Combination therapy with liposomal doxorubicin and liposomal vaccine containing E75, an HER-2/neu-derived peptide, reduces myeloid-derived suppressor cells and improved tumor therapy

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\textbf{ABSTRACT}

Myeloid-derived suppressor cells (MDSCs) are immunosuppressive cells causing resistance to immunotherapies in cancer tumors. In the current study, various immunogenic and therapeutic features of the combination therapies with non-liposomal Doxorubicin (Dox) and the E75 immunogenic peptide (Pep), derived from the human epidermal receptor-2 (HER-2), are investigated in parallel with their liposomal formulations (Lip-Dox (Doxil\textsuperscript{®}) and Lip-Pep). Therefore, triple injection doses of Lip-Pep were preceded with Dox and Lip-Dox injections in TUBO/breast tumor-bearing BALB/c mice. Chemotherapy with either Dox or Lip-Dox reduced the frequency of MDSCs, the level of reactive oxygen species (ROS), and MDSCs-associated genes of Arg1, iNOS, S100A8, S100A9. Whereas Lip-Pep + Dox and Lip-Pep + Lip-Dox treatments synergistically potentiated the immunized splenocytes to produce INF-γ and enhanced the frequency of the anti-tumor CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells as opposed to both chemotherapy and immunotherapy regimens. Chemo-immunotherapy increased the number of tumor-infiltrating lymphocytes (TILs) and reduced the level of CD25\textsuperscript{+}FoxP3\textsuperscript{+} T regulatory cells. Taken together, chemo-immunotherapy was the optimum treatment for the limitation of tumor progression as they targeted more cancer-related immune players.

\section{1. Introduction}

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature immune cells with an immune suppressive activity that potentiates tumor progression [1]. MDSCs inhibit both innate and adaptive immunity in the pathological sites, i.e. tumor environment. They produce some immunosuppressive cytokines that restrict the anti-tumor activity of the effector immune cells, i.e. the cytotoxic T lymphocytes (CTLs), dendritic cells (DC), and natural killer (NK) cells. In addition, they promote the generation of regulatory CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} T cells (Treg), which are known to suppress the anti-tumor activity of immune cells surrounding cancer cells [2]. Phenotypically, MDSCs are distinguished from the common immune cells by the expression of the myeloid cell markers on their cell surface (e.g. Gr-1 (Ly6G and/or Ly6C) and CD11b in mouse and CD33 and CD11b in men). They are also distinguished by the lack of the immunostimulatory markers i.e. CD3, CD14 and HLA-DR [3]. These cells are shown to mediate tumor growth, development, and malignancy through the secretion of inhibitory cytokines (e.g. TGF-β and IL-10) and the activation of multiple cell enzymes involved in the process of immune tolerance in the tumor microenvironment. These include arginase 1 (ARG1), inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), which results in the metabolites of nitric oxide (NO), peroxynitrates and reactive oxygen species (ROS) [4–6].

It is shown that the frequency of MDSCs in the tumor environment is associated with an increase in the tumor potency for invasion and malignancy and MDSCs’ activity poses an important obstacle to successful anti-tumor immunotherapy [7]. Therefore, both the elimination...
of these cells and the reduction of their activity are considered a major goal for the efficient cancer therapy. Conventional chemotherapy with agents like doxorubicin (Dox) is shown to reduce the number of MDSCs in the tumor tissues and combining with immunotherapy, it is shown to do so further [8–10].

Besides excellent anti-tumor activity, some studies suggest that Dox is able to promote anti-tumor immunity [11–13]. It is shown that Dox enhances the proliferation of tumor-specific CD8+ T cells [14], increases the tumor cell membrane permeability to granzyme B and perforin produced by these cells, and enhances their anti-cancer activity [15]. It is also shown that Dox selectively reduces the frequency and activity of MDSCs in the tumor and enhances the activity of NK cells [16]. However, the anticancer treatment with Dox is limited due to the insufficient dose delivery of Dox to tumor tissues upon intravenous (i.v.) injection [17], and the improvement of Dox delivery to the tumor might enhance the immune-related therapeutic benefits of Dox.

In this regard, a trademarked liposomal formulation of Dox (Doxil®) is shown to exclusively enhance Dox delivery to the tumor environment. Doxil and other similar nanoparticulated drug delivery systems have been shown to be accumulated in the tumor environment due to the exclusive leaky structure of vessels in the tumor and lack of lymphatic drainage, putatively known as the so-called “enhanced permeation and retention (EPR) effect” [18]. Several studies have suggested that Doxil® potentiates the host anti-tumor responses [19–21].

In addition to Dox and liposomal Dox, other liposome particles are found to improve the efficacy of cancer immunotherapy. In our previous study, CTL response was found to be improved in TUBO/breast cancer model of BALB/c mice, using liposomal formulations targeted with a cancer cell associated peptide. The peptide named E75 epitope (369–377, KIFGSLAFL) originally derived from the human epidermal growth factor receptor 2 (HER2/neu), which is overexpressed in the HER2 positive breast cancer cells. Of note, these liposomal formulations showed both therapeutic and prophylactic effects [22].

