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MPL nano-liposomal vaccine containing P5 HER2/neu-derived peptide pulsed PADRE as an effective vaccine in a mice TUBO model of breast cancer

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Abstract

Liposomal peptide-based vaccines can potentially suppress cancer cells proliferation in the host. To enhance the effectiveness of vaccination against cancer, additional strategies should also be employed. One strategy to promote peptide-based vaccine efficacy and induce powerful immune responses, is simultaneous activation of CD4+ and CD8+ T cells. To address this problem, we tested the efficacy of a nano-liposomal vaccine containing P5 peptide, a cytotoxic T lymphocytes (CTL) specific peptide derivative of rat HER2/neu protein, Pan HLA-DR (PADRE) peptide, a universal CD4+ T helper cell epitope and monophosphoryl lipid A (MPL) a toll-like receptor 4 ligand. We observed potent CD8+ T cell immune responses in TUBO mice vaccinated with liposomal P5 peptide in combination with PADRE and MPL. Also, this formulation remarkably improved anti-tumor effects against cells overexpressing HER2 in BALB/c mice compared to liposomal vaccine containing P5 only. Furthermore, we found that vaccination with Lip-P5-Integrated PADRE-MPL formulation significantly induced IFN-γ production, increased CD8+ T cells numbers and enhanced survival compared to other groups of treated mice. In conclusion, our study indicated that Lip-P5-Integrated PADRE-MPL, after further confirmatory investigations, could be employed as a promising vaccine to generate potent CTL anti-tumor immune responses that could be beneficial to treatment of HER2+ breast cancer.

Key words: Liposomal vaccine, HER2/neu peptide, PADRE peptide, Breast cancer, Cancer immunotherapy, Cancer vaccine
1. Introduction

During the last decade, immunotherapy has attracted considerable attention in cancer treatment. Cancer immunotherapy is rapidly advancing and it is considered as the “fifth pillar” of cancer therapy after surgery, chemotherapy, radiotherapy and targeted therapy [1]. The idea of modulation of the host’s immune system for cancer treatment was originated from the innate properties of the immune system as it is able to identify and eliminate malignant cells during initial transformation in a process called “immune surveillance” [2]. Active immunotherapy approaches such as vaccination with immunogenic peptides, such as tumor-associated antigens (TAAs), was able to stimulate the immune response [3, 4]. Cancer immunotherapy using peptide vaccines has the potential to prevent and inhibit cancer cells proliferation in the host. The use of peptide vaccines could be considered as a simple and attractive approach for activate of the immune system, due to the synthesis and purification of peptides are relatively simple with low cost. As well, they are currently available at both clinical and research grade [5].

Since human TAAs that are endogenously expressed by tumor cells, are poorly immunogenic, they are not detected by either the innate or acquired immune system. Therefore, further and effective stimulation of the immune system seems to be a beneficial approach to enhance the ability of tumor-recognizing cells and reduce the function of immunosuppressive cells, such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), in order to eventually produce a potent and strong immune response [6]. Since cellular immunity plays a pivotal role in elimination of solid tumors, effective induction of CD8+ cytotoxic T lymphocytes (CTLs) can result in effective recognition and killing of tumor cells [7]. For an effective and strong induction of CTL response that would lead to tumor inhibition, it is necessary that the antigen is presented to CD8+ CTLs via major histocompatibility complex (MHC) class I [8]. Since, immunization with naked peptides usually does not induce a potent immune response, successful delivery of TAAs into the cytoplasm of dendritic cells (DCs), antigen processing through MHC class I and induction of CTLs are essential to induce an effective and potent immune response required for complete tumor regression [5].

An enormous amount of recently published data has supported the role of nanoparticles as carriers for delivering antigens and adjuvants in cancer immunotherapy. Among them, liposomes attracted much attention as one of the most promising tools for cancer vaccine [9]. Liposomes are biodegradable, biocompatible, safe, nontoxic and non-immunogenic products that have been frequently used in vaccine development. Moreover, liposomes have significant potential to be readily manipulated and modified by different molecular ligands to effectively and specifically target antigen-presenting cell
(APCs). Therefore, liposomes are considered desirable antigen delivery systems for cancer vaccines and immunotherapy [10]. The potency of liposomes as a carriers to be used in cancer vaccines is affected by several physiochemical properties such as lipid composition, particle size, surface charge, rigidity of lipid bilayers and preparation method [11, 12]. One of the important parameters that affects the potency of liposomes as a carriers for immunotherapy, is their lipid composition. Dioleoylphosphatidylethanolamine (DOPE) is a pH-sensitive lipid that is able to form a hexagonal structure at low pH of endosomal compartment and introduce antigens into MHC class I pathway [13]. The importance of liposomal formulations containing DOPE to induce immune responses against incorporated or associated antigens, was reported by different studies [10, 14, 15]. Considerable evidence indicates that co-administration of antigen and a suitable immunostimulating molecule (i.e. adjuvant) can elicit more powerful responses compared with antigen given alone [16]. Since some of immunostimulating molecules are lipid or hydrophobic molecules, they can be easily incorporated into liposomal bilayer. Hydrophobic adjuvant can be dissolved in an organic solvent and embedded into lipid bilayer for preparation of liposome through dry lipid film methods [17]. One of hydrophobic immunostimulating molecules is monophosphoryl lipid A (MPL), a toll-like receptor 4 (TLR4) agonist. MPL is a potent and FDA-approved adjuvant in human vaccines [18, 19]. MPL is a detoxified form of lipopolysaccharide (LPS) that induces immunity responses via TLR4 stimulation [19]. Until now, several reports indicated the use of MPL in liposomal vaccines could promote CTL response induced by the immunogenic peptide against cancer cells in animal models [14, 20]. As well, one strategy to promote peptide-based vaccine efficacy to induce powerful immune responses is the activation of CD4+ T helper cell immune responses. Therefore, a vaccine that can specifically stimulate antigen-specific CD4+ T cells to enhance vaccine capacity seems to be beneficial. Pan HLA-DR epitope peptide (PADRE) is one of the best molecules to induce CD4+ responses in human and mouse [21]. Recent studies reported that PADRE acts as a potent immunogenic and is capable of enhancing vaccine potency when co-administered with other forms of vaccines [22-24]. In addition, other advantages of PADRE peptide as a T cell epitope, are as follows: it can be easily synthesized with high purity, antibodies are not produced against it and it was found safe and well-tolerated in a number of clinical trials [23, 25, 26]. In our previous studies, we reported that P5 peptide, a CTL specific peptide derivative of rat HER2/neu protein, can effectively induce CTL responses in mice bearing HER2-positive tumors [20, 22, 27, 28]. To investigate whether liposomal P5 peptide in conjunction with PADRE and MPL adjuvant are efficient in inducing CTL responses and anti-tumor immunity, we designed a liposomal vaccine composed of DMPC:DMPG:Chol:DOPE containing MPL, P5 and PADRE peptides to generate a
strong CTL response. P5 peptide containing 21 amino acid (ELAAWCWRGFLALLPPGIAG), was covalently conjugated with the liposomes surface. The prophylactic and therapeutic efficacy of P5-conjugated nanoliposomes composed of MPL and PADRE, in induction of CTL response was then evaluated in vitro and in vivo, using a breast cancer model in BALB/c mice (Fig. 1).

