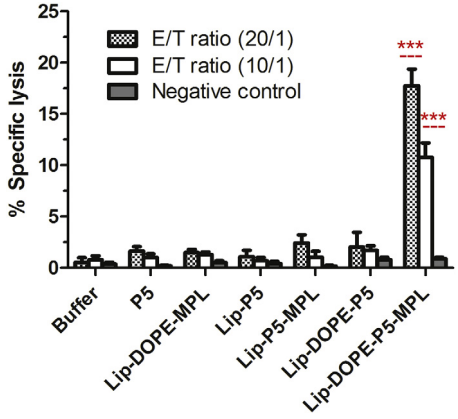

Fig. 4. Antigen-specific CTL response induced by various formulations at two different ratios of effector to target cells (E/T) was assessed using an in vitro CTL activity assay. Splenocytes isolated from mice (four in each group) were incubated with Calcein AM-loaded rHER2/neu-expressing TUBO tumor cells and rHER2/neu-expressing negative CT26 cells (see method).The data indicate the mean ± S.E.M. (n = 4). *** denotes significant difference from all other formulations at both various effector-to-target ratios.

Table 3
Therapeutic efficacy data of different liposomal vaccine formulations in TUBO tumor mice model (n = 6).

Formulation	MST[a] (day)	TTE[b] (day ± SD)	TGD[c] %
Buffer	52.5	47.97 ± 8.81	–
P5	56	55.67 ± 13.44	18.58
Lip-DOPE-MPL	63	58.03 ± 8.36	31.90
Lip-P5	66.5	62.67 ± 12.54	35.31
Lip-P5-MPL	59.5	57.03 ± 13.18	23.57
Lip-DOPE-P5	70	69.10 ± 11.90	54.16
Lip-DOPE-P5-MPL	Indefinable	175.45 ± 31.16***	80.41

*** Denotes significant difference from all other formulations.
[a] Median survival time.
[b] Time to reach end point.
[c] Tumor growth delay.

cytotoxicity activity induced by Lip-DOPE-P5-MPL was specific against rHER2/neu. CTL response was not observed against rHER2/neu-expressing negative CT26 cells (Fig. 4).

Anti tumor effects of Lip-DOPE-P5-MPL vaccination in BALB/c

Lip-DOPE-P5-MPL formulation had a superior tumor growth inhibition in the TUBO tumor mice model (Fig. 5A). The survival time was also significantly prolonged in mice following Lip-DOPE-P5-MPL vaccination compared to the other formulations (Fig. 5B). Since none of the mice vaccinated with Lip-DOPE-P5-MPL died during the experiment, median survival was indefinable in this group. Median survival time (MST) as well as TTE and %TGD for each treatment group are summarized in Table 3.

Discussion

In the present study, we attempted to enhance immunogenicity and adjuvanicity of P5 peptide, a synthetic peptide containing CTL multi-epitope from rHER2/neu protein, by conjugating with different liposomal formulations. The results showed P5 peptide conjugating with liposomes alone (Lip-P5) was incapable of inducing any immune response. Although some studies reported liposomes alone could elicit CTL response against encapsulated peptide antigens [25,26], this formulation could have been used exclusively as a carrier for antigen delivery with no potential to conduct antigens into the cytosol. Lip-P5 formulation had a particle size of 110–150 nm that is needed for efficiently draining to lymph nodes where CD8+ lymphoid DCs are present [24,27].

Inclusion of a pH sensitive lipid like DOPE in the liposome structure has been frequently demonstrated as an efficient strategy to introduce antigens into MHC class I pathway [28–31]. After endocytosis, at the pH of the endosomal compartment, the transition of DOPE from lamellar to hexagonal phase occurs. It induces the fusion of liposomes with the endosomal membrane and subsequently liposomal antigens are released toward the cytosol [32–34]. However, as results have shown, inclusion of DOPE alone in liposomal formulation (Lip-DOPE-P5) was not sufficient to elicit an effective immune response. Lip-DOPE-P5 formulation might have been able to deliver P5 antigen to MHC class I molecules successfully, however, once the peptide antigen was presented to CD8+ lymphocytes by APCs, presence of co-stimulatory molecules on the APCs was also required for activation of CD8 cells to produce CTLs [35–37]. MPL can induce intracellular signaling pathways leading to production of these co-stimulatory molecules through TLR4 stimulation [35]. Co-formulation of MPL and DOPE in liposomes (Lip-DOPE-P5-MPL) induced an effective cellular immune response characterized with the higher IFN-γ producing CD8+ cells and CTL activity. As

