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A new strategy for enhancing antitumor immune response using dendritic cells loaded with chemo-resistant cancer stem-like cells in experimental mice model

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ABSTRACT

Background and aim: Cancer stem cells (CSCs) are rare cell population present in the tumor bulk that are thought to be the reason for treatment failure following chemotherapy in terms of their intrinsic chemo-resistance. Our study aimed to develop an effective therapeutic strategy to target chemo-resistant cancer stem – like cells population in solid Ehrlich carcinoma (SEC) mice model using dendritic cells (DCs) loaded with enriched tumor cells lysate bearing CSC-like phenotype as a vaccine.

Materials and methods: Ehrlich carcinoma cell line was exposed to different concentrations of cisplatin, doxorubicin, or paclitaxel. Drug treatment that resulted in drug surviving cells with the highest expression of CSCs markers ($CD44^+/CD24^-$) was selected to obtain enriched cell cultures with resistant CSCs population. Dendritic cells were isolated from mice bone marrow, pulsed with enriched CSC lysate, analyzed and identified (CD11c, CD83 and CD86). SEC-bearing mice were treated with loaded or unloaded DCs either as single treatment or in combination with repeated low doses of cisplatin. IFN- γ serum level and p53gene expression in tumor tissues were determined by ELISA and real-time PCR, respectively.

Results and conclusion: The results revealed that vaccination with CSC loaded DCs significantly reduced tumor size, prolonged survival rate, increased IFN-γ serum levels, and upregulated p53gene expression in SEC bearing mice. These findings were more evident and significant in the group co-treated with CSC-DC and cisplatin rather than other treated groups. This study opens the field for combining CSC-targeted immunotherapy with repeated low doses chemotherapy as an effective strategy to improve anticancer immune responses.

1. Introduction

Conventional chemotherapy is one of the most common treatment options for cancer. Unfortunately, the efficacy of chemotherapy is limited and tumor recurrence may occur after treatment. Tumor immunotherapy has been reported to be effective and represent a promising strategy which acts as a trigger for mobilizing the body's immune cells to attack tumor cells (Salem et al., 2015).

Several experimental studies illustrated the presence of a very small percentage of tumor resistant self-renewing cells in the heterogeneous cells populations of the tumor mass that are able to escape conventional therapy and regenerate the tumor bulk (Nandi et al., 2008; Dallas et al., 2009; Lopez-Ayllon et al., 2014). This cell population are known as cancer stem cells (CSCs) or cancer-initiating cells which are characterized by unique properties such as self-renewal, capability of *in vivo* tumorigenesis, metastasis, and high resistance to chemotherapy and apoptosis (Ma et al., 2010; Lu et al., 2015).

Traditional treatment of tumors with chemotherapeutic agents leads to the destruction of non-CSCs in the tumor bulk which in turn lead to an increase in the relative CSCs percentage following treatment. The remaining CSCs after tumor treatment is thought to be responsible for recurrence and drug resistance (Lu et al., 2015). Therefore, it is likely that the elimination of drug - resistant CSCs could significantly improve the therapeutic efficacy of conventional chemotherapies which can be achieved by CSC-targeted immunotherapy (Weng et al., 2013).

A prior study demonstrated the existence of a rare population of

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CSCs in the heterogeneous pool of Ehrlich carcinoma (EC) cell line - identified utilizing flow cytometric analysis as $CD44^+/CD24^-$ - showing high tumorigenicity (Goltsev et al., 2013). Due to CSCs high chemotherapeutic drug resistance, we hypothesized that CSCs can be enriched and subsequently isolated from EC cells by *in vitro* drug selection method as previously reported (Ma et al., 2010; Levina et al., 2008; Nguyen et al., 2015).

In our study, enrichment of tumor cells with chemo-resistant cancer stem – like cells was achieved by exposing EC cell cultures to different concentrations of cisplatin, doxorubicin, or paclitaxel *in vitro*. Drug surviving cells following drug treatment of cell cultures were harvested from EC cell line and the cells populations exhibiting the highest percentage of CSC associated surface markers ($CD44^+/CD24^-$) as analyzed by flow cytometry were selected as an enriched source of drug - resistant cancer cells with CSC like phenotype. These cells are referred in our current study as CSCs.

