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Cell cytotoxicity, immunostimulatory and antitumor effects of lipid content of liposomal delivery platforms in cancer immunotherapies. A comprehensive *in-vivo* and *in-vitro* study

Amin Reza Nikpoor[a], Mahmoud Reza Jaafari[b,c], Parvin Zamani[d,e], Manouchehr Teymouri[f], Hamed Gouklani[g], Ehsan Saburi[h], Shahrzad Amiri Darban[c,e], Ali Badiee[e,c], Ali Bahramifar[i], Mahdi Fasihi-Ramandi[j], Ramezan Ali Taheri[k,*]

[a] *Molecular Medicine Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran*
[b] *Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran*
[c] *Department of Pharmaceutical Nanotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran*
[d] *Department of Medical Biotechnology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[e] *Nanotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran*
[f] *Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran*
[g] *Infectious and Tropical Diseases Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran*
[h] *Immunogenetic and Cell Culture Department, Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[i] *Trauma Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran*
[j] *Molecular Biology Research Center, System Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran*
[k] *Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Liposome is one of the promising technologies for antigen delivery in cancer immunotherapies. It seems that the phospholipid content of liposomes can act as immunostimulatory molecules in cancer immunotherapy. In the present study, the immunological properties of different phospholipid content of liposomal antigen delivery platforms were investigated. To this aim, F1 to F4 naïve liposomes (without tumor-specific loaded antigens) of positively charged DOTAP/Cholesterol/DOPE (4/4/4 mol ratio), negatively charged DMPC/DMPG/Cholesterol/DOPE (15/2/3/5), negatively charged DSPC/DSPG/Cholesterol/DOPE (15/2/3/5) and PEGylated HSPC/mPEG2000-DSPE/Cholesterol (13/110) liposomal compositions were administered in mice bearing C26 colon carcinoma to assess tumor therapy. Moreover, *In-vitro* studies were conducted, including cytotoxicity assay, serum cytokines measurements, IFN-γ and IL-4 ELISpot assay, T cells subpopulation frequencies assay. The liposomes containing DOTAP and DOPE (F1 liposomes) were able to stimulate cytotoxic T lymphocytes signals such as IFN-γ secretions. In parallel, the aforementioned phospholipids stimulated secretion of IL-4 and IL-17 cytokines from T helper cells. However, these liposomes did not improve survival indices in mice. As conclusion, DOTAP and DOPE contained liposomes (F1 liposomes) stimulate a mixture of Th1 and Th2 immune responses in a tumor-specific antigens-free manner in mice bearing C26 colon carcinoma. Therefore, phospholipid composition of liposomes merits consideration in designing antigen-containing liposomes for cancer immunotherapy.

1. Introduction

In the last few decades, immunotherapy has attracted much attention in treatment of various infectious diseases and cancers (Couzin-Frankel, 2013), in which different players of the host immune system are exploited to eliminate pathogens and cancer cells (Farkona et al., 2016; Khademi et al., 2018a). Among the immune system player, dendritic cells (DCs), putatively known as professional antigen-presenting cells (APCs), are important target cells for immunotherapy. These cells start and activate antigen (Ag)-specific immune responses leading (Veglia and Gabrilovich, 2017) to a cascade of immune actions, which eventually induces immune system to attack abnormal cancer cells and biotic agents. When a specific Ag is recognized and taken up by an APC, it is first subjected to a multi-step internal processing of

* Corresponding author.
E-mail address: taheri@bmsu.ac.ir (R.A. Taheri).

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Table 1
Physicochemical characteristics of synthetized liposomal formulations.

Formulations	Molar ratio	Total lipid concentration (mM)	Z-average (nm)	PdI[c]	Z-potential (mV)
F1: DOTAP, DOPE, Cholesterol	4, 4, 4	12 ± 3[b]	123 ± 23	0.05 ± 0.03	+48.4 ± 2.8[d]
F2 : DMPC, DMPG, Cholesterol, DOPE	15, 2, 3, 5	25 ± 4[a]	131 ± 26	0.09 ± 0.05	−41.3 ± 2.0[d]
F3: DSPC, DSPG, Cholesterol, DOPE	15, 2, 3, 5	25 ± 3	114 ± 33	0.09 ± 0.01	−35.8 ± 3.2[d]
F4:2 HSPC, mPEG2000-DSPE, Cholesterol	13, 1, 10	24 ± 3	120 ± 17	0.13 ± 0.03	−14.8 ± 1.0

[a] Data are shown as mean ± standard deviation of three independent measurements.
[b] Indicates significant difference as compared to other liposome formulations.
[c] Polydispersity index below 0.2 is regarded as the best uniform size distribution.
[d] Z-potential > +30 mV and < −30 mV is considered significantly positive and negative, respectively.

degradation in endosome, then it is exposed on the cell membrane and introduced to other immune cell players. The resultant fragments of Ag (i.e. immunogenic epitopes) bind to major histocompatibility complex (MHC) class I and class II on DCs and then, the activated DCs bearing antigenic epitopes move through the draining lymph nodes, where they interact with $CD8^+$ T or $CD4^+$ T cells. Finally, the $CD8^+$ T cells are induced to develop the ability to specifically recognize and destroy cancerous and infected cells, which is then called Ag-specific cytotoxic T lymphocytes (TCLs), (Mellman et al., 2011).