Taken together, we hypothesized that the combined chemo- and immuno-therapy with liposomal formulations of Dox and immunogenic peptide E75 might provide a strong tumor-related immunosuppressive activity against tumor cells. Therefore, we investigated the therapeutic efficacy of Dox and E75 peptide in both non-liposomal and liposomal forms in a tumor model of BALB/c mice, when they were administered in chemotherapy, immunotherapy, and the combined chemo-immunotherapy approaches. Non-liposomal Dox, liposomal dox (Doxil®), non-liposomal E75 immunogenic peptide, and the liposomal E75 peptide are shown as Dox, Lip-Dox, Pep and Lip-Pep in the study, respectively. For this aim, the female BALB/c mice tumored with TUBO breast cancer cells were subjected to chemotherapy, immunotherapy and chemo-immunotherapy with these treatments. The efficacy of Dox and Pep was examined in parallel with Lip-Pep and Lip-Dox formulations to understand the liposome’s effect in the treatment modalities. The frequency of MDSCs, some effector T cells and Tregs, as well as to their ability to produce cytokines were examined in the treatment modalities. Finally, the therapeutic outcome of these treatments was assessed in terms of tumor growth and event-free survival.

2. Materials and methods

2.1. Materials

E75 peptide was purchased from China Peptides Co. (Shanghai, China). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (maleimide-PEG2000-DSP), 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and Dioleoyl phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipid (Alabaster, USA). DCFDA/ H2DCFDA cellular reactive oxygen species (ROS) detection assay kit was obtained from Abcam (UK). Mouse anti-IFN-γ and Mouse anti-IL-10 ELISpot kits were purchased from Mabtech AB, (Stockholm, Sweden). Anti-CD11b-APC, anti- Ly-6C- PE, anti- Ly-6G- PerCP, and other antibodies were purchased from BioLegend (San Diego, USA). All solvents and reagents were in chemical grade. The commercially available pegylated liposomal doxorubicin (Doxil®) was supplied from Behestan Darou Company (Tehran, Iran).

2.2. Conjugation of the peptide to maleimide-PEG2000-DSP

E75 (Ac-CGGGKIFGSLAFL, purity > 97.15%) was linked covalently to maleimide-PEG2000-DSP as described previously [22]. Briefly, the E75 peptide, dissolved in dimethyl sulfoxide (DMSO), was added to maleimide-PEG2000-DSP, dissolved in chloroform, at a molar ratio of 1:2:1 (peptide: maleimide) and DMSO: chloroform volumetric ratio of 1:1. The mixture was mixed continuously for 48 h at 37 °C for the peptide-lipid conjugation reaction. The peptide-lipid conjugation was monitored on thin-layer chromatography (TLC) plate (silica gel 60 F254, Merck, USA) with a developing solvent of chloroform/methanol/water (90/10/2 v) and iodine vapor exposure against the pure agents during the incubation period. Subsequently, the solvents were removed with a rotary evaporator (Heidelberg, Germany) and freeze-dryer (VD-800F, Taitech, Japan) overnight. Then, the resulting powder was suspended in deionized water (2 mg/ml) and injected to an Elite RP-HPLC (Shimadzu, Japan) equipped with a reverse phase C18 column (250 mm × 4.6 mm, Shiseido capcell pak). The chromatograms were collected at 220 nm using a solvent gradient condition as follows: ‘0.1% Trifluoroacetic acid (TFA) in water’ and ‘0.1% TFA in acetonitrile’ as mobile phase, started from 20% of the acetonitrile to 60% within 7 min, followed by 20 min extra elution with 60% acetonitrile, and with a flow rate of 1.0 ml/min at 35 °C.

2.3. Preparation and physicochemical characterization of Pep-Lip

The Pep-Lip was prepared using a thin lipid film hydration and extrusion method [23]. Briefly, an appropriate amount of the lipids were added to a round-bottomed flask as a chloroform stock solution (Table 2). The solvent was removed with the rotary evaporator and freeze-dried overnight. Then, the dried lipid film was hydrated with HEPES buffered dextrose aqueous medium (Dextrose 5% w/v, HEPES 10 mM, pH 7.2) at 25 mM total lipid concentration. The mixture was then passed successively through polycarbonate membranes with 0.4, 0.2, ad 0.1 μm pore size, using an extruder device (Avestin, Canada). Finally, the prepared liposome was supplemented with E75-mPEG2000-DSPE micelle medium at a concentration of 100 μg peptide/ml liposome and incubated for 4 h at 55 °C under continuous agitation (250 RPM, Innova 4080 Incubator shaker) [24].

The micelle-to-liposome transition that leads to the post-insertion of the peptide-lipid in the liposomes and the elimination of micelle particle population was determined by measuring the particle size distribution of the Lip-Pep formulation with a dynamic light scattering (DLS) instrument (Malvern Instruments, Malvern, UK). In addition to particle size distribution, the polydispersity index (PDI) and surface charge (zeta-potential) of the liposome formulation were also measured with the DLS. The morphology of the liposomes were also determined using transmission electron microscopy (TEM Zeiss, Jena, Germany). Finally, the total content of the peptides and lipids was measured with the HPLC and the Bartlett phosphate assay, respectively [25].

2.4. Cell culture

TUBO, a cloned cell line that overexpresses rHER2/neu protein, is generated from a spontaneous mammary gland tumor from a BALB-neuT mouse [26]. This cell line was procured from the Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), and 20% fetal bovine serum (FBS).
2.5. Animal and ethics statement

Female BALB/c mice, aged 4-6 weeks with a weight range of 16-20 g, were purchased from Pasteur Institute and housed under a specific pathogen-free condition. All animal experiments received human care and conducted in compliance with institutional guidelines of the Pharmaceutical Research Center (Mashhad, Iran) according to animal welfare guidelines (Project code: MUMS 950941).