Dendritic cells (DCs) are potent antigen presenting cells that plays a critical role in mediating immune responses (El-Zamarany et al., 2015). DCs are characterized by their superior ability to acquire and present antigens to effector T cells with subsequent induction of immune responses (Salem et al., 2016a; Sabado et al., 2017). In the present study, we used resistant CSC- enriched cell population isolated from EC cell line as an antigen source to pulse DCs to prepare CSC-DC based vaccine.

Recent approaches are directed to combine chemotherapy and immunotherapy as a promising strategy to treat cancer. It was reported that chemotherapy pre-condition the tumor microenvironment for immunotherapy by eliminating some of the immuno -suppressive networks (Salem et al., 2015; Peng et al., 2015; Konduri et al., 2016).

This study aimed to propose an effective strategy for cancer management based on targeting both resistant cancer – stem like cells and non-CSCs through combining both CSC-targeted immunotherapy and chemotherapy in the clinical settings. We therefore investigated the effects of a new vaccination strategy based on administration of DCs *in vitro*-loaded with lysate of chemo-resistant CSCs, in association with low doses of chemotherapy on cancer progression as well as antitumor immune response.

2. Materials and methods

2.1. Animal model and cell line

Six-week old Swiss female albino mice (CD-1 strain) weighing 22 ± 3 g were purchased from National Research Center, Cairo, Egypt. Upon arrival, the mice were randomly transferred to clean, dry plastic cages containing sawdust bedding, and allowed to acclimatize for two weeks before the start of the experiments. They were housed under standard laboratory conditions of temperature, humidity and light and fed with rodent pellets and tap water *ad libitum*. Study protocol, experimental procedures and guidelines for care and use of laboratory animals were approved by the Research Ethics Committee, Faculty of Pharmacy, Tanta University, Egypt.

Ehrlich ascites carcinoma (EAC) tumor cell line was purchased from National Research Center, Cairo, Egypt. EAC cell line is an aggressive and malignant breast carcinoma cell line isolated originally from female CD1 mice with a high transplantable capability and rapid proliferation which is considered as an appropriate model for the evaluation of experimental cancer treatments (Khedr and Khalil, 2015). Ehrlich carcinoma (EC) cell line has two subtypes: ascites and solid according to the method of tumor cells inoculation (Aysan et al., 2013). The EC cell line was maintained in our laboratory by weekly transplantation of 2.5×10^6 viable tumor cells in 0.2 ml of sterile saline by intraperitoneal injection into female Swiss albino mice. The cell viability was assessed using trypan blue exclusion assay and counted by hemocytometer before injection into naïve CD1 mice for solid tumor induction (Osman et al., 2015).

2.2. Preparation of CSCs - enriched EC cell cultures by chemotherapeutic drug resistance selection method

EC cells were cultured in 25 mL tissue culture flasks at a density of 2×10^6 cells/flask in RMPI (Roswell Park Memorial Institute)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (the inactivation was done by heating at 57 ^{0}C for 30 min), 2 mM L-glutamine, mixture of 100 g/mL streptomycin and 100 U/mL penicillin (P/S), and 0.5 g/mL fungizone (Nguyen et al., 2015). Cells were incubated at 37 ^{0}C, 5% CO_2 in a horizontal position in a humidified incubator for 24 h. After reaching 80% confluence, cells were treated with varying concentrations of cisplatin (0, 10, 20, 30, 40, 50 ug/mL), paclitaxel (0, 3, 6, 12, 14, 16 ug/mL) or doxorubicin (0, 2, 4, 6, 8, 10 ug/mL) for 72 h. Each concentration of chemotherapy was repeated in three different flasks. The concentration range of the used chemotherapeutic drugs was based on IC50 for each drug which are 32 µg/mL, 4.5 µg/mL and 12 µg/mL for cisplatin, doxorubicin, and paclitaxel respectively (Verma and Prasad, 2012; Elbialy and Mady, 2015; Arican and Arican, 2006).