**Fig 1.** Schematic illustrations of liposomal vaccine formulations used for immunization of BALB/c mice to induce CTL response against P5 peptide and treatment of TUBO tumor mice. (A) Linking of peptide to maleimide-PEG$_{2000}$-DSPE through covalent binding between the thiol group of C terminal cysteine residue of peptide and the pyrrole group of maleimide. (B) The maleimide-PEG$_{2000}$-DSPE anchored the peptides to the lipid bilayer. MPL served as an adjuvant. Immunization and therapeutic models of BALB/c mice induce CTL response to P5 peptide mice.
2. Materials and methods

2.1 Materials

Dioleoylphosphatidylethanolamine (DOPE), Dimyristoylphosphoglycerol (DMPG), dimyristoylphosphatidylcholine (DMPC) and distearoylphosphoethanolamine-N (Maleimide-PEG\textsubscript{2000} DSPE) were purchased from Avanti Polar Lipid (Alabaster, USA). Cholesterol and Monophosphoryl lipid A (MPL) were purchased from Sigma-Aldrich (Steinheim, Germany). Mouse anti IFN-γ and Mouse anti IL-10 ELISPot kits were purchased from Mabtech AB, (Stockholm, Sweden). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Invitrogen (Carlsbad, CA). PMA/ ionomycin cocktail, anti-CD8a-PE-cy5, anti CD4-PE-cy5, anti-IL-10-APC antibody, anti-IFN-γ- FITC and antiIL-4-PE antibodies were purchased from BD Biosciences (San Diego, USA). All other used solvents and reagents were chemical grade.

2.2 peptides

P5 peptide containing linker sequence (\textemdash GGC) (ELAAWCRWGFLLALLPPGIAGGGC, purity > 97% and molecular weight (MW) of 2471.98 Dalton (Da)), PADRE peptide (AKFVAAWTLKAAGGC, purity > 97.95%, and MW of 1347.64 Da), PADRE peptide containing linker sequence (\textemdash GGGC) (AKFVAAWTLKAAGGGC, purity > 95.29% and MW of 1621.93 Da) were purchased from China Peptides Co. (Shanghai, China).

2.3 Animal

Female BALB/c mice, four to six weeks old, were purchased from Pasteur Institute (Tehran, Iran). All animals received humane care in compliance with institutional Guidelines under the approval of the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences (MUMS), according to animal welfare guidelines (Project code: MUMS 940815).

2.4 Cell lines and media

TUBO is a cloned cell line that overexpresses rHER2/neu protein on the cell membrane, generated from a spontaneous mammary gland tumor from a BALB-neuT mouse [29]. TUBO cell line was kindly provided by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy), and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented 20% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco,UK). CT26, a murine colon carcinoma cell line that rHER2/neu negative were used as negative control,
purchased from the Pasteur Institute and cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, UK). The cells were cultured at 37°C in an incubator containing 5% CO₂.

2.5 Conjugation of P5 and PADRE peptides with Maleimide-PEG₂₀₀₀-DSPE

P5 and PADRE peptides were separately linked to maleimide-PEG₂₀₀₀-DSPE through covalent binding of the thiol group of the C terminal of cysteine residue (-GGGC) of peptides to the pyrrole group of maleimide, as previously described (Fig. 1) [20]. Briefly, P5 and PADRE peptides, dissolved in dimethyl sulfoxide (DMSO), were conjugated with maleimide-PEG₂₀₀₀-DSPE (dissolved in chloroform), at a molar ratio of 1.2:1 (peptide:maleimide) in DMSO:chloroform (1:1) solution at 37 °C for 48 h. The mixture was dried using a rotary evaporator (Heidolph, Germany) and freeze-dryer (VD-800F, Taitech, Japan), which was followed by hydration with sterile water and bath-sonication for 5 min at 25°C. Thin layer chromatography (TLC) (silica gel 60 F254, Merck, USA) was carried out to confirm the formation of P5-P₂₀₀₀-DSPE and PADRE-P₂₀₀₀-DSPE. The conjugation of peptides with maleimide-PEG₂₀₀₀-DSPE was confirmed using HPLC. KNAUER smart line HPLC (Berlin, Germany) was equipped with a Nucleosil C18, 5 µm, 150 × 4.6 mm, 100Å° column (KENAUER) and a UV detector (KENAUER S2600) set at 220 nm. The mobile phases employed for analysis were A (water + 0.1% Trifluoroacetic acid (TFA)) and B (acetonitrile + 0.1% TFA). Elution program for P5 peptide was a gradient starting with 100% A and increasing concentrations of B to 30% in 2 min, 60% in 10 min and 90% in 2 min. The flow rate was 1 ml/min. The gradient elution program for PADRE peptide was started with 100% A followed by increased concentrations of C to 20% in 2 min and 80% A in 2 min. The flow rate was 1 ml/min.

2.6 Nano-liposome preparation

Liposomes were composed of DMPC:DMPG:Chol:DOPE at a molar ratio of 60:8:12:20, with a lipid concentration of 50 mM prepared using lipid film hydration method as previously described [30]. Briefly, lipids were first dissolved in chloroform and then combined in sterile glass tubes. In order to prepare liposomes containing MPL, 0.25 mg/ml monophosphoryl lipid A (MPL) was added to the lipid solutions. Rotary evaporator (Heidolph, Germany) and freeze-drier (VD-800F, Taitech, Japan) were used to remove the solvents from the lipid solutions. The lipidic thin film were then hydrated with HEPES buffer, 5% dextrose (10 mM, pH 7.2) and vortexed to completely disperse the lipids into the buffer. The resulting multilamellar vesicles (MLVs) were extruded through 1000, 400, 200 and 100 nm
polycarbonate membranes using an extruder (Avestin, Canada) to form 100 nm small unilamellar vesicles (SUVs) of uniform size.

To prepare liposomal formulation containing P5 and PADRE peptides, P5-mPEG$_{2000}$-DSPE and PADRE-mPEG$_{2000}$-DSPE micelles at concentration of 100µg/ml were post-inserted into the liposomes at 55°C at 250 RPM (Innova 4080 Incubator shaker) and shaken for 4h under argon gas. Moreover, in order to prepare liposomal formulation containing PADRE (integrated PADRE), 100 µg/ml of PADRE peptide (AKFVAAWTLKAAA) dissolved in HEPES buffer, 5% dextrose (10 mM, pH 7.2) was added to the liposomes at 55°C at 250 RPM and shaken for 4h under argon gas. The final liposomal formulations were sterilized by filtration through a 0.22 µm microbial syringe filter.

2.7 Liposome characterization

The amount of phospholipids was determined by the Bartlett phosphate assay method [31]. The MPL content of liposomal formulations were assayed by the Limulus amebocyte lysate (LAL) chromogenic endpoint assay (QCL-1000, Lonza, Walkersville, MD) [32]. The particle characterization (size (nm), polydispersity index (PDI) and zeta potential (mV)) was done by a dynamic light scattering (DLS) instrument (Nano-ZS; Malvern, Southborough, UK). Also, the morphological characteristics of liposomes were determined using transmission electron microscopy (TEM) (Zeiss, Jena, Germany) [33]. The percent of encapsulation efficiency of PADRE peptide into liposomes were measured with a commercial BCA Protein Assay Kit with a detection range of 20 to 2000 µg/mL (Thermo Scientific, Rockford, IL, USA) as described previously [34]. Before performing BCA assay unencapsulated peptides were removed by dialysis using a 300-kDa molecular weight cut-off (MWCO) dialysis membrane (Spectrum, Houston, TX, USA) against HEPES buffer, 5% dextrose (10 mM, pH 7.2). The encapsulation efficiency of PADRE was calculated by the following formula:

Encapsulation efficiency = \[ \frac{\text{peptide content after dialysis}}{\text{peptide content before dialysis}} \times 100 \]

2.8 In vitro cellular uptake

In order to confirm the uptake of liposomes containing antigenic peptide, mature DCs were generated from mouse bone marrow as previously describe [22]. Briefly, the mouse bone marrow cells were harvested from the femur and tibia bones of mice and cultured in Iscove’s modified Dulbecco’s media (IMDM) (Invitrogen) supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin. Subsequently, granulocyte monocyte colony-stimulating factor (GM-CSF) and IL-4 were added into the medium at a final concentration of 25 ng/ml and 5 ng/mL, respectively and incubated at
37°C in an incubator with 5% CO₂ for 7 days. On days 3 and 5, the cultured medium was replaced with the fresh medium and cells were seeded in new plates. On day 7, cells were stimulated with 1 μg/ml lipopolysaccharide (LPS) and incubated at 37°C for 6 h. Next, the mature DCs were obtained.