2.6. Tumor inoculation and the animal follow-up

The experiment was performed in a total of 63 female BALB/c mice tumored with TUBO cells. For tumor inoculation, the BALB/c mice were first anesthetized by an intraperitoneal (i.p.) injection of the ketamine-xylazine solution (ketamine 100 mg/kg xylazine 10 mg/kg) [27]. Then, TUBO cells (5 × 10^5 viable cells/mouse, suspended in 50 μl of phosphate-buffered saline or PBS) were injected subcutaneously (s.c.) into the right flank of the anesthetized mice. Ten days after the cell injection, when the tumors were palpable, mice were randomly divided into seven treatment groups (n = 9) as follows:

i) The untreated group injected i.v. with HEPES buffer, 5% dextrose (10 mM, pH 7.2).
ii) Dox treated group (3-4 mg/kg, i.v.).
iii) Lip-Dox treated group (3-4 mg/kg, i.v.).
iv) The Pep-treated group injected i.v. with 10 μg/mouse of the peptide, dissolved in the HEPES-buffered dextrose solution.
v) Lip-Pep treated group injected i.v. with 0.1 ml of the peptide-conjugated liposomes at concentration 10 μg/mouse.
vi) Lip-Pep + Dox-treated group injected with the liposomal peptide (10 μg/mouse) and Dox (3-4 mg/ml).
vii) Lip-Pep + Lip-Dox treated group injected with the liposomal peptide (10 μg/mouse) and Lip-Dox (3-4 mg/ml).

The animals' chemotherapy was carried out at 10, 16, 22 days post-tumor inoculation with the Lip-Dox and Dox and their immunotherapy was at 13, 19, 25 days post-tumor inoculation with Pep and Lip-Pep. The liposome dose of 5 μmol per mouse was used for each injection. Each mouse received 10 μg of the peptide in a total volume of 100 μl liposomes.

The animals were inspected every day and their tumor sizes were monitored every 3 days. Tumor volume was calculated as follows: (height × width × length) × 0.5, by measuring the three orthogonal dimensions of the tumor with a digital caliper. The euthanasia criteria included animal weight loss of >20% of the initial weight, tumor volume > 1000 mm^3, lethargy, and inability to feed. Time to reach end-point (TTE), i.e. the day when the animals met the euthanasia criteria was calculated for every mouse. Tumor growth delay, expressed as the percent of tumor volume reduction as opposed to those of the untreated animals (%TGD), median survival time (MST), and increased life span (%ILS) were also calculated for each group [24].

2.7. Cell isolation from spleen and tumor

Twelve days after the last treatment (on day 34 and 37), three out of nine mice in the groups were sacrificed by cervical dislocation. Spleens and tumors were immediately removed and washed (×2) with cold PBS, under sterile condition. The spleen tissue was homogenized and passed through a 70μm cell strainer to harvest single-cell suspension. For tumor, the tissue was first washed (×2) with cold PBS and cut into tiny pieces with a sterile scalpel in a warm (37 °C) collagenase type I solution (2 mg/ml in PBS) and then, the digest was quenched by adding RPMI-1640 medium supplemented with 10% FBS. Subsequently, the cells were harvested with the cell strainer and washed excessively with cold PBS. In addition, erythrocytes were lysed with ACK lysis buffer (0.15 M NH4Cl, 1.0 M KHCO3, 0.1 mM Na2EDTA). Finally, the viable cells were determined using trypan blue (0.4%, w/v, Gibco, USA) dye exclusion method [28]. The cells were re-suspended in RPMI-1640 medium supplemented with 10% FBS.

2.8. Determination of MDSCs frequency in the splenocytes

The splenocyte suspension was stained with the myeloid-specific antibodies and then, the frequency of MDSCs was determined in the suspension with a flow cytometer (BD FACSCalibur™, BD Biosciences, San Jose, USA). For this, 1.0 × 10^6 splenocytes were transferred into the flow cytometry tubes and washed (×2) with a PBS containing 2% v/v washing solution. Subsequently, the splenocytes were incubated with the specific antibodies (2 μl/tube), i.e. anti-CD11b-APC antibody, anti-Ly-6C- PE antibody, and anti-Ly-6G- PerCP antibody, at 4 °C for 30 min. The cells were washed again with the washing solution (×2). Finally, the fluorescent intensity of the cell population was measured with the flow cytometry.

2.9. Measurement of ROS

The intracellular level of ROS was measured using the DCFDA Cellular ROS Detection Assay Kit (Abcam) according to the manufacturer’s instruction. Briefly, 3 × 10^5 splenocytes were first resuspended in a staining buffer (2% FCS in PBS containing the cell-permeant reagent 2′,7′′-dichlorofluorescin diacetate (DCFDA, 20 μM) and incubated at 37 °C for 30 min. Then, the level of ROS was measured using flow cytometry.