Therefore, delivery of Ag to APCs is the essential rate-limiting step needed for an effective immune response. This it is considered as an important factor in cancer immunotherapy, which necessitates strategies to improve the Ag delivery. In this regard, various kinds of nano-carriers, including nanopolymers, nanogels, carbon nanomaterials, have already been utilized as promising technologies for Ag delivery in cancer immunotherapy (Khademi et al., 2018b; Khademi et al., 2018c; Park et al., 2018; Yuba, 2018). Among these systems, liposomes are more widely used as a delivery vehicle platform for Ag delivery (Yuba, 2018; Zamani et al., 2018; Zamani et al., 2019). Liposomes are bilayer spherical vesicles formed upon self-assembly of phospholipid molecules in an aqueous medium. They have exhibited multiple beneficial properties suitable for drug formulation, including high biocompatibility, biodegradability, safety, high loading capacity and easy preparation, which make them one of the most popular type of nano-carriers in immunotherapies (Nikoofal-Sahlabadi et al., 2018; Schwendener, 2014; Zamani et al., 2018; Zamani et al., 2019).

Phospholipids and cholesterol are the major backbone of liposomes offering various biological and physicochemical properties. Our recently published papers reported that the lipid components of liposomal formulations might act as immune stimulating agents as well as to the major immunogenic agent used in the formulation (Jafari et al., 2018; Razazan et al., 2017; Zamani et al., 2018). In this regard, the immunogenic properties of liposomal formulations are associated with the physiochemical properties such as types of phospholipid, particle size, liposome surface charge and modifications (Tao Liang et al., 2006; Zamani et al., 2018).

In our previous studies, we reported that some naive liposomal formulations (empty liposomes) were able to induce immune response in mouse models of tumors (Arab et al., 2018; Mansourian et al., 2014; Razazan et al., 2017; Shariat et al., 2014; Talesh et al., 2016; Zamani et al., 2018). This encouraged us to investigate the relationship of some lipid compositions with the level of induced immune response and with the type of immune reaction. For this purpose, four characteristic liposomal formulations with various physicochemical properties were prepared without being loaded with tumor-specific antigens, and they were administered in a mice model of C26 colon carcinoma.

2. Materials and methods

2.1. Materials

The phospholipids used in the present study were 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-Dimyristoyl-*sn*-glycero-3-phosphorylglycerol (DMPG), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) hydrogenated soya phosphatidylcholine (HSPC), methoxypolyethelene glycol (Mw 2000)-distearylphosphatidylethanolamine (mPEG2000-DSPE), 1,2-distearoyl-*sn*-glycero-3-(phospho-rac-(L-glycerol)) (sodium salt) (DSPG) and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), which were obtained from Lipoid (Ludwigshafen, Germany). Cholesterol was purchased from Sigma–Aldrich (St. Louis, MO). All other chemical solvents and reagents were of chemical grade. C26 colon carcinoma cell line was purchased from Cell Lines Service (Eppelheim, Germany).

2.2. Liposome preparation

The nomenclature and lipid compositions of the liposomes used in the study are given in Table 1. The liposomes were prepared via thin film hydration and extrusion method (Nikpoor et al., 2015). Briefly, appropriate amounts of phospholipids were added to a round-bottomed flask, previously prepared as chloroform stock solution and the solvent was removed with a rotary evaporator and one-night freeze drying. The resulting film was hydrated and mixed with phosphate-buffered saline (PBS, 300 mM, pH: 7.4) at 60 °C for 30 min under argon atmosphere followed by one-night incubation at 4 °C. The resulting multi-lamellar liposomes were then passed through respective 400, 200 and 100 nm pore size polycarbonate membranes. Finally, liposomes were sterilized for further experiments using 0.45 µm sterile syringe filters.

2.2.1. Liposome physicochemical characterization

The liposome properties, including particle size mean (shown as Z-average), particle size distribution (Polydispersity Index or PdI) and particle charge (Z-potential) were measured by a Dynamic Light Scattering (DLS) analyzer (Nano-ZS; Malvern, UK). The particle size and Z-potential measurements were done in PBS (10 mM, pH: 7.4) and 3-N-morpholinopropane sulfonic acid (MOPS, 10 mM, pH: 7.4) buffers, respectively at 50-fold dilution of liposomes. Phosphorus content of liposomes was also measured spectrophotometrically at 800 nm according to Bartlett phosphate assay (Bartlett, 1959).

2.3. Examination of apoptotic activity of liposomes

The apoptotic effect of the liposomes was tested on primary immune cells using Annexin V and Propidium iodide (PI). To this end, splenocytes were harvested from the spleen tissue of BALB/c mice after tissue digestion in collagenase type I solution (0.5% w/v in PBS) supplemented with 3 mM calcium chloride and cell harvest through 70 µm cell strainer. Subsequently, 1×10^6 cells/ml were cultured in a 24-well culture plate. The splenocytes (10^6 cells/well) were incubated with varying concentrations of liposomes as shown in Table 2 for 24 h in a humidified 5% CO_2 incubator at 37 °C. The cells were then washed twice with PBS and the rate of apoptosis was measured as follows: 1×10^5 cells/100 µl were incubated with 5 µl of Annexin V-FITC (Biolegned, USA) for 35 min at 37 °C. Subsequently, 10 µl of PI solution was added to the cells. After 5 min incubation, the rate of apoptosis was

Table 2
The apoptotic effects of liposomal formulations on mouse splenocytes during 24-h culture with liposomes.