Drug surviving cells (DSCs) were allowed to grow for additional 48 h in drug- free medium. Then, all of the media was removed and adherent tumor cells were washed with PBS and harvested using 0.25% trypsin-EDTA detachment solution and subsequently stained with trypan blue dye for cell viability and counting. The number of cells per flask were counted using a haemocytometer under digital inverted microscope. DSCs were washed twice with PBS and stored at – 80 ^{0}C until use. The highest concentration of chemotherapy in which cells could survive and grow was used to select cells with drug resistant phenotype.

2.3. Flow cytometric analysis of the expression of CSCs surface markers ($CD44^+/CD24^-$) in DSCs populations

We assessed the percentage of CSCs ($CD44^+/CD24^-$) in DSC populations using flow cytometric analysis of cultures from parental and drug-treated EC cells. Single cell suspensions of the harvested DSCs were prepared and counted as previously mentioned and then incubated with fluorescent-conjugated monoclonal antibodies anti-mouse CD44-FITC and anti-mouse CD24-PE (Miltenyi Biotec Gmb, Germany) at 4 ^{0}C for 30 min. Cells were washed twice with PBS, harvested after centrifugation and then re-suspended in 300 µL FACS buffer and analyzed by BD FACSCanto™ II flow cytometer (BD biosciences, USA). The obtained data were analyzed by the FlowJo software package (Tree star, Ashland, USA). DSCs were analyzed using flow cytometry to determine the suitable chemotherapy and the optimal dose that could enrich cells populations with the highest percentage of $CD44^+$/CD24- cells to be used in further experiments.

2.4. Preparation of CSC- enriched cell lysate

CSC lysates were prepared from selected DSCs with the highest percentage of CSC surface markers as previously described. In brief, cells were re-suspended in sterile PBS at a concentration of 5×10^6 cells per mL. Cells were lysed by four rapid freeze and thaw cycles (37 ^{0}C water bath and – 80 ^{0}C), Cell lysates were centrifuged at 2000 rpm for 7 min at 4 ^{0}C (Salem et al., 2016a). The supernatants were collected in sterile vials and stored at – 80 ^{0}C until use.

2.5. Generation of mice bone marrow – derived dendritic cells in vitro and identification of their phenotypical characteristics using flow cytometry

DCs were generated from bone-marrow (BM) derived mononuclear cells of healthy CD1 mice. BM cells were flushed from the marrows of femur and tibia bones of 13 male mice under aseptic conditions. The harvested BM cells was depleted of RBCs by incubation with ammonium chloride potassium lysis buffer and were then cultured at a

concentration of 1×10^6 / mL in complete medium of RPMI-1640 supplemented with 10% heat inactivated FBS, 1% P/S, 2 mM L-glutamine in addition to 20 ng/mL GM-CSF and 20 ng/mL IL-4. On day 4, the cultures were fed with freshly prepared complete medium containing 20 ng/mL of GM-CSF and IL-4. On day 6, immature DCs were supplemented with either PBS (unloaded DCs) or loaded with previously prepared CSC-lysate (loaded DCs) at ratio 1:1 (Li et al., 2014) and incubated overnight at 37 °C and 5% CO_2. Maturation stimulus TNF-α was added to the cultures at a concentration of 20 ng/mL on day 7.

Activated mature DCs were harvested on day 9 by careful pipetting and collected in 50 mL centrifuge tubes for counting and phenotypic analysis using flow cytometry. Aliquots of generated DCs (Loaded DCs and unloaded DCs) were directly stained using fluorescent conjugated mouse monoclonal antibodies including PE-CD 11c, FITC-CD 83 and FITC-CD 86 (eBioscience, USA) as described previously. Cells were analyzed using BD FACSCanto™ II Flow cytometer (Becton Dickinson®, USA). The obtained data were analyzed by the using FlowJo® software package (Tree star, Ashland, USA). Loaded DCs and unloaded DCs will be used as vaccines as specified in the subsequent experiments. Each mouse was treated with 2×10^6 cells in 200 µL sterile PBS per vaccine as will be mentioned in the treatment protocol.