DiI-labeled liposomes were prepared by adding % 0.2 mole ratio of the DiI fluorescent dye to basic liposomal composition (DMPC:DMPG:Chol:DOPE, at a molar ratio of 60:8:12:20, with a lipid concentration of 50 mM). Liposomes were prepared using lipid film hydration method according to the above procedure. The particle size and zeta potential of the resulting liposomes were analyzed. Liposomes were post-inserted with P5-mPEG₂₀₀₀-DSPE and PADRE-mPEG₂₀₀₀-DSPE micelles. For the uptake assay, mature DCs at a density of 10⁶ cells/ml were incubated with DiI–labeled liposomes (100 nmol/mL) at 37 and 4°C for 1 hr. Untreated DCs were used as control groups. Liposomes uptake by DCs were analyzed by flow cytometry on the FL2 channel and the mean fluorescence intensity was measured.

2.9 Immunization of BALB/c mice

2.9.1 Immunization schedule

One hundred BALB/c mice were randomly divided into 10 experimental groups (10 mice per group) and subcutaneously vaccinated with different liposomal formulations for three times with two-week intervals. Each liposomal formulation with lipid dose of 5 μmol per mice, was administered. Free P5 peptide (10 μg/mouse) and free p5/PADRE peptides (10 μg/mouse at 1:1 ratio) in HEPS-dextrose 5% buffer, were given to control groups.

2.9.2 Splenocytes isolation

Two weeks after the last vaccination, three mice per group were sacrificed by injection of 100μl of ketamine-xylazine solution (100 mg/kg ketamine and 10 mg/kg xylazine) [35]. Spleens were collected and homogenized by cell strainer under sterile conditions. The splenocytes were aseptically harvested by using ACK buffer (0.15 M NH₄Cl, 1.0 M KHCO₃, and 0.1 mM Na₂EDTA). Viable splenocytes were counted using trypan blue (0.4% w/v) (Gibco) and re-suspended in RPMI-1640 medium supplemented with 10% FBS.

2.10 In vitro study

2.10.1 Enzyme-linked immunospot (ELISpot) assay

The ELISpot assay was used for the evaluation of IFN-γ and IL-10 expression in the splenocytes in response to the P5 peptide. ELISpot assays were carried out using mouse basic ELISpot kits from
Mabtech AB, (Stockholm, Sweden) according to the manufacturer’s instructions. Briefly, one day before mice sacrifice, ELISpot 96-well plates (PVDF-paltes, type MSIP, Mabtech) were coated with anti-IL-10 and anti-IFN-γ antibodies and incubated overnight at 4 °C. Splenocytes isolated from sacrificed mice, were cultured in triplicate (3 × 10^5 cells/200 µL per well) in plates pre-coated with medium supplemented with 10% FBS, and stimulated with P5 peptide (10 µg/ml). Control cultures done in triplicate (1 × 10^5 cells/200 µL per well), were incubated with medium alone, or mitogens (phytohemagglutinin (PHA) 10 µg/mL). Cells were incubated at 37°C with 5% CO₂ for 24 h at 37 °C for IFN-γ and 48 h at 37 °C for IL-10 determination. After incubation period, plates were prepared for detection of spots according to the manufacturer’s instructions. When the spots appeared, counting was performed using Kodak 1D image analysis software (version 3.5, Eastman Kodak, Rochester, New York).

2.10.2 Evaluation of intracellular cytokines using flow cytometric analysis

Flow cytometry was performed on the isolated splenocytes to evaluate their response to the P5 peptide, using a BD FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ, USA). To measure intracellular cytokines, splenocytes (10^6 cells/ml) were cultured in RPMI-1640 medium supplemented with 10% FBS. The cells were then activated by P5 peptide (10 µg/ml) 12 h at 37 °C. After incubation period, brefeldin A solution (2 µl/ml) (BioLegend, San Diego, CA) was added to cell media and incubated for 4 h at 37 °C. As positive control, the splenocytes (10^5 cells/ml) in medium containing 1 µl/ml GolgiPlug™ were stimulated with 2 µl/ml PMA/ionomycin cocktail for 4 h at 37 °C. After stimulation, splenocytes (10^5 cells) were washed with staining buffer (2% FBS in PBS) and stained with flow cytometry antibodies (BD Biosciences) as previously describe [14]. Briefly, 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 staining 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 and anti-IL-10-APC 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 staining buffer for flow cytometric analysis (BD FACSCalibur™, BD Biosciences, San Jose, USA).

2.10.3 In vitro cytotoxicity assay

Two weeks after the final vaccination, mice were sacrificed (three mice per group) and splenocytes were harvested. The target cells, TUBO tumor cells and CT26 cells (used as negative control) were
incubated with calcein acetoxymethyl (Calcein-AM, Invitrogen, USA) (12.5 µM), then, incubated for 1 h at 37 °C in the dark. After incubation, targeted cells (1.2 × 10^5 cell/well) were co-cultured with different concentrations of the splenocytes (as effector cells). Triton X-100 (2%) and culture medium were added to the wells with maximum and minimum release, respectively and incubated for 4 h at 37°C. Fluorescence intensity was measured at excitation of 485 nm and emission of 538 nm by a fluorescent plate reader (FLx 800, Bio-Tek Instruments Inc., and Beverly, Massachusetts). The mean percentage of specific lysis of triplicate wells was calculated using the following formula: \[
\text{(release by CTLs - minimum release by targets)/(maximum release by targets - minimum release by targets) \times 100}
\]

2.11 In vivo study

2.11.1 TUBO tumor challenge in prophylactic model

Two weeks after the last booster injection, the immunized mice (seven mice per group) were challenged with 5 × 10^5 TUBO cells in 50 µl PBS buffer, subcutaneously administered to the right flank. Weight of mice and tumor size were monitored and calculated regularly. Tumor volume were calculated according to the formula: \((\text{length} \times \text{width} \times \text{height}) \times 0.52\) [36]. The mice were discarded if the weight loss was <15% of their initial weight or size of tumors was ≥1000 mm^3. Time to reach the end-point (TTE) for each mouse was calculated based on the equation of the line obtained by exponential regression of the tumor growth curve, and tumor growth delay (% TGD) for each treatment group was calculated based on the following equation: \(\% \text{TGD} = (\text{the mean TTE of treatment group - the mean TTE of the buffer group}) / \text{the mean TTE of the buffer group} \times 100\). Also, median survival time (MST), and increased life span (%ILS: MST of treated group/MST of buffer group ×100) – 100) were calculated for each mouse group.