Formulations	Dose[a]	Fully apoptosis %	Middle apoptosis%	Early apoptosis%	Live%
F1: DOTAP, DOPE, Chol	0.6	23.8 ± 2.7[c]	0.7 ± 0.3	0.1 ± 0.0	75.3 ± 2.7[c]
F2: DMPC, DMPG, Chol, DOPE	5.0	18.8 ± 1.8[b]	0.4 ± 0.2	0.2 ± 0.1	80.6 ± 2.1
F3: DSPC, DSPG, Chol, DOPE	5.0	18.6 ± 1.6	0.8 ± 0.1	0.1 ± 0.0	80.4 ± 1.6
F4: HSPC, mPEG2000-DSPE, Chol	2.4	16.1 ± 2.1	1.5 ± 0.3	0.5 ± 0.3	82.0 ± 1.8
Culture media	0.0	16.3 ± 4.9	0.7 ± 0.3	0.1 ± 0.0	82.0 ± 2.6

[a] The liposome dose used is expressed as total lipid μmole/well.
[b] Data presented as mean ± standard deviation of three independent samples.
[c] Indicates significant difference as compared to controlled culture media.

evaluated using a BD FACSCalibur flow cytometry (BD Biosciences, USA) in FL-1 H and FL-2 H channels in a logarithmic mode.

2.4. Tumor inoculation, challenge and treatment

The liposome tumor challenge was conducted *in vivo* in female C26 tumor-bearing BALB/c mice. The animals were kept in standard condition in an animal house under controlled light cycle (12 h light/12 h dark) and fed at libitum. All procedures were performed according to specific national ethical guidelines for biomedical research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran (Approval code: 91002182). For this purpose, mice aged 6–8 weeks old were inoculated subcutaneously (SC) with C26 colon carcinoma cells (3×10^5 cells/mouse) in the right flank (Nikpoor et al., 2017).

Seven days after tumor induction when the tumor size exceeded 3 mm^3, mice were randomly allocated to four cages and treated with liposomes (five mice per group). Mice were received F1 (0.6 μmole/50 μl/mouse), F2 (5 μmole/200 μl/mouse), and F3 (5 μmole/200 μl/mouse) liposomal formulations subcutaneously weekly for three consecutive weeks. F4 liposome (2.4 μmole/100 μl/mouse) was injected intravenously (*i.v.*) weekly for three consecutive weeks. After one week, mice were sacrificed with cervical dislocation and their splenocytes were harvested as described previously for the following experiments.

2.5. Measurement of serum cytokines

The level of different cytokines, e.g. IFN-γ, IL-2, IL-4, IL-10, IL-17, and TNF-α, were measured in sera of mice treated with liposomes using an Enzyme-Linked ImmunoSorbent Assay (ELISA kit, Hangzhou Eastbiopharm, China) according to the manufacturer's protocol. For this purpose, blood samples were collected into polypropylene tubes at the end of the treatment period via heart puncture and the sera was drawn and stored at −70 °C until cytokine assay.

2.6. Measurement of IFN-γ and IL-4 secretion from splenocytes

The cell potential of IFN-γ and IL-4 secretion from mice splenocytes was examined with an Enzyme-Linked ImmunoSpot (ELISpot) kit (Mabtech AB, Sweden) as per the manufacturer's protocol following immunization with liposomes. In brief, the corresponding detection antibodies were coated onto the surface of ELISpot wells of 96-well plates (PVDF-plate, type MSIP, Mabtech AB, Sweden). Subsequently, the harvested splenocytes were seeded into the wells at 1×10^5, 2×10^5, and 3×10^5 cells/well suspensions and the plates were incubated at 37 °C in the cell culture incubator for 24 and 48 h for IFN-γ and IL-4 measurement, respectively. The liposomes were then added to the wells at the total lipid concentrations expressed in Table 2. For positive control, the polyclonal activator of Phytohaemagglutinin (10 μg/ml) was added to a few other wells. After incubation for 24 h, the spots, which are the representative of spot-forming units (SFU), were counted in every well.

2.7. Flow cytometric examination of cell surface markers and generated cytokines

The frequency of cell surface markers and intracellular cytokines were determined in lymphocytes after staining with fluorescent antibodies via flow cytometry. In brief, 10^6 splenocytes/well were treated with liposomes at total lipid dose/concentrations expressed in Table 2 and 1 μl/million cells BD GolgiPlug™ protein transport inhibitor (BD Biosciences, USA) for 24 h in a 24-well plate. Subsequently, the wells were washed twice with PBS containing 2% v/v fetal bovine serum (FBS) washing solution. First, 1×10^6 splenocytes were added to the flow cytometry tubes and surface antigens were stained with anti-mouse CD4-PE-Cy5, anti-mouse CD8-PE-Cy5 and anti-mouse CD25-FITC antibodies (BD Biosciences, California, USA) at 37 °C for 30 min. The stained cells were then fixed using cytofix/cytoperm™ kit (BD Biosciences, USA). In parallel, the intracellular cytokine staining was performed using anti-mouse IFN-γ –FITC, anti-mouse IL-4-PE and anti-mouse FoxP3-PE antibodies (BD Biosciences, USA) at 4 °C for 30 min. The stained cells were washed twice with the washing solution and suspended in 0.3 ml of the staining buffer. Finally, the frequency of cell markers and cytokines were measured in the cell population using flowcytometry.