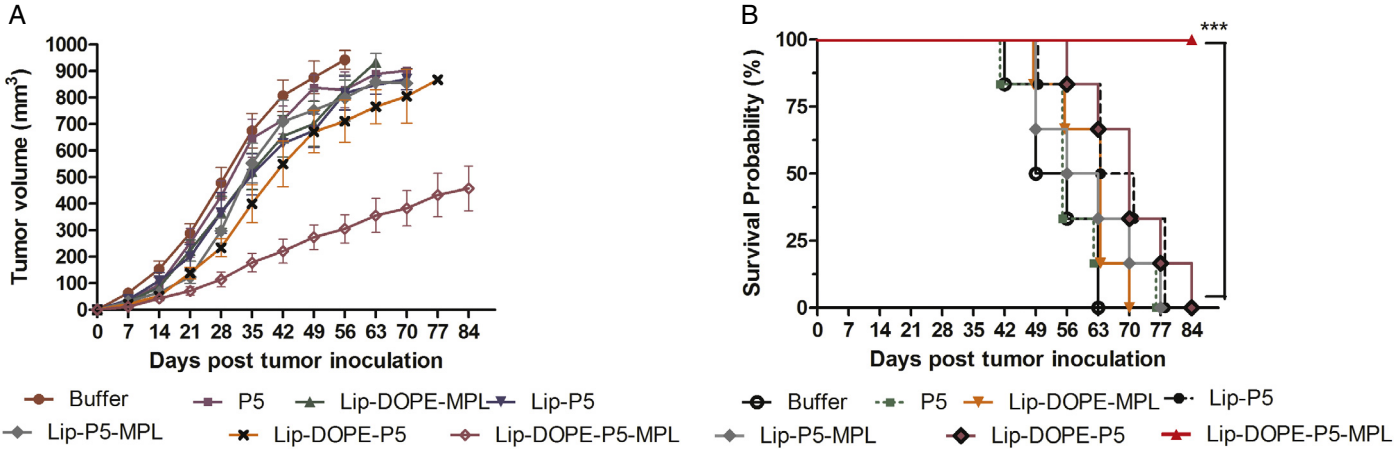

Fig. 5. Protective effects of vaccination with different formulations in BALB/c mice against a TUBO tumor model. (A) Immunized mice (six in each group) were challenged 14 days post last booster with 5×10^5 TUBO cells. Tumor size was calculated based on three dimensions. The values are means of tumor size ± S.E.M. (n = 6). (B) Effects of immunization on survival time were monitored for a period of 84 days among BALB/c mice (n = 6). *** denotes significant difference from all other formulations.

results have shown, the pair of MPL and DOPE used in liposomal formulation had synergic effect on promoting vaccine efficacy while using one of them individually in the lipid carrier (Lip-P5-MPL or Lip-DOPE-P5 formulations) was impotent to induce immune response. Due to the lipidic structure of MPL and DOPE which facilitated incorporation to liposomes and the safe use of anionic liposomes containing MPL in humans [13], Lip-DOPE-P5-MPL formulation might be superior to vaccine delivery systems such as cationic liposomes containing CpG ODN or LPDs employed for induction of CTL response against HER2-derived peptide antigens [16,38].

The success of Lip-DOPE-P5-MPL formulation to elicit a robust CTL response against P5 peptide containing just MHC class I epitopes of HER2/neu without the use of T-helper epitope can be accordant with the claim that MPL has the stimulatory action on APCs [10,35]. Although formulations containing both CTL and T helper epitopes derived from Her2/neu antigen in combination with GM-CSF could induce effective CTL responses [39,40] and confirmed the claim that CD4 T cell help was required to activate CTLs [41,42], our findings demonstrated that this requirement might be dependent on how peptide vaccines were formulated. MPL has been shown to be capable of provoking DCs to produce co-stimulatory molecules and secrete inflammatory cytokines such as IL-6, IFN-γ and IL-12 which are essential for activation of T cells [10,35,43–45]. CTL induction due to the stimulatory action of TLR agonists on APCs was previously observed about CpG ODN adjuvant [38].