2.10. Enzyme-linked immunospot (ELISpot) assay of cytokine-secreting cells

The frequency of IFN-γ- and IL-10-secreting cells was measured in response to the E75 peptide using the mouse Basic ELISpot kits from Mabtech (Stockholm, Sweden) as described previously [25]. Briefly, splenocytes, 3 × 10^5 cells/well, were cultured in triplicate in pre-coated plates and stimulated with 10 ng/ml of the peptide (in 10 mM PBS, pH 7.4). For positive control culture, 1 × 10^5 splenocytes/well were incubated with 10 μg/ml phytohemagglutinin (PHA) and for negative control culture, the splenocytes were incubated only with the culture medium. For IFN-γ determination, the cells were incubated in a cell culture incubator at 37 °C for 24 h for and for IL-10, they were incubated for 48 h. Subsequently, the cells were removed by emptying and washing (× 5) the ELISpot plate with PBS. The ELISpot plates were stained with the biotinylated detection antibody (in PBS/FBS, 100/5 v/v) for 2 h at room temperature. The plates were washed again (× 5) and incubated with the streptavidin-horseradish peroxidase (HRP) diluted 500 times with the PBS/FCS for 1 h at room temperature. After washing the plates, tetramethylbenzidine (TMB) HRP substrate was added and the plates were left for the spots to appear. Finally, the spots were counted with the aid of Kodak® 1D image analysis software (Version 3.5, Eastman Kodak, USA).

2.11. Intracellular cytokine assay

Flow cytometry was performed on the isolated splenocytes to measure intracellular cytokines as described previously [25]. Briefly, 1.0 × 10^6 splenocytes/ml/well were cultured in RPMI1640 medium supplemented with 10% FBS in a 12-well plate and stimulated with the addition of the peptide (10 μg/ml of E75 peptide in PBS) to the well for 12 h at 37 °C. Subsequently, 2 μl of Brefeldin A Solution (BFA, BioLegend, San Diego, USA) was added to the wells and incubated for a further 4 h at 37 °C. In addition, 1.0 × 10^5 cells/ml of splenocytes in the medium containing GolgiPlug TM (1 μl/ml) was stimulated with PMA/ionomycin cocktail (2 μl/ml) for 4 h at 37 °C. Subsequently, the splenocytes were transferred into flow cytometry tubes and washed (×2) with PBS/FBS (98/2 v/v). The splenocytes were stained with 2 μl anti-CD8a-PE-cy5 antibody and anti-CD4-PE-cy5 antibody in separate tubes for 30 min at 4 °C. Then, they were washed with the PBS/FCS and
fixed by the addition of Cytofix/Cytoperm solution (for 30 min). Then, they were washed (×2) with Perm/Wash TM buffer (554,723/BD Biosciences, USA) and finally, they were stained with 1 μl anti-IFN-γ-FITC antibody and anti-IL-10-APC antibody for 30 min at 4 °C. In addition, for CD4+ cell detection, the cells were also stained with 1 μl anti-IL-4-PE antibody, washed with Perm/Wash TM buffer, collected in 300 μl of the PBS/FCS medium in the flow cytometric tubes.

2.12. Tumor-infiltrated lymphocytes

The tumor-infiltrated lymphocytes (TILs) were measured in the tumor cell suspension using staining with anti-mouse CD3, CD4, CD8, and CD25 antibodies. For this, 1.0 × 10^6 cells/ml were cultured in RPMI 1640 with 10% FBS in 24-well plates. The cells were then stained with 2 μl of anti-CD8a-PE-cy5 antibody, anti-CD4-PE-cy5 antibody, anti-CD3-APC and anti-CD25-FITC antibody in separate tubes for 30 min at 4 °C. Finally, TILs were measured by flow cytometry for the expression of surface markers CD3, CD4, CD8, and CD25.

2.13. Quantitative-reverse transcription-PCR (qRT-PCR)

The gene expression profile of MDSCs (Table 1) was evaluated with a qRT-PCR method. For this purpose, total RNA was first extracted from the isolated splenocytes and tumor cells using Total RNA Purification Kit (Jena Bioscience, Germany) according to the manufacturer’s protocol. Then, the extracted RNA was converted to cDNA product using the PrimeScript 1st Strand cDNA Synthesis kit (Takara, Japan) and then, the real-time RT-PCR was performed with the forward and reverse primers listed in Table 1, using One-Step SYBR® PrimeScript™ RT-PCR Kit according to manufacturer’s instructions (Takara, Japan). In addition, the gene expression of β-actin was also evaluated as reference together with other genes.

2.14. Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 6 (GraphPad Software, San Diego, CA) at different level of significance (P < 0.05, P < 0.01, P < 0.001 and P < 0.0001). Two-way analysis of variance (ANOVA) plus Tukey's post-test was used to find out significant differences between different groups. Survival data, expressed as survival probability, were analyzed by log-rank test to compare the survival curves associated with the treatments in terms of the significant differences.

3. Results

3.1. Peptide to DSPE-mPEG2000 conjugation and physicochemical characterization of the liposomes

Prior to the preparation of the liposomes, the linkage of the E75 peptide to maleimide-PEG2000-DSPE was confirmed by TLC and HPLC as described in [22], in which the attachment of the peptide to lipid resulted in a product that differed with the unattached peptide in terms of mobility on TLC and retention time on HPLC chromatogram. Subsequently, the peptide-lipid micelle was used in the preparation of the liposomes.

The phospholipid concentration of the liposomal formulations was determined 20–22 mM by the phospholipid assay. Both the empty liposome and the peptide-conjugated liposome (Lip-Pep) showed a discoidal vesicular shape in TEM (Fig. 1). These liposomes exhibited a size of about 100–130 nm in TEM and according to DLS data (Table 1), the average size (Z-average) of the liposomes was found at about 130 nm. They were extremely narrow in size distribution given PDI values < 0.1. With respect to the liposome surface charge, they were considered slightly negative given zeta potential values at about −30 mV.