2.8. In-vivo therapeutic studies

The therapeutic effect of liposomes were examined using C26 colon carcinoma BALB/c mice model. Mice (five per each group) were treated with liposomes at total lipid concentrations stated in Table 2 at three subsequent consecutive weeks after tumor inoculation. Mice were monitored regularly by measuring tumor volume and weight and they were euthanized if either the tumor volume exceeded 1000 mm^3 or the body weight loss was > 20% or mice were lethargic and unable to feed (Nikpoor et al., 2017). The tumor volume was calculated according to the following formula: [(tumor height × tumor width × tumor length) × 0.5] mm^3. Time to reach end point (TTE), i.e. when the tumor size reaches 1000 mm^3, increased life span (%ILS) and tumor growth delay (%TGD) indices were also calculated as described previously in (Arabi et al., 2015).

2.9. Statistical analysis

Statistical analysis was conducted using a GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). Descriptive statistics, One-way ANOVA and post hoc test or their alternative nonparametric tests if applicable were used. Moreover, the survival time of mice groups were statistically analyzed using Mantel-Cox method. *P* values below 0.05 were considered significant.

3. Results

3.1. Liposomes of different net surface charges exhibited similar size distribution

Physicochemical characteristics of liposomes were given in Table 1. Total lipid concentrations of liposomes were, 12, 25, 25 and 24 mM for F1, F2, F3 and F4 formulations. The liposomes exhibited monomodal and similar particle size distribution given the values of Z-average and PdI, which were not significantly different between liposomes. However, Z-potential of the liposomes was different between the formulations; the surface charge of DSPG- and DMPG-containing liposomes were noticeably negative, of DOTAP-containing liposome was positive, and of F4 formulation was nearly neutral as expected (liposome surface charge between −30 and +30 mV is considered as the range of neutral charge).

3.2. Only F1-DOTAP liposome induced apoptosis in splenocytes

The liposomal formulations exerted no cytotoxic effect on the splenocytes, except for F1-DOTAP-containing liposomes that reduced the percent of the live splenocytes and increased the percent of the cells subjected to fully apoptosis (Table 2 and Fig. 1). Other liposome formulations did not show any cytotoxicity at the concentrations tested. After 24-h treatment, the highest and lowest percent of cell apoptosis were found in F1 and F4 treated cells, respectively.

3.3. F1-DOTAP liposomes significantly enhanced the Th2- and Th17-related cytokines

As depicted in Fig. 2, the maximum change of cytokines serum level compared to the buffer group was related to the F1 formulation, which significantly increased the IL-4 and IL-17 cytokines. The F1 formulation also significantly increased IL-4 cytokine level compared to the other liposomal formulations, with the highest differences in comparison with the F3 and F4 formulations. In addition, the F1 formulations significantly induced the serum level of IL-17 compared with the F4 formulation.

3.4. F1-DOTAP liposome induced the splenocytes to secrete IFN-γ and IL-4

The production of IFN-γ and IL-4 by mice splenocytes is shown in response to stimulation with liposomal formulations in Fig. 3. There was no significant difference in the secreted cytokines of the splenocytes between the non-stimulated cell groups. F1 injection increased significantly the level of the secreted IFN-γ from the splenocytes as compared to the other liposomal formulations as well as control PBS-stimulated cells (Fig. 3A). Moreover, the results showed that the stimulated mice splenocytes with the F1 formulation had the highest IL-4 production among others (Fig. 3B).

3.5. F1-DOTAP- and DOPE-containing liposome was the most effective formulation in inducing CD8$^+$ CTL-IFN-γ-producing cells

The frequencies of CD4$^+$ T and CD8$^+$ T lymphocytes as well as T lymphocytes subtypes including Th1, Th2, and Treg were investigated in splenocytes of treatment groups mice. As shown in Fig. 4, the IFN-γ secretion from CD8$^+$ T lymphocytes was statistically different among mice groups which treated with the F1 and F2 with other liposomes treated mice groups, indicating increased cytotoxic T lymphocytes (CTLs) responses in these two groups. However, the CD4$^+$ T, CD8$^+$ T and Treg lymphocytes frequencies showed no statistically significant differences among treated mice groups. Also, no significant differences were observed between the IFN-γ or IL-4 secretions by CD4$^+$ T lymphocytes in the mice groups which treated with the liposomal formulations.

3.6. In-vivo therapeutic studies

The liposomes showed neither a noticeable cancer therapy nor side-effect-associated death event in terms of tumor growth and lifespan of mice (Fig. 5A and B). Although TGD% and ILS% were negative for all the liposomes, they were non-significant as opposed to those of the PBS group (Table 3).