2.12 Assessment of therapeutic effects in a mouse model of breast cancer

2.12.1 TUBO challenge in therapeutic model

The therapeutic experiment was performed in a total of 90 female BALB/c mice (4–6 weeks old). Mice were challenged with 5 × 10^5 TUBO cells in 50 µl PBS buffer, subcutaneously administered to the right flank. Ten days after tumor inoculation, when the tumors were palpable and reached a size of 3 mm, mice were randomly divided into 10 treatment groups (n=9 mice per group). Mice were immunized with different liposomal formulations for three times with two-week interval. Mice were monitored regularly and tumor volume was calculated according to the formula mentioned above.
### 2.12.2 Flow cytometry analysis of splenocytes and tumor-infiltrated lymphocytes

Analysis of tumor-infiltrated lymphocytes (TILs) and intracellular cytokine were performed by a FACSCalibur flow cytometer. Two weeks after the last vaccination, three mice per group were sacrificed. Spleens and tumors were immediately removed and washed with PBS under sterile conditions. Splenocytes were isolated according to the above-noted methods. Also, the tumor tissues were collected, and washed twice with 10 mM PBS (pH 7.4); then, tissue pieces were manually minced using a scalpel, followed by a 60 min enzymatic digestion using 2 mg/ml collagenase type I solution (Gibco) diluted with 10 mM PBS (pH 7.4) (1:1 ratio), at 37 °C with gentle mixing. After incubation period, digest was quenched by adding RPMI-1640 medium supplemented with 10% FBS. Then, digested tissue was filtered through a cell strainer and centrifuged at 1500 RPM for 10 min. The resulting pellet was resuspended in flow cytometry staining buffer and total tumor cells were counted using trypan blue staining (0.4%, w/v). The TILs and splenocytes were analyzed by flow cytometry for the expression of T cells’ surface and intracellular markers. Moreover, for the experimental groups that were euthanized due to the tumor volume exceeding 1000 mm$^3$, the spleens and tumors were harvested and splenocytes and tumor cells were analyzed by flow cytometry.

### 2.13 Statistical analysis

Statistical analysis was carried out by two-way analysis of variance (ANOVA) and Tukey's post-test. Survival data were analyzed by log-rank (Mantel-cox) test to compare survival curve. A $P < 0.05$ were considered significant. Graph Pad Prism 6 Software was used to analyze all data.

### 3. Results

#### 3.1 Conjugation of P5 and PADRE peptides with PEG$_{2000}$-DSPE

Formation of P5-mPEG$_{2000}$-DSPE and PADRE-mPEG$_{2000}$-DSPE through binding the activated maleimide with thiol group of the peptides were identified by TLC [Fig. S1A](#). Disappearance of maleimide-PEG$_{2000}$-DSPE spot from the reaction mixture was confirmed by iodine vapor. As shown in Fig S1A, maleimide-PEG$_{2000}$-DSPE spot (spots 3 and 6) completely disappeared from the reaction mixture (spots 1 and 4) which indicates an almost complete reaction between maleimide group of PEG$_{2000}$-DSPE and thiol on P5 and PADRE peptides.

The efficiency of the peptides conjugation and amount of unconjugated (free) peptides were determined using HPLC analysis. Free peptides (P5 and PADRE), used as a reference were also
analyzed by HPLC. The extent of unconjugated peptides indicated almost complete reaction between maleimide group of PEG<sub>2000</sub>-DSPE and thiol in P5 and PADRE peptides (Fig. S1B and C).

### 3.2 Characteristics of nano-liposomal particles

In this study, two different liposomal formulations DMPC:DMPG:DOPE:Chol (at molar ratio of 60:8:20:12, respectively) and DMPC:DMPG:DOPE:Chol:MPL (at molar ratio of 59.80:7.97:19.93:11.96:0.32, respectively) were prepared with a lipid concentrations of 50 mM. The phospholipid concentrations of the liposomal formulations, as determined by phospholipid assay, were 39 mM and 40 mM for liposomal formulation in the absence and presence of MPL, respectively, in the final products. Also, based on BCA assay the encapsulation efficiency percentage of PADRE peptide was between 29.36% - 34.13%.

The particle characteristics (size (nm), polydispersity index (PDI) and zeta potential (mV)) were determined and presented in Table 1. Liposomal formulations ranged from 120 to 140 nm in size and had a negative zeta potential due to the presence of negatively-charged lipid DMPG in the formulation, which is desirable for the stability of formulations as it prevents from aggregation. Meanwhile, the negatively-charged liposomes are more potent vaccine delivery systems compared to neutral liposomes [37]. The homogeneity of the liposomal formulations was confirmed by the PDI < 0.2. Moreover, based on the TEM images (Fig. 2) the liposomes having almost spherical shape and are relatively uniform with the size of around 120 to 140 nm which show a high compatibility with data in Table 1.

It should be noted that the physicochemical characteristics of liposomal vaccines such as lipid content, liposomal size, as well as surface charge have an important role in the produced immune response [38, 39].

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Liposomal composition</th>
<th>Lipid Molar ratio</th>
<th>Z average&lt;sup&gt;a&lt;/sup&gt; (nm) ±SD</th>
<th>Zeta potentials (mV) ±SD</th>
<th>PDI&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Empty liposome</td>
<td>DMPC:DMPG:DOPE:Chol</td>
<td>60:8:20:12</td>
<td>138.3 ± 0.9</td>
<td>-24.3 ± 0.1</td>
<td>0.01 ± 0.002</td>
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<tr>
<td>Lip-P5</td>
<td>DMPC:DMPG:DOPE:Chol:P5-mPEG&lt;sub&gt;2000&lt;/sub&gt;-DSPE</td>
<td>59.95:7.99:19.98:11.99:0.08</td>
<td>149.4 ± 0.2</td>
<td>-20.5 ± 0.5</td>
<td>0.05 ± 0.004</td>
</tr>
<tr>
<td>Liposome Type</td>
<td>Composition</td>
<td>Z-average (nm)</td>
<td>Polydispersity Index</td>
<td>Standard Deviation (SD)</td>
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<td>Lip-P5-Linked PADRE</td>
<td>DMPC:DMPC:DOPE:Chol:</td>
<td>59.88:7.98:19.96:11.97:0.08:0.12</td>
<td>136.5 ± 0.2</td>
<td>-17.1 ± 1.2</td>
<td>0.06 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>P5-mPEG_{2000}-DSPE:PADRE-mPEG_{2000}-DSPE</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Lip-P5- Integrated PADRE</td>
<td>DMPC:DMPC:DOPE:Chol:</td>
<td>59.95:7.99:19.98:11.99:0.08</td>
<td>128.1 ± 0.7</td>
<td>-16.8 ± 0.9</td>
<td>0.04 ± 0.002</td>
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<tr>
<td></td>
<td>P5-mPEG_{2000}-DSPE:PADRE-mPEG_{2000}-DSPE :MPL</td>
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<tr>
<td>Lip-P5-Linked PADRE-MPL</td>
<td>DMPC:DMPC:DOPE:Chol:</td>
<td>59.68:7.95:19.89:11.93:0.08:0.12:0.32</td>
<td>144.6 ± 0.5</td>
<td>-36.8 ± 0.9</td>
<td>0.03 ± 0.009</td>
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<td>P5-mPEG_{2000}-DSPE:PADRE-mPEG_{2000}-DSPE :MPL</td>
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<td></td>
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<tr>
<td>Lip-P5- Integrated PADRE-MPL</td>
<td>DMPC:DMPC:DOPE:Chol:</td>
<td>59.75:7.96:19.92:11.95:0.08:0.32</td>
<td>130.5 ± 0.9</td>
<td>-33.8 ± 2.2</td>
<td>0.02 ± 0.001</td>
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<td></td>
<td>P5-mPEG_{2000}-DSPE:PADRE-mPEG_{2000}-DSPE :MPL</td>
<td></td>
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</table>