2.6. Solid Ehrlich carcinoma tumor induction, treatment protocol and sample collection

Murine EAC cells were collected from the ascitic fluid of a female Swiss (CD-1) albino mouse bearing 7-day old tumor ascetic fluid purchased from the National Cancer Institute (Cairo, Egypt). The ascitic fluid was diluted with saline at ratio 1:10. Solid tumors were implanted by subcutaneous injection of 0.2 mL of the diluted ascitic fluid (containing about 2.5×10^6 viable EAC cells) in the thigh of the right hind limb of each CD-1 mouse (Bassiony et al., 2014). Solid tumors were formed at day 14 post-inoculation. Solid Ehrlich carcinoma (SEC) bearing mice were divided into 6 groups. Each experimental group included six mice. The control group was injected with PBS (untreated control group), chemotherapy group (chemo group) was injected with cisplatin (CIS; 2 mg/kg) intraperitoneally on day 14, 18, 22, 26, and 30 as five cycles with 3-day intervals (Gorbach et al., 2014), unloaded DC group were intraperitoneally treated with small dose of CIS (2 mg/kg) as a single dose on day 14 once a solid tumor appeared then unloaded DCs administered twice on days 15 and day 23 (DC group), CSC-loaded DC group (Vacc group) was similarly treated as the DC group except for using CSC-loaded DCs instead of unloaded DCs as a vaccine, mice in the last two groups received repeated doses of CIS (2 mg/kg) intraperitoneally similarly as the chemotherapy group in five cycles with 3-day intervals and were vaccinated with either unloaded DC (DC + chemo group) or CSC-loaded DCs (Vacc + chemo group) *via* intravenous injections through the tail vein on day 15 and day 23. Fig. 1 illustrates the study treatment protocol.

2.7. Determination of tumor volume and survival rate in SEC bearing mice

Tumor volumes were measured twice a week. The long and short diameters of tumor mass were measured with a caliper, starting on day 14, and calculated using the formula: tumor volume = ($width^2$ x length)/2. Survival was monitored and was calculated according to the following equation as described (El-Ashmawy et al., 2017) by El-Ashmawy et al. (2017): Survival rate of tumor bearing mice = number of surviving mice in a group on the final day of the study × 100 / number of animals in the same group at the beginning of the study. On day 31, mice were anesthetized under light ether and blood samples were collected by cardiac puncture. Serum samples were separated and stored at – 80 °C until use. Mice were sacrificed by cervical dislocation and tumor masses were excised, divided and stored at – 80 °C for subsequent analysis.