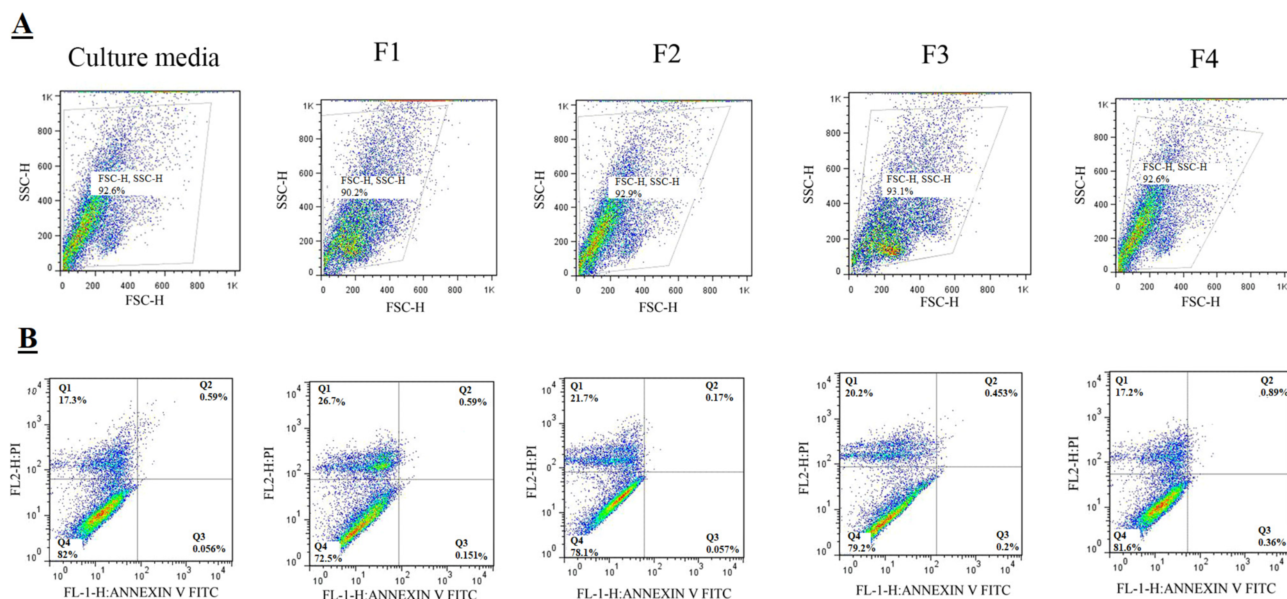

Fig. 1. The apoptotic effects of liposomal formulations on BALB/c splenocytes during 24-h culture with liposomes was studied using Annexin V and Propidium iodide (PI). To this aim, the splenocytes were extracted from BALB/c mice spleens and were cultured. The splenocytes were treated for 24 h with liposomes. Then, 10^5 cells/100 µl with 5 µl Annexin V-FITC incubated for 35 min at 37 °C. The PI dye was then added to the samples five minutes before running; apoptosis was evaluated using a BD FACSCalibur flow cytometry. A: The Live cells were gated through FSC-H and SSC-H parameters. B: The frequencies of live cells (lower left side of quadrant), early apoptotic cells (lower right side of quadrant), middle apoptotic cells (upper right side of quadrant) and fully apoptotic cells (upper left side of quadrant) of stained cells were calculated using FL-1 H and FL-2 H channels in a logarithmic mode.

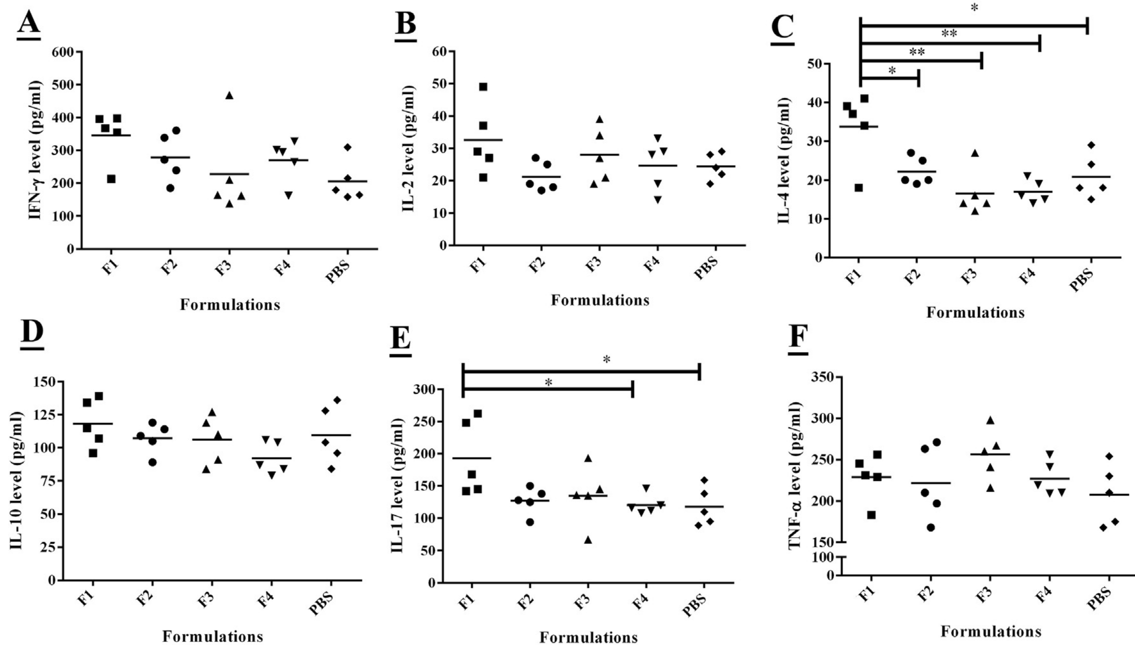

Fig. 2. Evaluation of the serum cytokines induced by liposomal formulations in mice model of C26 colon cancer. Liposomal formulation injection slightly increased the Th-1 related responses (A, B, and F for IFN-γ, IL-2 and TNF-α, respectively). The injection also slightly decreased the level of the regulatory cytokine, IL-10 (D). F1-L injection significantly increased the level of Th-2-related cytokine of IL-4 (C) and Th-17-related cytokine of IL-17 (E). Data are presented as individual value and mean (n = 5). Statistically significant differences are shown as follows: *p < 0.05, **p < 0.01.