\(^a\) The size of liposomes (Z average).
\(^b\) Polydispersity index.
\(^c\) The concentration of integrated PADRE was 100µg/ml

The particle size of liposomes can impact the pharmacokinetic profile of vaccines as smaller liposomes were shown to be cleared faster from the site of injection compared to larger liposomes. Larger liposomes can be retained at the site of injection and they more effectively enter the lymph nodes and induce more effective immune responses [38, 39]. The doses of antigenic peptide and MPL adjuvant plays an important role in liposomal vaccine efficacy and T cell immune response induction [40]. Therefore, the dose of antigenic peptide and adjuvant were accurately determined for different liposomal formulations. The MPL content was 24 µg per mouse and the peptide content (for P5 and PADRE separately) were precisely determined 10 µg per mouse for each liposomal formulation.
Fig. 2. Transmission electron microscopy (TEM) images of negatively stained liposomal formulations. A) Empty liposome, B) Lip-P5, C) Lip-P5-Linked PADRE, D) Lip-P5- Integrated PADRE, E) Lip-P5-Linked PADRE- MPL, F) Lip-P5-Integrated PADRE–MPL.

3.3 In vitro cellular uptake
The liposomal uptake by mouse DCs was evaluated in vitro. The results showed that mature DCs could uptake liposomes at 37°C. As illustrated in Fig. S2, the endocytic activity of DCs for liposomes at 37°C was greater than that observed at 4°C, with mean fluorescence intensity of 14 and 5.02, respectively. These results indicated that liposomes could be effectively up-taken by mature DCs in vitro.

3.4 IFN-γ and IL-10 secretion assay by ELISpot
To determine the induction of immune responses, splenocytes were isolated 14 days after administration of the last booster. The splenocytes were then stimulated with the P5 peptide (+P). IFN-γ and IL-10 secretion from splenocytes was measured by ELISpot assay. Splenocytes which were not activated by P5 peptide and incubated with medium alone, were considered controls (-P5). The results indicated that splenocytes isolated from the mice immunized with formulations containing the CD4+ T helper epitope PADRE, released higher amounts of IFN-γ in comparison to P5 group (P<0.0001) (Fig. 3A).

As shown in Fig. 3A, the response against free PADRE or P5 peptide was not significant and the immunization with PADRE or P5 could not induced IFN -γ response in mice. While, co-administration of PADRE with P5 was able to induce IFN -γ response in mice (P<0.001).
Collectively, these data showed that co-administration of P5 peptide with PADRE peptide in the liposomal formulation, could significantly enhance the induction of IFN-γ release compared to the other formulations. Also, we found significant differences between integrated and linked PADRE peptide in liposomal formulation. The IFN-γ secretion induced by liposomal formulations Lip-P5-Integrated PADRE-MPL and Lip-P5-Integrated PADRE were generally higher than that of Lip-P5-Linked PADRE and Lip-P5-Linked PADRE-MPL formulations (P<0.0001).

As shown in Fig 3.B, no significant differences were observed between splenocytes isolated from the mice immunized with Lip-P5-Integrated PADRE-MPL, Lip-P5-Integrated PADRE and Lip-P5-Linked PADRE and P5 group with respect to IL-10 release. While splenocytes isolated from the mice immunized with Lip-P5 and Lip-P5-Linked PADRE-MPL were significant different from P5 group in terms of IL-10 secretion (P<0.001) (Fig. 3B).

Fig. 3. The efficacy of different formulations in inducing IFN-γ (A) and IL-10 (B) production in vaccinated mice. In vitro IFN-γ and IL-10 production were analysed in vaccinated mice 2 weeks after the last booster by ELISpot assay. Spleens of mice (n=3) were harvested and activated with peptide (+ P5) and the induced immune responses were then evaluated using ELISpot assays. Splenocytes which were not activated with peptide and incubated with medium alone used as control (-P5).
IFN-γ (C) and IL-10 (D) expression in the splenocytes in response to the P5 peptide on the ELISpot plate. Positive and negative controls containing mitogens (Phytohemagglutinin (PHA)) at 10 µg/mL and culture medium alone, respectively. All groups were compared to P5 group. The data indicate the mean ± SD (n = 3). Statistically significant differences designated as follows: ns: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.

3.5 Intracellular cytokine production assay by flow cytometry

To evaluate the immune response induced in the vaccinated mice, secretions of intracellular cytokines (IL-4, IL-10 and IFN-γ) were assayed in CD4+ and CD8+ T cell populations by flow cytometry. Data revealed that the mice vaccinated with Lip-P5-Integrated PADRE-MPL showed a significantly higher level of IFN-γ compared to P5 group (P <0.0001) (Fig. 4 D), and indicated larger CD8+ T cell population compared to the free P5 peptide group (P <0.0001) (Fig. 4A). These findings showed that immunization with P5, MPL, and PADRE induced a potent CTL immune response (Fig. 4).

As shown in Fig. 4 F, no statistically significant differences in the production of IL-4 from CD4+ T-cells were seen between the different groups (P > 0.05).

Also, no statistically significant differences in IL-10 production by CD4+ T-cells were seen between different groups and free P5 peptide group (P > 0.05) (Fig. 4G).

Although there was no statistically significant difference in the level of IL-10 in CD4+ cells’ population, in the Lip-P5-Integrated PADRE-MPL group, this amount was lower than that measured in the free P5 peptide and buffer groups (P > 0.05, Fig. 4G).
Fig. 4. Flow cytometry assay with splenocytes of immunized mice. The frequency of CD8+ cells population (A), CD4+ cells population (B), CD4+/CD25+/FOXP3+ cells population (C). Geometric mean fluorescence intensity (MFI) of IFN-γ in gated CD4+ cells (D), IFN-γ in gated CD8+ cells (E) IL-4 in gated CD4+ cells (F) and IL-10 in gated CD4+ cells (G). Fourteen days after the last booster spleens of mice (three per group) were isolated and stained with PE labeled CD8 or CD4 surface markers and then stained with anti-IFN-γ-FITC or anti-IL-4-PE antibodies, respectively. The frequency of IFN-γ producing cells within the CD8+ or CD4+ population as well as IL-4 and IL-10 producing cells within the CD4+ population were then determined by FACSCalibur Cell analyzer. All groups were compared to P5 group. Data represent mean ± SEM (n = 3). Statistically significant differences designated as follows: ns: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.