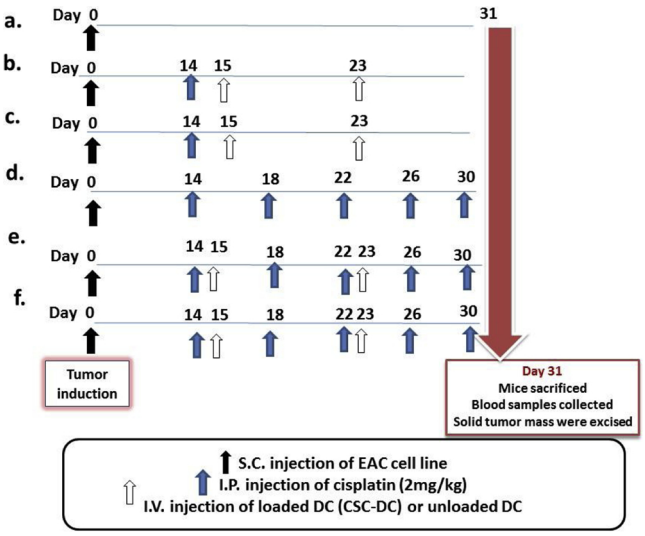

Fig. 1. Timeline of experimental protocol. Solid tumors were induced in mice by subcutaneous injection of 2.5×10^6 cells on day 0. Mice received small dose of cisplatin (CIS) (2 mg/kg) by intraperitoneal injection on day 14. Vaccine groups received either loaded or unloaded DCs by intravenous injection in tail vein on day 15 and 23. Mice were sacrificed on day 31 to perform further analysis. The groups shown are as follows: **a, control** (untreated group); **b & c, Vacc and DC groups, respectively**: mice in the 2 groups were intraperitoneally treated with small dose of CIS (2 mg/kg) as a single dose on day 14 once a solid tumor appeared then vaccinated by intravenous injection with either CSC-loaded (group b) or unloaded DC (group c) twice on days 15 and 23; **d, chemo group**: mice in this group were injected with CIS (2 mg/kg) intraperitoneally on days 14, 18, 22, 26, and 30 in five cycles with 3-day intervals; **e & f, Vacc + chemo and DC + chemo groups, respectively**: mice in the last 2 groups received CIS (2 mg/kg) intraperitoneally on days 14, 18, 22, 26, and 30 as five cycles with 3-day intervals and vaccinated by intravenous injection with either CSC-loaded (group e) or unloaded DCs (group f) twice on days 15 and 23.

2.8. Determination of IFN- γ serum levels by ELISA assay

ELISA assay was performed to determine interferon-γ (IFN-γ) serum level using mice IFN-γ ELISA kits obtained from Sun Red Biotechnology® (China), according to the manufacturer instructions. IFN-γ serum level was calculated from the generated standard ELISA curve.

2.9. Total RNA extraction and semi-quantitive real time RT-PCR analysis of p53 relative gene expression

Frozen tumor tissues were disrupted and homogenized using TissueLyser II (Qiagen, Germany) with subsequent total RNA extraction using Simply P total RNA extraction kit (Cat # BSC52S1) (BioFlux®, China) following the manufacturer's instructions. Total RNA concentration and purity was determined using Nanodrop (Thermo Scientific™, USA). Complementary DNA was synthesized from 1 µg of total RNA by reverse transcription using Quantitect reverse transcription kit (Cat # 205,311) (Qiagen®, Germany). The reverse-transcription master mix was composed of 1 µl of Quantiscript reverse transcriptase, 4 µl of 5X Quantiscript RT buffer, 1 µl of RT primer mix and 14 µl of template RNA (after genomic DNA elimination reaction) in a total volume of 14 µl. The tubes were transferred to thermo shaker (Biometra®, Germany) and incubated at 42 °C for 15 min. Then, the Quantiscript reverse transcriptase was inactivated by incubation at 95 °C for 3 min and chilled on ice. The synthesized cDNA concentration was calculated for each sample as well as A_{260}/A_{280} ratio to detect the purity of cDNA samples. Purity is indicated by $A_{260}/A_{280} = 1.8 - 2.0$. The resultant cDNA was then stored at −20 °C until further use.

Synthesized cDNA was used as a template for amplification of p53 using SYBR green-based real-time PCR and was detected by Piko real-time PCR instrument (Thermo Fisher Scientific Co., Finland). Gene expression of p53 was measured using SensiFAST™ SYBR No-ROX Kit (Cat # BIO-980050) (Bioline, Germany). P53 primer (Invitrogen, USA) sequences are: forward: 5′- GTTCCGAGAGCTGAATGAGG-3′, reverse: 5′- TTTTATGGCGGGACGTAGAC-3′. The thermal cycling conditions were as follows: intial polymerase enzyme activation at 95 °C for 2 s, then 40 cycles of 95 °C for 5 s, annealing at 55 °C for 10 s and extension at 72 °C for 20 s. The data represent the average of 3 independent experiments. The relative fold change in gene expression of p53 was calculated using Livak method ($2^{-\Delta\Delta CT}$ method). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a reference gene for mRNA level normalization. No-template sample was used as a negative control.