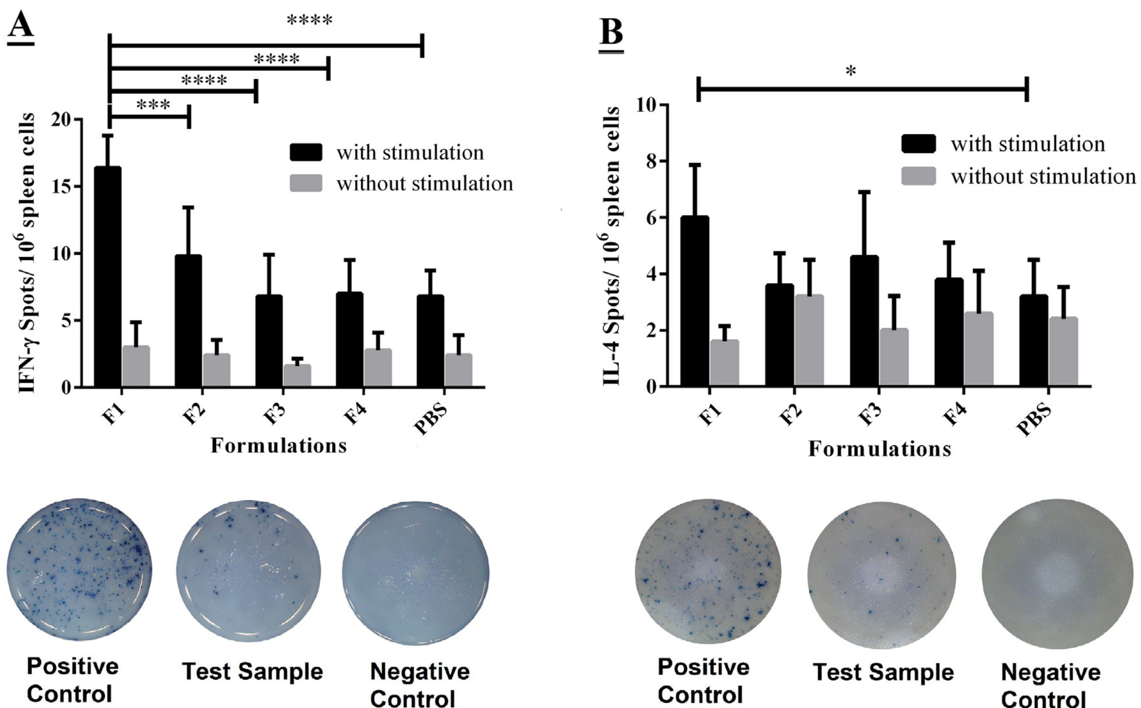

Fig. 3. *In-vitro* secretion assay of IFN-γ (A) and IL-4 (B) from mice splenocytes following immunization with liposomal formulations using ELISpot. Phytohaemagglutinin (PHA) with the concentration of 10 μg/mL was also used as a polyclonal activator of splenocytes and as a positive control. The number of spots per well, denoting cytokine-secreting colony-forming units (CFU)/10^6 splenocytes, were increased significantly in F1-L-treated mice. At the bottom of the figures, as an example of the ELISpot assay wells are shown. 3×10^5 splenocytes was stimulated by PHA as Positive control, liposomal formulation as sample and culture media as negative control. The number of spots were then count after staining. Data are presented as mean ± SD (n = 5). The spots statistically significant differences are shown as follows: *p < 0.05, ***p < 0.001, ****p < 0.0001.

As shown in Fig. 5B, survival analysis (up to 32 days) indicated the slowest tumor growth in PBS group. The log-rank (Mantel-cox) survival analysis revealed no statistically differences between the survival rates of the mice groups.

4. Discussion

In the current study, four common liposomal platforms used in cancer immunotherapies were investigated in terms of cytotoxicity,

Fig. 4. $CD4^+$ T and $CD8^+$ T lymphocytes frequencies as well as subtypes of T lymphocytes, i.e. Th1, Th2, and Treg were measured using flow cytometry. A represents respectively the lymphocyte gating in terms of CD4 cell marker followed by the quadrants of IFN- γ and IL-4-producing and non-producing cells, the lymphocyte gating in terms of CD8 class followed by quadrant of IFN- γ producing and non-producing cells, and the quadrant of Treg based on the cell markers of CD25-FITC and Foxp3 PE. None of the liposome formulation changed the frequency of $CD4^+$ cell frequency (B), $CD4^+$ IFN- γ- (C) and $CD4^+$ IL-4-producing cells (D). The liposome formulations also did not change the frequency of $CD8^+$ cell frequency (E). However, F1 and F2 significantly increased the proportion of the $CD8^+$ IFN-γ-producing cells as opposed to others, including the control PBS (F). None of the liposomes changes the frequency of Treg cell population (G). Data are presented as individual values and mean (n = 5). Statistically significant differences are shown as follows: $^{**}p < 0.01$, $^{***}p < 0.001$.