3.2. Chemo-immunotherapy reduced the frequency of MDSCs and the level of ROS

The frequency of MDSCs (CD11b+Gr-1+) was measured in the spleen and tumor tissue of TUBO tumor-bearing mice with flow cytometry (Fig. 2). TUBO tumor growth is associated with substantial MDSC expansion, compared with the basal level of MDSCs (CD11b+Gr-1+) in the non-tumored mice and the treatments limited the number and activity of these cells (Fig. 2).

Among the treatment groups, combination therapy with Lip-Dox and Lip-Pep was the most effective treatment in terms of the reduction of DMSCs in spleen and tumor tissue. The frequency of MDSCs (CD11b+Gr-1+ cells) was found to be noticeably high in the spleen of the TUBO tumor-bearing mice as compared to the healthy non-tumored mice (Fig. 2A). The frequency of MDSCs in the healthy non-tumored mice was < 10%, which was significantly lower than those of tumor-bearing mice left untreated (P < 0.0001). Dox and Lip-Dox significantly reduced the frequency of MDSCs as compared to the untreated group (P < 0.0001) in both spleen and tumor tissues (Fig. 2A and B). Lip-Dox reduced MDSCs more effectively than Dox (P < 0.05). It is noteworthy that Lip-Dox and Dox in combination with Lip-Pep decreased the MDSCs population compared to Lip-Pep, while immunotherapy with Lip-Pep and the free peptides (Pep) failed to decrease the number of these cells compared to those of the untreated group.

Besides the decrease in the number of MDSCs, the treatments also decreased the level of ROS production in both spleen and tumor tissues (Fig. 2 C and D). Correlating to the frequency of MDSCs in the splenocytes, the level of ROS was reduced in the splenocytes of the treatment groups compared to those of the untreated group (Fig. 2 C, P < 0.0001). Also, the level of ROS was noticeably lower in the splenocytes samples of the non-tumored group than that of tumor-bearing mice. Chemo-immunotherapy with Lip-Pep and Lip-Dox effectively reduced ROS production as compared to immunotherapy with either liposomal or non-liposomal peptides (Fig. 2 B, P < 0.0001). While the immunotherapy was ineffective. Moreover, chemo-immunotherapy with Lip-Pep + Lip-Dox or Lip-Pep + Dox effectively reduced the level of ROS in tumor cells compared to those of the untreated group (Fig. 2 D).

3.3. Chemo-immunotherapy reduced the expression of the genes in MDSCs

Since the immunosuppressive function of MDSCs depends on multiple mechanisms, the expression of different related genes such as S100A8, S100A9, Arg1 and iNOS were measured in the splenocytes by real-time RT-PCR (Fig. 3). Data indicated that chemo-immunotherapy and chemotherapy almost equally reduced the relative expressions of the selected genes compared to the untreated group, whereas immunotherapy with Pep and Lip-Pep showed no change in their expression.

| Table 1 |
The primers for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
<th>Ref.</th>
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<td>ACCATGCAGA6GAACTCCTGGA</td>
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<td>GCCTGTATTTCCCTCCATG</td>
<td>CCAGTTGGAACATCGTCCCAT</td>
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</table>
3.4. Enzyme-linked immunospot (ELISpot) assay of cytokine-secreting cells

Upon stimulation with the peptides, a higher number of IFN-γ-secreting cells were counted in the samples harvested from the mice treatment groups than those of the untreated group, while a lower number of IL-10-secreting cells were found in the splenocytes samples of the treated mice \( (P < 0.0001) \), Fig. 4).

With respect to IFN-γ-secreting cells, the highest number of these cells were found in the cell samples from mice received the chemio-immunotherapy, while they were fewer in the chemotherapy and immunotherapy groups \( (P < 0.0001, \) Fig. 4A). Moreover, the data indicated that Lip-Dox was more effective than Dox in combination with Lip-Pep in terms of the frequency of IFN-γ-secreting cells \( (P < 0.001) \). A higher number of IFN-γ-secreting cells were isolated from Lip-Pep + Dox and Lip-Pep + Lip-Dox in the splenocytes compared to other treated groups \( (P < 0.0001) \).

With respect to IL-10-secreting cells, a higher number of IL-10-secreting cells was found in the cell samples from mice received the chemo-immunotherapy, while they were fewer in the chemotherapy and immunotherapy groups \( (P < 0.0001, \) Fig. 4A). Moreover, the data indicated that Lip-Pep was more effective than Dox in combination with Lip-Pep in terms of the frequency of IL-10-secreting cells \( (P < 0.001) \). A higher number of IL-10-secreting cells were isolated from Lip-Pep + Dox and Lip-Pep + Lip-Dox in the splenocytes compared to other treated groups \( (P < 0.0001) \).

With respect to IL-10-secreting cells, a higher number of IL-10-secreting cells was found in the splenocytes isolated from mice in untreated groups. Immunotherapy with the liposomal and non-liposomal peptides led to an increase in the frequency of these cells \( (P < 0.0001, \) Fig. 4B). Interestingly, chemotherapy with Lip-Dox and Dox led to a decrease in IL-10-secreting cells in the spleen in comparison with the frequency of these cells in the spleens of the untreated mice \( (P < 0.0001, \) Fig. 4B). Chemo-immunotherapy also led to the decreased number of IL-10-secreting cells as compared to the untreated mice \( (P < 0.0001, \) Fig. 4B).