2.10. Statistical analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) version 19. Results were expressed as mean ± SD. To determine significant differences between two groups, P values were calculated by unpaired student t-test. Comparison between 3 or more studied groups was performed with one-way ANOVA (F-testing). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Enrichment of tumor cells bearing stem cell like characteristics from parental EC cell line based on their chemotherapeutic drug resistance

In order to develop a method to enrich chemotherapeutic drug resistant tumor cells

bearing CSC characteristics from EC cell line *in vitro*, we cultured EC cells for 24 h until reaching about 80% confluence and treated them with varying concentrations of cisplatin (CIS) (0, 10, 20, 30, 40, 50 μg/mL), paclitaxel (PACLI) (0, 3, 6, 12, 14, 16 μg/mL) or doxorubicin (DOX) (0, 2, 4, 6, 8, 10 μg/mL) at 37 ^{0}C, 5% CO_2 in a horizontal position in a humidified incubator for further 72 h.

The vast majority of cells died with increasing doses of chemotherapeutic drugs.

EC showed relatively slow growth in low doses of applied chemotherapeutic agents and showed a significant change in cell morphology **(**Fig. 2a**)** and cell density **(**Fig. 2b**).** Alternatively, at higher doses of chemotherapeutic drugs, cancer cell growth greatly decreased and most of the cells died and floated in the culture medium within 72 h of exposure to the chemotherapeutic drugs. After 72 h of exposure to chemotherapeutic drugs, some cells were able to survive, grow and form resistant cells population termed as “drug surviving cells (DSCs)”. We assumed that these DSCs were cancer stem cells (CSCs). To verify this, DSCs were counted using trypan blue staining method and analyzed for CSC surface markers ($CD44^+$ /$CD24^-$) using flow cytometry to select the concentration of one of the applied chemotherapeutic drugs that showed a significantly high percentage of $CD44^+$ /CD24- cells to be used in the preparation of CSC-based DC vaccine.

3.2. Flow cytometric analysis of CSC surface markers identified as $CD44^+$/$CD24^-$ cells

To verify whether that the DSCs population selected after 72 h of different concentrations of the applied chemotherapies possessed the hypothesized chemotherapeutic-resistant phenotype with the characteristic features of stem cell associated surface markers $CD44^+$/$CD24^-$ expression corresponding to the previously identified CSCs phenotype, we assessed the percentage of CSCs ($CD44^+/CD24^-$) in parental and drug-treated cell cultures by flow cytometry. Subsequently, the concentration of chemotherapeutic agent with the highest percentage of $CD44^+$/CD24- cells was selected to obtain enriched cell cultures with chemo-resistant cancer stem –like cells population to be used as an antigen source for pulsation of dendritic cells in further experiments. Gating strategy is illustrated in Fig. 3.

Fig. 4 illustrates the results of flow cytometric analysis of DSCs after treatment with different concentrations of cisplatin, doxorubicin, and paclitaxel on $CD44^+/CD24^-$ expression. The analysis of DSCs populations in drug-treated EC cell cultures differed in $CD44^+/CD24^-$ percentages ranging from (0.26 ± 0.03% to 14.7 ± 0.25%) *versus* 0.3 ± 0.05% in parental EC cell line as shown in **(**Fig. 4a**).** The percentage of $CD44^+/CD24^-$ expression in DSCs population of cisplatin-treated cultures increased from 0.45 ± 0.05%–14.7 ± 0.25% with increasing cisplatin dose. Cultures treated with cisplatin 50 μg/mL showed the highest percentage of $CD44^+/CD24^-$ expression that increased significantly ($P < 0.01$) as compared with other cisplatin-treated groups.