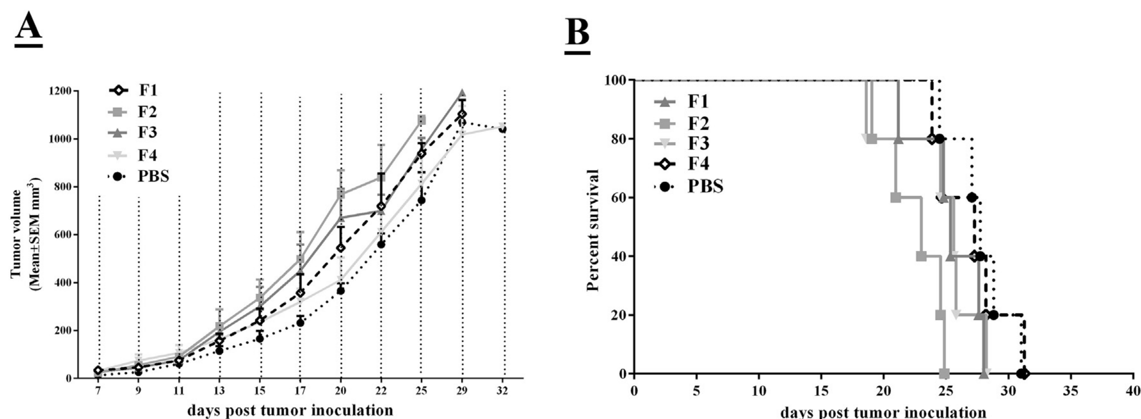

Fig. 5. Therapeutic effect of the liposomal formulations in BALB/c mice bearing C26 colon carcinoma. Seven days after inoculation of C26 cells, when the dimension of tumor reached about 3 mm^3, all mice (five mice in each group) were treated with liposomal formulations. None of the formulation exerted therapeutic effect in terms of tumor growth restriction (A) and event-free survival (B). The data are shown as the mean ± SEM (n = 5) for tumor size analysis and percent survival for survival analysis.

Table 3
Therapeutic efficacy indices of liposomal formulations in mice bearing C26 tumor.

Treatment group	MST[a](day)	Average of TTE[b]	TGD (%)[d]	ILS (%)[e]
F1	25.35	25.39 ± 2.44	−8.74	−8.66
F2	23.02	22.50 ± 2.2[c]	−19.16	−17.05
F3	25.60	24.56 ± 3.21	−11.74	−7.75
F4	27.27	27.05 ± 2.65	−2.79	−1.73
PBS	27.75	27.83 ± 2.14	–	–

[a] Median survival time.
[b] Time to reach end point.
[c] Data are shown as mean ± standard deviation (n = 5).
[d] Tumor growth delay.
[e] Increase life span.

immunogenicity and therapeutic properties. The liposome ingredients (lipids) are varied in chemical structure, molecule net charge and crystalline-to-gel phase transition temperature or Tm. HSPC with the primary lipid component of DSPC and DSPG are solid at the body temperature, given their Tm = 55 ^{0}C, while DMPC, DMPG, DOTAP and DOPE have respectively Tm of 24, 23, 4, and −11 °C. Accordingly, it could be inferred that F1- and F2 liposomes had fluid membrane in the body, while F3- and F4 liposomes were solid assemblies in the body (Anderson and Omri, 2004; Chen et al., 2013).

Moreover, it is determined that the liposomes of different size, surface charge, membrane stability and composition exhibited varied level of immunogenic responses as well as to immune cell polarization (Brewer et al., 1998). In the current study, the liposomes exhibited similar particle size distribution (given the Z-average and PdI parameters in Table 1); however, they were different in terms of liposome net surface charge and membrane fluidity. It was found out that the positively charged F1-DOTAP- and DOPE-containing liposome was the most effective liposomal formulation to induce immune system in our different immunogenic assessments of the liposomes. F1-L induced the serum lymphocytes to produce IL-4 and IFN-γ and splenocytes to secrete these cytokines. It also polarized the lymphocyte population toward $CD8^{+}$ CTL- IFN-γ –producing cells.

At the second place, it was F2-DOPE-containing liposome that produced the abovementioned results as for F2-L, which is surmised to have fluid membrane at body temperature (37 °C). In contrast, the corresponding F3-DOPE-containing liposome displayed no immunogenic activity or immune system polarization as with the PBS control and F4-PEGylated liposomes. It is imagined that substitution of DMPC and DMPG lipids with the respective DSPC and DSPG lipids results in a liposomal membrane with high stability at the body temperature and low tendency to induce lymphocytes, thereby leading to F3-L immune inactivity as opposed to the corresponding F1-L.

DOTAP and DOPE lipids, as well as other lipids in these categories, are shown to enhance the transfection efficiency to immune and non-immune cells and they are utilized as lipid helpers. On the other hand, it is found that cholesterol, which is known to stabilize the liposome membrane, reduces the transfection efficiency (Ramezani et al., 2009). Whether or not a corresponding F3-choesterol-free liposome formulation could exhibit improved immune activity as compared to the present F3-liposome formulation remains an unanswered question in the current study.