In conclusion, our results show that Lip-DOPE-P5-MPL is an effective formulation for the preparation of a peptide-based HER2/neu vaccine. Liposomes composed of DMPC:DMPG:Chol:DOPE containing MPL can deliver peptide antigen to the cytosol and induce CTL response that can be used prophylactically to reduce tumor growth. The benefits of this formulation (easy manufacturing process and safe use in human) can make it a potential candidate to alternative ones for developing liposomal vaccines in terms of antitumor therapies in breast cancers in which HER2/neu antigen overexpresses.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] J. Couzin-Frankel, Breakthrough of the year 2013. Cancer immunotherapy, Science 342 (2013) 1432–1433.
[2] H.L. Hanson, D.L. Donermeyer, H. Ikeda, J.M. White, V. Shankaran, L.J. Old, et al., Eradication of established tumors by CD8+ T cell adoptive immunotherapy, Immunity 13 (2000) 265–276.
[3] K. Matsui, L.A. O'Mara, P.M. Allen, Successful elimination of large established tumors and avoidance of antigen-loss variants by aggressive adoptive T cell immunotherapy, Int. Immunol. 15 (2003) 797–805.
[4] R.M. Steinman, Decisions about dendritic cells: past, present, and future, Annu. Rev. Immunol. 30 (2012) 1–22.
[5] K. Palucka, J. Banchereau, Human dendritic cell subsets in vaccination, Curr. Opin. Immunol. 25 (2013) 396–402.
[6] J. Parkin, B. Cohen, An overview of the immune system, Lancet 357 (2001) 1777–1789.
[7] D.S. Watson, A.N. Endsley, L. Huang, Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens, Vaccine 30 (2012) 2256–2272.
[8] C.R. Alving, Liposomes as carriers of antigens and adjuvants, J. Immunol. Methods 140 (1991) 1–13.
[9] P. Nordly, H.B. Madsen, H.M. Nielsen, C. Foged, Status and future prospects of lipid-based particulate delivery systems as vaccine adjuvants and their combination with immunostimulators, Expert Opin. Drug Deliv. 6 (2009) 657–672.
[10] J.T. Ulrich, K.R. Myers, Monophosphoryl lipid A as an adjuvant. Past experiences and new directions, Pharm. Biotechnol. 6 (1995) 495–524.
[11] C.R. Alving, Lipopolysaccharide, lipid A, and liposomes containing lipid A as immunologic adjuvants, Immunobiology 187 (1993) 430–446.
[12] C.R. Casella, T.C. Mitchell, Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant, Cell. Mol. Life Sci. 65 (2008) 3231–3240.
[13] E. Vacchelli, L. Galluzzi, A. Eggermont, W.H. Fridman, J. Galon, C. Sautes-Fridman, et al., Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy, Oncoimmunology 1 (2012) 894–907.
[14] Y. Yarden, Biology of HER2 and its importance in breast cancer, Oncology 61 (Suppl. 2) (2001) 1–13.
[15] C.N. Baxevanis, N.N. Sotiriadou, A.D. Gritzapis, P.A. Sotiropoulou, S.A. Perez, N.T. Cacoullos, et al., Immunogenic HER-2/neu peptides as tumor vaccines, Cancer Immunol. Immunother. 55 (2006) 85–95.
[16] S.A. Jalali, M. Sankian, J. Tavakkol-Afshari, M.R. Jaafari, Induction of tumor-specific immunity by multi-epitope rat HER2/neu-derived peptides encapsulated in LPD nanoparticles, Nanomedicine 8 (2012) 692–701.
[17] A.A. Levin, A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides, Biochim. Biophys. Acta 1489 (1999) 69–84.
[18] S. Shariat, A. Badiee, M.R. Jaafari, S.A. Mortazavi, Optimization of a method to prepare liposomes containing HER2/neu- derived peptide as a vaccine delivery system for breast cancer, Iran. J. Pharm. Res. 13 (2014) 15–25.
[19] P. Harmon, D. Cabral-Lilly, R.A. Reed, F.P. Maurio, J.C. Franklin, A. Janoff, The release and detection of endotoxin from liposomes, Anal. Biochem. 250 (1997) 139–146.
[20] G.R. Bartlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[21] R. Lichtenfels, W.E. Biddison, H. Schulz, A.B. Vogt, R. Martin, CARE-LASS (calcein-release-assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity, J. Immunol. Methods 172 (1994) 227–239.
[22] Z. Huang, M.R. Jaafari, F.C. Szoka Jr., Disterolphospholipids: nonexchangeable lipids and their application to liposomal drug delivery, Angew. Chem. Int. Ed. Engl. 48 (2009) 4146–4149.
[23] T. Schluep, J. Hwang, J. Cheng, J.D. Heidel, D.W. Bartlett, B. Hollister, et al., Preclinical efficacy of the camptothecin-polymer conjugate IT-101 in multiple cancer models, Clin. Cancer Res. 12 (2006) 1606–1614.
[24] M.F. Bachmann, G.T. Jennings, Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns, Nat. Rev. Immunol. 10 (2010) 787–796.
[25] A.H. Hale, H-2 antigens incorporated into phospholipid vesicles elicit specific allogeneic cytotoxic T lymphocytes, Cell. Immunol. 55 (1980) 328–341.
[26] L. Raphael, B.H. Tom, Liposome facilitated xenogeneic approach for studying human colon cancer immunity: carrier and adjuvant effect of liposomes, Clin. Exp. Immunol. 55 (1984) 1–13.
[27] R.M. Steinman, L. Bonifaz, S. Fujii, K. Liu, D. Bonnyay, S. Yamazaki, et al., The innate functions of dendritic cells in peripheral lymphoid tissues, Adv. Exp. Med. Biol. 560 (2005) 83–97.
[28] R. Reddy, F. Zhou, L. Huang, F. Carbone, M. Bevan, B.T. Rouse, pH sensitive liposomes provide an efficient means of sensitizing target cells to class I restricted CTL recognition of a soluble protein, J. Immunol. Methods 141 (1991) 157–163.
[29] S. Nair, F. Zhou, R. Reddy, L. Huang, B.T. Rouse, Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro, J. Exp. Med. 175 (1992) 609–612.
[30] J.S. Chang, M.J. Choi, H.S. Cheong, K. Kim, Development of Th1-mediated CD8+ effector T cells by vaccination with epitope peptides encapsulated in pH-sensitive liposomes, Vaccine 19 (2001) 3608–3614.
[31] L. Luo, Y. Li, J.S. Chang, S.Y. Cho, T.Y. Kim, M.J. Choi, et al., Induction of V3-specific cytotoxic T lymphocyte responses by HIV gag particles carrying multiple immunodominant V3 epitopes of gp120, Virology 240 (1998) 316–325.
[32] D.P. Siegel, R.M. Epand, The mechanism of lamellar-to-inverted hexagonal phase transitions in phosphatidylethanolamine: implications for membrane fusion mechanisms, Biophys. J. 73 (1997) 3089–3111.
[33] J. Connor, M.B. Yatvin, L. Huang, pH-sensitive liposomes: acid-induced liposome fusion, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 1715–1718.
[34] N. Düzgünes, R.M. Straubinger, P.A. Baldwin, D.S. Friend, D. Papahadjopoulos, Proton-induced fusion of oleic acid-phosphatidylethanolamine liposomes, Biochemistry 24 (1985) 3091–3098.
[35] D.J. Marciani, Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity, Drug Discov. Today 8 (2003) 934–943.
[36] B. Guy, The perfect mix: recent progress in adjuvant research, Nat. Rev. Microbiol. 5 (2007) 505–517.
[37] V.E. Schijns, E.C. Lavelle, Trends in vaccine adjuvants, Expert Rev. Vaccines 10 (2011) 539–550.
[38] W.M. Li, W.H. Dragowska, M.B. Bally, M.P. Schutze-Redelmeier, Effective induction of CD8+ T-cell response using CpG oligodeoxynucleotides and HER-2/neu-derived peptide co-encapsulated in liposomes, Vaccine 21 (2003) 3319–3329.