The percentage of $CD44^+/CD24^-$ expression in DSCs population of doxorubicin-treated cultures increased from 0.26 ± 0.03% –1.4 ± 0.15%. Treatment with DO X 10 μg/mL showed a significant increase ($P < 0.01$) in $CD44^+/CD24^-$ expression compared to other DOX-treated groups. $CD44^+/CD24^-$ expression in DSCs populations of paclitaxel-treated cultures ranged from 0.17 ± 0.02%–6.25 ± 0.26%. A significant increase ($P < 0.01$) in $CD44^+/CD24^-$ percentage in cultures treated with PACLI 16 μg/mL compared to other paclitaxel-treated groups.

These findings revealed that $CD44^+/CD24^-$ expression of EC cell line treated with 50 μg/mL cisplatin showed the highest percentage among drug-treated EC cell cultures. The $CD44^+/CD24^-$ expression was significantly increased in cisplatin (50 μg/mL) treated cultures by ($P < 0.01$, ↑57.5%) and ($P < 0.01$, ↑90.5%) when compared to PACLI (16 μg/mL) and DOX (10 μg/mL), respectively **(**Fig. 4b**).** The cisplatin (50 μg/mL) showed the highest expression of $CD44^+/CD24^-$ among cell cultures and was selected to obtain enriched drug resistant cells with CSCs phenotype in order to be used in subsequent preparation of CSC-DC vaccine.

3.3. Identification and analysis of loaded DCs and CSC-loaded DCs using flow cytometry

In this study, we investigated the microscopic and phenotypic characteristics of DCs generated from bone marrow (BM) – derived mononuclear cells of naive mice. The BM cell count was $27.7 \pm 4.3 \times 10^6$ cells per mouse with 98% viability using trypan blue staining method. Microscopic observation of cultured BM – derived DCs showed significant changes in morphology throughout days of incubation. On day 1, most cells were round and small in size. On day 6, cells with dendrites significantly increased in number indicating the differentiation of monocytes and their transformation into immature DCs. On day 6, most immature DCs still adhered to flask bottom with semi-adherent and floating BMDCs (bone marrow derived dendritic cells). On day 6, immature DCs were loaded with CSCs lysates followed by the addition of maturation stimulus TNF-α on day 7. BMDCs showed an evident increase in cells populations and formation of large sized colonies of BMDCs at various sites with branched and extended morphology and adhered macrophages were also observed. On day 9 of culture, morphological observations showed a higher proportion of loosely adherent DCs with more pronounced dendrites and presence of veils around dendrites **(**Fig. 5**).**

To evaluate the phenotype of the *in vitro* generated mature BMDCs, mature DCs were analyzed by flow cytometry for their cell surface marker CD11c and co-stimulatory molecules CD83 and CD86 expression. The results of flow cytometric analysis of DCs - after incubation with GM-CSF, IL-4, and maturation with TNF-α -revealed the generation of mature DCs with characteristic phenotype CD11c+, CD83+, and CD86 + . The impact of antigen loading of DCs with tumor cell lysate enriched in cells expressing CSCs surface markers was illustrated