3.6 Cytotoxic effects of splenocytes against TUBO cells

The effect of vaccination was evaluated in splenocytes by cell cytotoxicity assay. A higher specific cytotoxicity was observed in groups immunized with Lip-P5-Integrated PADRE-MPL, Lip-P5-Integrated PADRE and Lip-P5-linked PADRE-MPL compared to those treated with P5 (P<0.0001) (Fig. 5). As shown in Fig. 5, at 20 and 40 ratios of effector (splenocytes) to target (TUBO) cells (E/T ratios), the percentage of TUBO cells killed by CD8+ cells were greatest with Lip-P5-Integrated PADRE-MPL formula. This indicates that vaccination with formulations containing P5, integrated PADRE and MPL, induced a significant CTL immune response. CTLs did not kill HER2/neu-negative CT26 cells, indicating that the toxicity of splenocytes was specific to HER2+cells. Also, we found significant differences between integrated and linked PADRE peptide in liposomal formulation. CTL response induced by liposomal formulations Lip-P5-Integrated PADRE-MPL and Lip-P5-Integrated PADRE were generally greater than that produced by Lip-P5-linked PADRE and Lip-P5-linked PADRE-MPL formulations (P<0.0001). These findings indicated that the effector cells generated by liposomes with a co-integrated CD4+ T cell epitope PADRE, could efficiently lyse target cells.
Fig. 5. Antigen specific CTL response. Induction of specific CTL response for tumor cell killing by the different formulations was assessed by in vitro CTL activity assay. Two different ratios of effector to target cells (E/T) were tested. HER2/neu-expressing TUBO cells and CT26 cells (as a negative control) were labelled by Calcein AM and co-incubated with different ratios of splenocytes. All groups were compared to P5 group. The data are expressed as means ± SD (n = 3). E: effector cells and T: target cells. Statistically significant differences are shown as follows: ns: P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.7 In vivo prophylactic efficacy in TUBO tumor model of mice

The prophylactic efficacy of the immunization schedule in the mouse model that comprised TTE, % TGD, MST and %ILS is summarized in Table 2.

Table 2.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TTE(^\text{a}) (days±S.D.)</th>
<th>TGD(^\text{b})%</th>
<th>MST(^\text{c}) (day)</th>
<th>ILS(^\text{d}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>58.1±4.2</td>
<td>-</td>
<td>55.1</td>
<td>-</td>
</tr>
<tr>
<td>P5</td>
<td>59.5±6.8</td>
<td>2.3</td>
<td>60.1</td>
<td>3.8</td>
</tr>
<tr>
<td>PADRE</td>
<td>55.1±3.3</td>
<td>-5.0</td>
<td>57.9</td>
<td>5.0</td>
</tr>
<tr>
<td>P5-PADRE</td>
<td>57.7±3.7</td>
<td>-0.6</td>
<td>56.3</td>
<td>2.2</td>
</tr>
</tbody>
</table>
The log-rank (Mantel-cox) survival analysis (up to 100 days) indicated statistically significant differences in the survival rate among the groups (P < 0.0001). Survival analysis also revealed that the Lip-P5-Integrated PADRE-MPL and Lip-P5-linked PADRE-MPL formulations were the most effective formulations for vaccination and they could significantly reduce tumor growth of the Her2/neu-expressing TUBO cell line (Fig. 6C). Also, Lip-P5-Integrated PADRE-MPL and Lip-P5-linked PADRE-MPL formulations had the longest survival times and significantly prolonged TTE compared to the buffer. The lower tumor size, longer MST and higher percentage of increase of life span (%ILS) were seen in the Lip-P5-Integrated PADRE-MPL group (MST = 100 days, and %ILS = 81.4%), compared to those observed for the Lip-p5 group (MST = 55.1 days and %ILS = 0.1%) (Table 2 and Fig. 6). These data suggest that prophylactic effect of Lip-P5-Integrated PADRE-MPL group had a %TGD of 60.3% which plays an important role in long-term survival.
Fig. 6. Protective effects of vaccination with different liposomal formulations in BALB/c mice against a TUBO tumor model. Two weeks after the last booster, seven mice in each group were challenged subcutaneously with $5 \times 10^5$ TUBO cells. Mice were observed for tumor growth and survival. (A) Tumor volume (mm$^3$) of each mouse in each vaccinated group were evaluated and compared with Buffer groups. The dotted line represents the last day that all mice have died in the buffer group. (B) Average tumor volume in all treated groups. The values are means of tumor size ± SEM. (C) Survival analysis of all vaccinated groups were monitored by the multiple comparison log-rank (Mantel–Cox) test. Effects of immunization on survival time were monitored for a period of 100 days among BALB/c mice ($n = 7$). All groups were compared to P5 group. Statistically significant differences are shown as follows: ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.8 In vivo therapeutic efficacy in TUBO tumor model of mice

The therapeutic efficacy of liposomal formulations in mice is summarized in Table 3 with respect to MST, TTE, %TGD and ILS.
Table 3: Therapeutic efficacy data of different liposomal vaccine formulations in TUBO tumor mice model (n = 6).

<table>
<thead>
<tr>
<th>Groups</th>
<th>TTEa (days±S.D.)</th>
<th>TGDb (%)</th>
<th>MSTc (day)</th>
<th>ILSd (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>41.3±4.9</td>
<td>-</td>
<td>37.4</td>
<td>-</td>
</tr>
<tr>
<td>P5</td>
<td>44.9±5.5</td>
<td>8.7</td>
<td>41.7</td>
<td>11.5</td>
</tr>
<tr>
<td>PADRE</td>
<td>44.0±5.5</td>
<td>6.3</td>
<td>43.3</td>
<td>15.7</td>
</tr>
<tr>
<td>P5-PADRE</td>
<td>44.5±6.1</td>
<td>7.6</td>
<td>44.9</td>
<td>20.0</td>
</tr>
<tr>
<td>Empty liposome</td>
<td>45.6±6.7</td>
<td>10.2</td>
<td>42.3</td>
<td>13.1</td>
</tr>
<tr>
<td>Lip-P5</td>
<td>45.0±3.0</td>
<td>8.7</td>
<td>43.8</td>
<td>17.2</td>
</tr>
<tr>
<td>Lip-P5-Linked PADRE</td>
<td>55.5±6.3</td>
<td>34.2</td>
<td>54.7</td>
<td>46.3</td>
</tr>
<tr>
<td>Lip-P5-Integrated PADRE</td>
<td>60.7±8.2</td>
<td>46.8</td>
<td>60.8</td>
<td>62.4</td>
</tr>
<tr>
<td>Lip-P5-Linked PADRE- MPL</td>
<td>69.3±6.2</td>
<td>67.6</td>
<td>70.5</td>
<td>88.4</td>
</tr>
<tr>
<td>Lip-P5-Integrated PADRE- MPL</td>
<td>71.3±8.1</td>
<td>72.4</td>
<td>73.2</td>
<td>95.7</td>
</tr>
</tbody>
</table>

a Time to reach end-point.
b Tumor growth delay (in comparison with buffer group).
c Median survival time.
d Increase life span.

As shown in Fig. 7, survival analysis (up to 80 days) indicated that the lowest tumor growth and the longest survival time were seen in Lip-P5- linked PADRE-MPL and Lip-P5- Integrated PADRE-MPL groups and the maximum volumes of tumors were >1000 mm³, 75 days post-tumor inoculation for Lip-P5- linked PADRE-MPL. Two mice in Lip-P5- integrated PADRE-MPL group survived till the end of the test. While, 48 days post-tumor inoculation, all mice in the buffer group died and were excluded from the study.

The survival analysis results revealed statistically significant differences in the survival rates among different groups (P < 0.0001). Survival data showed that the greatest MST and %ILS were seen in the Lip-P5- integrated PADRE-MPL group (MST = 73.2 days and %ILS = 95.7%), compared to those obtained for the Lip-p5 group (MST = 43.8 days, and %ILS = 17.2%) (Table 3 and Fig. 7).