3.5. Chemo-immunotherapy changed the frequency of various T lymphocytes in splenocytes and TILs

Chemo-immunotherapy and chemotherapy regimens were shown to change the frequency of various T lymphocytes in the splenocytes of mice and TIL 12 days after the last treatment (Fig. 5).

In the spleen, mice in the chemo-immunotherapy regimen, i.e. Lip-Pep + Dox and Lip-Pep + Lip-Dox, showed the highest increase in the percentage of CD8\(^+\) T and CD4\(^+\) T cells compared to those of other treatment groups (Fig. 5A and B). These treatments led to a significant decrease of the Treg (CD4\(^+\)/CD25\(^+\)/FoxP3\(^+\)) cells to the same level as those of mice subjected to chemotherapy (Fig. 5C). Treatment with liposomal peptides (Lip-Pep) enhanced the frequency of CD8\(^+\) and CD4\(^+\) T cells significantly as opposed to those of the untreated group, while it was ineffective to alter the frequency of Treg cells. Similarly, treatment with the non-liposomal peptides (Pep) did not alter the frequency of Treg cells and overall, it led to the least alteration in the frequency of the mentioned three T lymphocytes.

As with the cases of spleens, the most change in the frequency of the different TILs was found in the tumors of mice subjected to the chemo-immunotherapy regimen (Fig. 5D-F). The flow cytometric analysis showed an increase in CD8\(^+\) T lymphocytes (8%) in the tumors of the Lip-Pep + Lip-Dox group and about 5% increase of CD4\(^+\) T cells in this group (Fig. 5D & E). Furthermore, the treatment reduced the level of the immune-regulatory CD25\(^+\) FoxP3\(^+\) TILs in the tumors of mice subjected to chemo-immunotherapy as compared to that of the untreated group (Fig. 5F). Chemotherapy alone also increased significantly the level of CD8\(^+\) TIL although only Lip-Dox increased the level of CD4\(^+\) TILs compared to the untreated group. Moreover, the chemotherapy reduced the percentage of the immune-regulatory CD25\(^+\) FoxP3\(^+\) TILs to the same level as chemo-immunotherapy (see the results for Dox and Lip-Dox vs. Lip-Pep + Dox and Lip-Pep + Lip-Dox in Fig. 5F). As with the cases of spleens, the immunotherapy failed to change the frequency of TILs as opposed to the untreated group.

3.6. Chemo-immunotherapy led to increased frequency of IFN-γ- secreting cells

In addition to the increased frequency of splenocytes capable of secreting IFN-γ and IL-10, flow cytometric analysis of the splenocytes showed that the frequency of CD8\(^+\) and CD4\(^+\) IFN-γ-secreting cells is also increased, especially in the spleen of mice subjected to the chemo-immunotherapy regimen; but nor IL-4-producing cells neither IL-10-

### Table 2

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Liposomal composition</th>
<th>Lipid molar ratio</th>
<th>Z average (nm) ± SD</th>
<th>Zeta potentials (mV) ± SD</th>
<th>PDI b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty liposome</td>
<td>DSPC:DSPG:DOPE:Chol</td>
<td>60:8:20:12</td>
<td>117.3 ± 5.9</td>
<td>−31.3 ± 0.1</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td>Lip-Pepc</td>
<td>DSPC:DSPG:DOPE:Chol: E75-mPEG2000-DSPE</td>
<td>59.83:7.94:19.95:11.96:0.32</td>
<td>130.4 ± 4.1</td>
<td>−34.5 ± 0.2</td>
<td>0.02 ± 0.001</td>
</tr>
</tbody>
</table>

Values are presented from triplicate measurements of each formulation. The data are represented as mean ± Standard deviation (S.D).

a The size of liposomes (Z average).

b Polydispersity index.

c Lip-E75.
producing cells was changed with treatments (Fig. 6). Chemo-immunotherapy was more effective than both chemotherapy and immunotherapy in enhancing CD8+ and CD4+ INF-γ-secreting cells (Fig. 6A and B). On the other hand, none of the treatments led to a change in the level of CD4+ IL-4- and IL-10-producing cells (Fig. 6C and D).

3.7. Chemo-immunotherapy with Lip-Pep and Lip-Dox was the most effective therapy

To evaluate the in vivo efficacy of different treatment strategies anti-tumor activity was assessed in a murine model of breast cancer. For this purpose, tumor size and survival were monitored over time. The tumor growth rate of different formulations was calculated by measuring the tumor in three dimensions and depicted in Fig. 7A. Data indicated that mice treated with the chemo-immunotherapy approach (Lip-Pep + Lip-Dox and Lip-Pep + Dox) showed significantly lower tumor growth rates and highest survival time than other groups and significantly reduced tumor growth rate compared to chemotherapy and immunotherapy (P < 0.0001). Also, chemo-immunotherapy with Lip-Pep + Lip-Dox was more effective than chemo-immunotherapy with Lip-Pep + Dox (P < 0.001).

Chemotherapy with Dox and Lip-Dox was more effective than immunotherapy with Pep and Lip-Pep (P < 0.0001) (Fig. 7A), as it increased the survival of the treated mice (Fig. 7B and Table 3). The rate of tumor growth decreased in the following order: Untreated, Pep, Lip-Pep, Dox, Lip-Dox, Lip-Pep + Dox, and Lip-Pep + Lip-Dox (Fig. 7A). Chemo-immunotherapy also improved effectively the survival rate of the tumored mice as compared to both chemotherapy and immunotherapy regimens with the Lip-Pep + Lip-Dox found as the best treatment (P < 0.05) (Fig. 7B). Two out of six cases in the Lip-Pep + Lip-Dox group and one mouse in the Lip-Pep + Dox group survived to the end of the test (day 80th), whereas 39 days post-tumor inoculation, all mice in the untreated group died. Liposomal formulations led to improved survival rate compared with the corresponding non-liposomal formulations (Lip-Pep vs Pep group and Lip-Dox vs Dox group).