The size of liposomes is a critical determining factor for the liposome uptake by DCs. Liposomes with small size (< 100 nm) are rapidly removed from the site of injection, while liposomes with large size (> 200 nm) are shown to enter the surrounding lymph nodes. This is the reason that the size of all of the liposomal formulations were adjusted below 150 nm and in the same range to insure that the particle size would not be a differential immunostimulatory factor for the liposomes in the present study. These liposomes are removed by reticulo-endothelial cells via clathrin-mediated endocytosis (Rejman et al., 2004; Saremi et al., 2018; Shen et al., 1997). Moreover, the type of induced immune responses (Th1, Th2 responses) is a key immunologic factor that play important role in immunotherapy. It is worth mentioning that the size of liposomes influences the type of immune response and different patterns of cytokines. Brewer et al. indicated that liposomes with size of ≥225 nm induce Th1 responses and promote production of IFN-γ and IgG2a; whereas liposomes with a mean diameter of ≤155 induce Th2 responses and enhance level of IL-5 cytokine. (Brewer et al., 1998). In the current study, all liposomal formulation (F1, F2, F3 and F4) have a size ranged from 120 to 135 nm and PdI less than 0.1 that indicates a homogeneous population of liposomes, able to induce Th2-related immunogenic responses. In contrast, F1-liposome also induced Th1-related cytokines, indicating that the liposomal composition and surface charge are also the factors that must be taken into account of designing a liposomal platform for a specific immune system polarization.

Since liposomes are mainly taken up by the immune cells especially by macrophages, the impact of liposome on the induction of apoptosis of immune cells is considered as an important factor in the liposome-based immunotherapies (Lechanteur et al., 2018; Peters et al., 2015; Vasievich et al., 2011). The Lipid concentrations of the liposomal formulations used in apoptosis assay were similar to those used in the *in-vitro* and *in-vivo* therapeutic studies. The result from apoptosis assay revealed that the cationic F1-liposome containing DOTAP and DOPE phospholipid with 0.6 μmole concentration exhibits a relatively high cytotoxicity against splenocytes (Table 2).

Similar to our investigation, several studies evaluated differential cytotoxicity of liposomal formulations as vaccine delivery systems. For instance, Filion et al. reported that liposomal formulations containing DOPE and cationic lipid DOTAP had toxic effect on macrophages in a relatively low concentrations of DOPE or DOTAP (Filion and Phillips, 1997). Aramaki et al. also reported that cationic liposomes were able to induce apoptosis in macrophages and the macrophage-like cell lines such as RAW264.7 through reactive oxygen species (ROS) pathway, which is associated with the liposomal surface charge (Aramaki et al., 1999). On the other hand, Chen et al. reported that liposomal formulations containing DMPC/DMPG/cholesterol had no apoptotic effect on human lymphocytes (Chen et al., 2009).

The most remarkable finding of the present study was that the liposomal formulations containing DOTAP and DOPE were able to stimulate cellular immunity and activated cellular immune responses such as the secretion of IFN-γ, IL-4 and IL-17. As previously reported, positive-charged liposomes are potentially able to bind to the negatively charged surface of APCs in comparison to negative-charge liposomal formulations. This is the reason that liposomes composed of positively charged lipid such as DOTAP can stimulate cellular immune responses by providing antigens through APCs and activating $CD8^{+}$ T cells (Askarizadeh et al., 2017; Nakanishi et al., 2000).

It suggests that the mentioned positively charged liposomes could stimulate a mixture of immune responses pathways without carrying any specific antigen. In this regard, DOPE, which is a pH-sensitive phospholipid, is shown to destabilize the liposome membrane integrity in the acidic endosomal compartment after liposome endocytosis, resulting in antigen escape to cytoplasm and the introduction of the antigen to MHC class I pathway (Lechanteur et al., 2018; Peters et al., 2015; Romoren et al., 2005; Vasievich et al., 2011).

In the present study, due to simulation of the generated immune responses by liposomal formulations, *i.v* and *s.c* routes were used for administration of liposomes. The idea of the different route of liposomes injection was that the F1 to F3 liposomes formulations commonly uses in vaccine researches due to their charge and lipid properties which are desire to antigen delivery to APCs. However, the reason for *i.v* injection of F4 liposomes in our study is that this rigid, negatively charged liposome is extensively used for *i.v* administered cytotoxic drug delivery to tumor such as Doxil. Studies reported that various administration routes of Ags with similar particle size leads to different levels

of immune responses (Balasse et al., 2008). Balasse et al. reported that the injection route of nanoparticles affected the quality of immune responses due to differences in frequency and population type of APCs at the injection sites. During intraperitoneal (*i.p*) and *s.c* immunization, the injected particles are mainly taken up by APCs and DCs of peritoneal cavity and subcutaneous, while in *i.v* administration minimum amount of the particles are immediately available for APC and DCs (Balasse et al., 2008). Accordingly, in our present study, F4 liposomal formulation which was administrated through intravenous route induced lower level of IFN-γ compared to F2- to F3-liposomes that administrated through subcutaneous route.

Nonetheless, the survival analysis study showed neither cancer immunotherapy nor significant side effect resulting in death event. The *in-vivo* results revealed that F1-liposome induces a phospholipid-specific cellular immune response, although the generated immune response against phospholipid content of liposome could not induce any targeted immunity against implanted tumor which leads to enhanced survival.

5. Conclusion

In the present study, four different liposomal formulation platforms were investigated in terms of cytotoxicity, immunogenicity and therapeutic activity. Among the liposomal formulations, the liposomal formulations containing DOTAP as a positively charged phospholipid and DOPE were able to stimulate cellular immunity and CTLs responses such as IFN-γ secretion as well as stimulation of IL-4 and IL-17. Taken together, fluid DOTAP and DOPE-containing liposomes can stimulate a mixture of Th1 and Th2-immune responses in an antigen-free manner.

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Declarations of Competing Interest

The authors declare that no competing interest, financial or otherwise exists in relation to the present study.

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