Based on these results, it could be concluded that liposomal formulation Lip-P5- integrated PADRE-MPL has a higher therapeutic efficacy, as compared to other formulations.
Fig. 7. Therapeutic effects of administered formulations in BALB/c mice against a TUBO tumor model. 10 days after inoculation of $5 \times 10^5$ TUBO cells to six mice in each group, different liposomal formulations were administered three times with two weeks’ intervals and tumor sizes were calculated. (A) Therapeutic efficacy of different liposomal formulations and tumor volume (mm$^3$) of each mouse in each treatment group were evaluated and compared with Buffer groups. The dotted line represents the last day that all mice have died in the buffer group. (B) Average tumor volume in all treated groups. The values are means of tumor size ± SEM. (C) Survival analysis of therapeutic groups were monitored by the multiple comparison log-rank (Mantel–Cox) test. Effects of treatments on survival time were monitored for a period of 80 days among BALB/c mice (n = 6). All groups were compared to P5 group. Statistically significant differences are shown as follows: ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

3.9 Flow cytometry analysis of splenocytes and tumor infiltrated lymphocytes

To investigate the immune response induced in the therapeutic model, intracellular cytokine and tumor-infiltrated lymphocytes (TILs) were analyzed by a flow cytometer. Intracellular cytokines (IL-4, IL-10 and IFN-$\gamma$) secretions were assayed in CD4$^+$ and CD8$^+$ T cell populations isolated from splenocytes. Results demonstrated that the mice treated with Lip-P5-integrated PADRE ($P <0.001$) and Lip-P5-integrated PADRE-MPL ($P <0.01$) had larger CD8$^+$ T cell population compared to the group treated with free P5 peptide (Fig. 8A). Also, the mice treated with Lip-p5-linked PADRE, Lip-P5-
PADRE, Lip-p5-linked PADRE MPL and Lip-P5- Integrated PADRE-MPL showed larger CD4⁺ T cell population compared to other groups (P <0.0001) (Fig. 8B). As shown in Fig. 8D, mice treated with Lip-P5- Integrated PADRE (P <0.0001), Lip-P5- Integrated PADRE-MPL (P <0.0001) and Lip-P5-linked PADRE-MPL (P <0.001) showed a significant level of IFN-γ compared to other groups. These data showed that treatment with P5, MPL, and PADRE induced a potent CTL immune response in tumor environment (Fig. 8).

There was no statistically significant difference among different groups in IFN-γ and IL-4 production by CD4⁺ T cells (P > 0.05). Also, no statistically significant differences were observed between different groups and free P5 peptide group in IL-10 production by CD4⁺ T cells (P > 0.05) (Fig. 8G).

Fig. 8. Flow cytometry assay with splenocytes isolated from treated mice. The frequency of CD8⁺ cells population (A), CD4⁺ cells population (B), CD4⁺/CD25⁺/FOXP3⁺ cells population (C). Geometric mean fluorescence intensity (MFI) of IFN-γ in gated CD4⁺ cells (D), IFN-γ in gated CD8⁺ cells (E) IL-4 in gated CD4⁺ cells (F) and IL-10 in gated CD4⁺ cells (G). Fourteen days after the last booster spleens of treated mice (three per group) were isolated and evaluated by flow cytometer. The frequency of IFN-γ producing cells within the CD8⁺ or CD4⁺ population as well as IL-4 and IL-10 producing cells within the CD4⁺ population were determined. All groups were compared to P5 group. Data represent mean ± SEM (n = 3). Statistically significant differences designated as follows: ns: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.

Tumor-infiltrated lymphocytes (TILs) analysis indicated a higher percentage of CD8⁺ T cells in the Lip-P5-Integrated PADRE groups (8.43 ± 0.5%) in the tumor area, while a lower percentage of CD8⁺ T
cells was seen in the PADRE group (4.47 ± 0.2%) (Fig. 9A). Results indicated that there were no statistically significant differences in %CD4+ cells of TIL among different groups (P > 0.05) (Fig. 9B). Also, no statistically significant differences were seen in %CD4+ CD25+ FoxP3 cells in the tumor area among different groups (P > 0.05) (Fig. 9C).

Fig. 9. Flow cytometry analysis of tumor-infiltrating lymphocytes (TILs). Two weeks after last vaccination, three mice per group were sacrificed and tumors cells were isolated. TILs of treatment groups with different liposomal formulations were analyzed. The TILs were analyzed by flow cytometry for the expression of % CD4+, CD8+, CD25+, and Foxp3. All groups were compared to P5 group. Data represent mean ± SEM (n = 3). Statistically significant differences designated as follows: ns: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001, ****: P<0.0001.

4. Discussion

In the current study, we attempted to promote immunogenicity and anti-tumor activity of P5 peptide, a CTL multi-epitope peptide derived from rHER2/neu protein, plus PADRE peptide, a CD4+ T cell activator, using a liposomal delivery system. For these reasons, we conjugated P5 peptide with maleimide-PEG2000-DSPE to improve peptide incorporation into liposomes composed of DMPC:DMPG:DOPE:Chol containing PADRE peptide and MPL; then, we investigated immune-responses in a mice model of TUBO tumor. As we expected, addition of PADRE peptide to P5 peptide-
linked liposomal formulations, remarkably improved immune responses which was generated by P5 peptide, compared to free P5 peptide or other liposomal formulations without PADRE and adjuvant. Cancer peptide vaccines containing TAA can stimulate and activate CTL-specific responses against tumors in the host. But, due to poor immunogenic properties of TAA, researchers are evaluating different approaches to increase vaccines’ immunogenicity to making them more potent and efficient. Some of these strategies are using long multi-epitope peptide, dendritic cells therapy pulsed peptide, MHC class I and II epitope hybrid vaccines, combination therapy, etc [6, 41]. P5 peptide is a 21-amino acid synthetic peptide, containing multiple epitopes that can activate various T cell populations and promote immune responses [27]. As previously reported, by comparing the effectiveness of different liposomal formulations, we observed that P5 peptide conjugated with liposomes (Lip-P5) was incapable of inducing appropriate anti-tumor immune responses [20].

One of the most important factors that is considered a strong antitumor approach is effective activation of CD4⁺ T-cell in an immune response which is necessary for generation and activation of CD8⁺ T cell as well as memory T cell responses [42, 43]. Moreover, PADRE is one of the most effective CD4⁺ T helper cell epitope that is used as a potent adjuvant to induce CD4⁺ T-cell responses [21]. Being safe, well-tolerated in a number of clinical trials, and producible along with not inducing the production of antibodies against PADRE, are the advantages of PADRE peptide compared to other conventional carrier proteins such as keyhole limpet hemocyanin (KLH) and tetanus toxoid-derived epitope [23, 25, 26]. Furthermore, recent studies reported that PADRE can act as a potent immunogen and is capable of enhancing vaccines’ potency when co-administered with other forms of vaccines [22-24].