Except for the Pep-treated group, time to reach endpoint (TTE) increased significantly as opposed to that of the untreated group in the treatment groups (Table 3). Among the treatments, Pep-treated and Lip-Pep-treated groups were found to have the minimum efficacy of TTE prolongation. Liposomal Dox (Lip-Dox) and Dox in combination with liposomal peptide (Lip-Pep + Dox) enhanced TTE significantly as opposed to that of Dox-treated group and Lip-Ppep + Lip-Dox-treated group increased it significantly further than the treatment with Lip-Dox. Lip-Pep + Lip-Dox enhanced MST of mice from 36 to 73 days and doubled the increased life span (ILS) of the tumored mice (103.7% for ILS of Lip-Pep + Lip-Dox, Table 3).

4. Discussion

MDSCs are a phenotypically heterogeneous cell population of immunosuppressive cells causing resistance to immunotherapy and tumor.

Fig. 2. The flow cytometric analysis of the frequency of MDSCs in the spleen (A) and tumor tissue (B) and mean fluorescent intensity (MFI) of ROS production in the splenocytes (C) and tumor cells (D) isolated from mice treated with different liposomal and non-liposomal formulations of the peptides and Dox. Data are shown as mean ± SD (n = 3). Statistically significant differences are designated as follows: * P < 0.05; ** P < 0.01; and *** P < 0.001, **** P < 0.0001.
It is shown that the frequency of these cells in the blood increased by 10-fold in breast cancer patients compared with the normal level. As a result, MDSC is considered as a major barrier to successful cancer immunotherapy, including breast cancer immunotherapy [32,33], and new treatment modalities are required to restrict the population and immunosuppressive function of MDSCs to

**Fig. 3.** Relative expression of some genes associated with MDSCs function. Chemo- and Chemo-immuno-therapies led to the reduced expression of Arg1 (A), iNOS (B), S100A8 (C), S100A9 genes (D) compared with those of the untreated mice. Data are shown as the mean ± SD (n = 3). Statistically significant differences are designated as follows: *\( P < 0.05 \); **\( P < 0.01 \); and ***\( P < 0.001 \).

**Fig. 4.** The frequency of IFN-γ (A) and IL-10 (B) secreting cells in the splenocytes isolated from mice subjected to different treatments. After the end of the treatment period, varying number of these cells were detected upon stimulation with the antigenic peptide (+Peptide) with IFN-γ and IL-10 ELISpot assays. The splenocytes were not stimulated with the peptide used as control (−Peptide). Data are shown as the mean ± SD (n = 3). Statistically significant differences are designated as follows: ns: \( P > 0.05 \); *\( P < 0.05 \); **\( P < 0.01 \); and ****\( P < 0.0001 \).
enhance the efficacy of the cancer immunotherapy.

In the current study, we investigated the chemo-immunotherapy efficacy of a dual drug regimen, composed of a chemotherapeutic agent (i.e. either Dox or Lip-Dox) and an immunogenic agent (i.e. either Pep or Lip-Pep). Both non-liposomal and liposomal formulations of these agents were examined in terms of cancer immunity and tumor treatment in the TUBO/breast tumor model. The immunogenic liposomal peptide formulation included a synthetic epitope (E75) derivative from HER2 protein, a receptor that is overexpressed in TUBO/breast cancer tumors. In the chemo-immunotherapy mouse group, the triple injections of the immunogen were preceded by the injections of the chemotherapeutic (Dox) to investigate whether the chemotherapy could restrict the development of tumor-immune tolerance and improve the efficacy of the immunotherapy?

Chemo-immunotherapy was found to affect multiple common immune players and factors involved in tumor resistance to cancer immunotherapy, where some could be associated with the effect of chemotherapy and the others with the effect of immunotherapy. First of all, it was found that chemotherapy with Dox and Lip-Dox reduced the frequency of MDSCs and the associated ROS production in the splenocytes regardless of the immunotherapy effect as chemotherapy + immunotherapy and chemotherapy regimens led to the equal reduction of the MDSCs sub-population and function (Figs. 2 & 3). Therefore, it is Dox, not the peptides that restrict MDSCs. Secondly, the mentioned fact was confirmed by the examination of the MDSCs specific genes. Since the chemo-immunotherapy and immunotherapy reduced equally the expression levels of the myeloid-related gene markers (i.e. S100A8 and S100A9) as well as the genes involved in ROS production (i.e. Arg1 and iNOS in Fig. 3), it could be inferred that Dox is responsible for the limitation of DMSCs sub-population and the restriction of cancer-supportive function.