[39] M.L. Disis, K.H. Grabstein, P.R. Sleath, M.A. Cheever, Generation of immunity to the HER-2/neu oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine, Clin. Cancer Res. 5 (1999) 1289–1297.
[40] K.L. Knutson, K. Schiffman, M.L. Disis, Immunization with a HER-2/neu helper peptide vaccine generates HER-2/neu CD8 T-cell immunity in cancer patients, J. Clin. Invest. 107 (2001) 477–484.
[41] S. Crotty, R. Ahmed, Immunological memory in humans, Semin. Immunol. 16 (2004) 197–203.
[42] F.G. Gao, V. Khammanivong, W.J. Liu, G.R. Leggatt, I.H. Frazer, G.J. Fernando, Antigen-specific CD4+ T-cell help is required to activate a memory CD8+ T cell to a fully functional tumor killer cell, Cancer Res. 62 (2002) 6438–6441.
[43] J.R. Baldridge, P. McGowan, J.T. Evans, C. Cluff, S. Mossman, D. Johnson, et al., Taking a Toll on human disease: toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents, Expert Opin. Biol. Ther. 4 (2004) 1129–1138.
[44] G. De Becker, V. Moulin, B. Pajak, C. Bruck, M. Francotte, C. Thiriart, et al., The adjuvant monophosphoryl lipid A increases the function of antigen-presenting cells, Int. Immunol. 12 (2000) 807–815.
[45] S. Mazumder, M. Maji, N. Ali, Potentiating effects of MPL on DSPC bearing cationic liposomes promote recombinant GP63 vaccine efficacy: high immunogenicity and protection, PLoS Negl. Trop. Dis. 5 (2011) e1429.