Our findings indicated that liposomal formulation containing P5 peptide combined with PADRE peptide (Lip P5-Integrated PADRE –MPL and Lip P5-Integrated PADRE) induced secretion of higher levels of IFN-γ and stronger CTL specific immune responses along with longer survival time against the Her2/neu-expressing TUBO cell line. The success of liposomal formulation containing PADRE and P5 to elicit a potent CTL response against TUBO tumors can be attributed to the existence of both CTL and T helper epitopes in the mentioned formulation. These data are consistent with our previous study which showed that the presence of PADRE peptide improves of the resulting immune response in a mice model of breast cancer [44]. Also, we previously employed PADRE peptide in combination with P5 and DC to improve the immune responses and demonstrated that mice vaccinated with DC loaded with PADRE+P5+CpG-ODN showed significantly greater CTL responses compared to control groups [22]. A recent study done by Wu et al. indicated that co-administration of DNA vaccine encoding HPV E7 antigen, a human papillomavirus antigen, with PADRE peptide led to highly effective E7-specific CTL immune responses and exhibited marked protective and antitumor effects against TC-1 tumors.
These data confirmed that induction of CD4+ T helper cells using the PADRE strategy may be beneficial in peptide-based vaccination in order to enhance the antigen-specific immune responses and antitumor effects generated by the long peptides. Several lines of evidence indicated that CD4+ T helper cells have an important role in activation of CD8+ T cell immune responses [45]. A previous report indicated that vaccine formulations containing both CTL and T helper epitopes derived from HER-2/neu antigen mixed with GM-CSF could induce potent CTL responses [46]. Moreover, Knutson et al. showed that a vaccine consisting of HER-2/neu derived T helper cells and CTL epitopes, was able to enhance generation of both CD4+ and CD8+ T-cell populations and induce effective HER-2-specific CD8+ T responses [47]. These data confirmed the claim that CD4+ T helper cells are essential for the activation of CTLs to produce antitumor immunity [48, 49].

However, the importance of CD4+ T helper cells for tumor cell-killing and inhibiting tumor growth are less well-characterized. Some studies suggest that CD4+ T helper cells are able to inhibit tumor growth and induce apoptosis of tumor cells [50]. Moreover, some reports demonstrated that CD4+ T helper cells via augmentation of the CD8+ T cell responses and recruitment of macrophages and Natural killer (NK) cells, may induce tumor protective effects [51]. Hung et al. also reported in their study that tumor growth can be inhibited in CD8-knockout mice in a CD4-dependent manner [52]. In parallel to mentioned studies, our data demonstrated that PADRE increases the CD4+ Th1 subset populations, and consequently, confer more potent protective and therapeutic antitumor effects against TUBO tumors in treated mice compared to control groups.

In the present study, we used liposome as a carrier system for vaccine development. A key advantage of liposomes compared with other materials which can deliver antigen or adjuvant, is that liposome-based vaccine delivery systems have high versatility and flexibility. Composition and preparation method of liposome can be chosen and readily manipulated to achieve desired features such as type of lipid composition, particle size, surface charge, entrapment and location of antigens or adjuvants. Depending on the chemical properties (hydrophobicity or hydrophilicity properties), antigens and adjuvants can be entrapped within the inner aqueous space of liposomes, incorporated into liposome bilayer or directly attached to the liposome surface. Therefore, liposomes of different characteristics can be prepared to make liposomal vaccines desirable and suitable for individual applications [53]. To date, different studies indicated that liposomes were able to serve as efficient vaccine delivery system, enhanced antigen uptake by DCs, improved antigen cross-presentation in tumor tissues and produced a strong anti-tumor immunity [9].
In contrast to a previous study which reported that PADRE peptide co-integrated or cross-linked to the liposome surface, did not enhance the CTL response [54], our findings demonstrated that liposomal formulations containing integrated PADRE (Lip P5-Integrated PADRE-MPL and Lip P5-Integrated PADRE) were more effective compared to formulations containing PADRE peptide linked to the surface of liposome (Lip P5-PADRE+MPL and Lip P5-PADRE). Another study indicated that physical attachment of peptides to surface of liposome is an economic, versatile, and less time-consuming approach to prepare liposomal vaccines [55]. In the present study, we expected that in addition to encapsulating of peptide in liposomes, the PADRE peptide could attach to the surface of liposomes through ionic interactions between the PADRE peptide cationic side groups and negatively-charged DMPG of the liposomes to release the PADRE peptide in an easier manner. Generally, antigens which bind to MHC class II molecules are generated via the endocytic pathway and commonly presented to CD4\(^+\) T cells, whereas endogenous antigens enter the cytosol and can be loaded onto MHC class I molecules in the endoplasmic reticulum and finally, MHC-antigen complexes can be recognized by CD8\(^+\) CTLs [56, 57].

The role of pH sensitive DOPE phospholipid in the liposome structure has been frequently demonstrated to introduce antigens into MHC class I pathway [13, 58, 59]. After endocytosis, endosomal compartment undergoes changing of pH that leads to the transition phase of DOPE from lamellar to hexagonal state and subsequently, fusion of liposomes with the endosomal membrane which causes the release of all components of liposomes to the cytosol [60, 61]. Therefore, it seems that physical attachment of PADRE peptide to surface of liposome could facilitate the release and delivery of peptide inside cells to be more readily taken up by DCs and presented to CD4\(^+\) T cells on MHC class II molecules. This method is economic, easier and less time-consuming compared to peptide linked to the maleimide-PEG\(_{2000}\)-DSPE.

In the present study, we used liposomal anti-cancer vaccine formulations containing MPL as an adjuvant. MPL is a safe and effective cancer vaccine adjuvant that was evaluated in several human clinical trials [62]. Also, MPL is a lipid-based adjuvant which has a biochemical structure similar to that of phospholipids that could be easily incorporated into the liposomal bilayer with 100% encapsulation efficiency. MPL can be added to the organic phase of basic liposomal formulation and directly incorporated into the lipid bilayer during preparation of liposome [17]. MPL, through activation of Toll-like receptor4 (TLR4), plays an important role in the induction of immune responses [62]. MPL can induce signaling pathways in APCs through TLR4 stimulation and lead to activation of the nuclear factor-κB (NF-κB), co-stimulatory molecules CD80 and CD86 and of the adaptive immunity [63]. Our results indicated that co-formulation of MPL and P5 peptide in liposomes induced
an effective cellular immune response with significantly higher production of IFN-γ and CTLs activity. However, free P5 peptide or non-MPL liposomal formulations had low prophylactic effects in terms of tumor growth inhibition and did not produce considerable therapeutic effects in a mice model of breast cancer. Finally, our findings demonstrated that co-administration of MPL, P5 and PADRE in liposomal formulations had synergic protective and therapeutic antitumor effects, while the liposomal formulations containing P5 or P5-PADRE only, were incapable to induce effective anti-tumor immune responses.

Conclusion
The present study demonstrated that P5 peptide, a HER2/neu derived protein conjugated on the surface of a liposomal formulation composed of DMPC:DMPG:Chol:DOPE and MPL adjuvant with integrated PADRE, enhances prophylactic and therapeutic anti-tumor immune responses. Moreover, the presence of PADRE, a CD4+ T cell activator can enhance antigen-specific CD4+ T cells activity and vaccine’s efficiency. This formulation can be applied prophylactically and therapeutically to reduce tumor growth in HER2/neu-overexpressing TUBO breast cancer model. Therefore, this formulation can be regarded as a potential candidate for developing a prophylactic and therapeutic HER2/neu breast cancer liposomal vaccine that merits further investigations.

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Conflict of interest
The authors declare that there is no conflict of interest.

Additional information
Supplementary Information: Supplementary Figures S1-S2.

Reference:


Graphical abstract:

Highlights

- Liposomal P5 plus PADRE and MPL effectively activated CD8+ T cell immune response.
- Liposomal P5, PADRE and MPL showed remarkable anti-tumor effects in HER2+ breast cancer.
- Integration of PADRE to liposome would provide potent immunity-inducing systems.