On the other hand, both the immunogen formulations (Pep and Lip-Pep) and the chemotherapeutic formulations (Dox and Lip-Dox) enhanced the frequency of the anti-tumor effector T cells as well as their proportion in the splenocyte population and in the cells isolated from the tumor tissues. The chemo-immunotherapy regimen enhanced significantly the level of effector T cells capable of producing INF-γ compared with the single-agent treatments (Fig. 6). In addition, the chemo-immunotherapy also significantly enhanced the frequency of CD8+ and CD4+ effector T cells in the splenocyte population compared with the single-agent treatments (Fig. 6). It seems that immunotherapy does not target immunosuppressive CD25+ FoxP3+ Treg cells and the decrease in these cells in the spleen and tumor tissues (among TILs) could be attributed to the supportive function of the chemo-agent-containing regimen (Fig. 5 C & F). However, for the effective stimulation of the anti-tumor immunity in the tumor tissues, both the chemo-agent and immuno-agent formulations must be included in the tumor treatment as the Lip-Pep + Dox and Lip-Pep + Lip-Dox treatments were able to increase the frequency of the CD8+ TILs and CD4+ TILs in the tumor tissues only (Fig. 5 D & E). The resultant flow cytometric analysis of the splenocytes showed that Dox (or Lip-Dox) and Pep (or Lip-Pep) could lead to the potentiation of CD8+ CTL responses and the secretion of the associated cytokines (e.g. INF-γ) and their combination potentiates them further (Fig. 6A).

Taking the mentioned features into account, it is plausible for mice in the chemo-immunotherapy groups to exhibit a reduced rate of tumor...
progression and survive more than all other groups (Fig. 7A and B). In this regard, mice treated with the liposomal formulations of the immunogenic peptides and Dox also exhibited reduced tumor growth and improved survival. Such an improvement in the tumor growth reduction could be attributed to the benefits of the liposomal nano-particulate systems which are accumulated in spleen and tumor tissues due to their exclusive leaky vasculature, leading to the increased and prolonged exposure of the drug cargo to the tumor cells and splenocytes.

Recently, some studies indicated that chemotherapeutic agents can enhance the function of CTL, NK cells, and antigen-presenting cells (APCs) and it can inhibit immunosuppressive cells such as Treg or MDSCs [34–37]. Several studies have well described the immunogenic effect of Dox in vitro and in vivo [13,38]. It is revealed that chemotherapeutic agents not only kill tumor cells but also enhance anti-tumor immunity through different mechanisms [34]. For instance, Mattarollo et al. reported that both the innate and adaptive immunity could be involved in the treatment efficacy of Dox. Further, the therapeutic effect of Dox was found to be mediated through various mechanisms, including the activation and proliferation of CD8+ T cells and increased production of IFN-γ in tumor tissues [35]. Consistent with these results, our study showed that treatment with Dox and Lip-Dox improved the anti-tumor immune responses through multiple pathways, including the enhancement of TILs, the enhancement of the secretion of IFN-γ, and the reduction of MDSC population.

It was shown that the Lip-Dox induced immune responses more effectively than the non-liposomal Dox. In the chemo-immunotherapy, it seems that chemotherapy can enhance the potency of the immune response through sensitizing tumor cells to immunotherapy, consequently resulting in cooperative action for tumor cell elimination.

Our previous study indicated that the liposomal formulation containing E75 peptide was quite effective to induce CTL responses in the cancer-bearing mice model. This liposomal formulation showed both therapeutic and prophylactic effects in a TUBO breast cancer model of BALB/c mice [22]. When an animal immunized with a liposomal vaccine containing antigenic peptide, it could effectively enter the draining lymph nodes and promote immune responses. It seems that liposomal formulation containing DOPE phospholipid was able to deliver antigenic peptide to the MHC class I pathway and induce CTLs that are essential to effective and potent CTL immune response [25]. Moreover, different studies have indicated that liposomal peptide vaccines possess a higher potency for eliciting the anti-tumor immune response [39].

5. Conclusions

Taking all into account, chemo-immunotherapy modality was found to be more efficacious than immunotherapy and chemotherapy.
approaches in treating cancer tumors and the prevention of the development of drug-resistant cancer cells. Our study showed that immunotherapy and chemotherapy-related targets could be both in common and specific to the treatments, where the combination therapy covers many more immune targets as well as to cancer cells, resulting in a synergistic anti-tumor treatment.
Table 3
The efficacy of the treatments in the TUBO tumor mice model in terms of survival.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TTE (%)</th>
<th>TGD (%)</th>
<th>MST (%)</th>
<th>ILS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>36.1 ± 1.5</td>
<td>–</td>
<td>36.2</td>
<td>–</td>
</tr>
<tr>
<td>Dox</td>
<td>52.4 ± 3.2</td>
<td>45.1</td>
<td>51.9</td>
<td>43.3</td>
</tr>
<tr>
<td>Lip-Dox</td>
<td>59.5 ± 3.9</td>
<td>64.8</td>
<td>60</td>
<td>65.7</td>
</tr>
<tr>
<td>Pep</td>
<td>39.0 ± 2.9</td>
<td>8.0</td>
<td>40.1</td>
<td>10.7</td>
</tr>
<tr>
<td>Lip-Pep</td>
<td>43.9 ± 3.5</td>
<td>21.6</td>
<td>44.1</td>
<td>21.8</td>
</tr>
<tr>
<td>Lip-Pep + Dox</td>
<td>66.5 ± 7.5</td>
<td>84.2</td>
<td>65.6</td>
<td>81.2</td>
</tr>
<tr>
<td>Lip-Pep + Lip-Dox</td>
<td>73.21 ± 6.0</td>
<td>102.7</td>
<td>73.6</td>
<td>103.3</td>
</tr>
</tbody>
</table>

* Time to reach end-point.
| Median survival time.
| Increase life span.

Declaration of competing interest

The authors attest that there are no reportable conflicts of interest.

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References