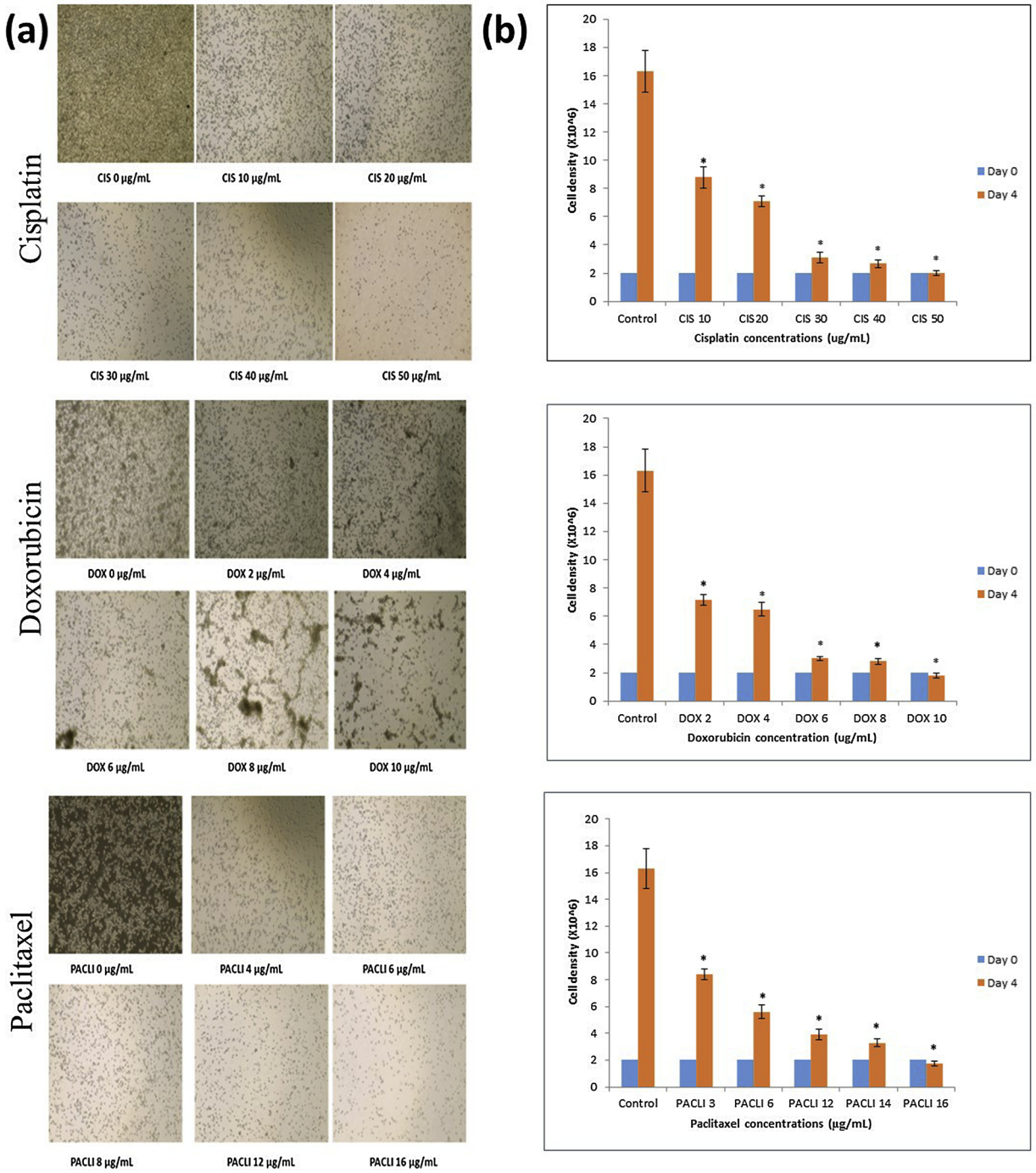

Fig. 2. a) Morphological appearance of parental and drug-treated cell cultures after *in vitro* treatment of EC cell line with different concentrations of cisplatin, doxorubicin, and paclitaxel. b) Cell density were detected by trypan blue exclusion assay after cell cultures treated with different concentrations of cisplatin, doxorubicin, and paclitaxel. Representative images showing the effect of the applied chemotherapies on the morphology of murine EC cell line after 72 h of incubation with CIS (10, 20, 30, 40, 50 µg/mL); DOX (2, 4, 6, 8, 10 µg/mL); PACLI (3, 6, 12, 14, 16 µg/mL). Data are presented as mean ± SD. *P < 0.01. EC cells were seeded at density 2×10^6 into T-25 tissue culture flask with three triplicates. After 24 h of plating, cells were treated for another 72 h with different chemotherapeutic agents. CIS, cisplatin; DOX, doxorubicin; PACLI, paclitaxel; EC, Ehrlich carcinoma.

in (Fig. 5). The percentage of unloaded DCs that expressed CD11c + was 57.3 ± 6.3%, CD83+ was 58.4 ± 4.7%, and CD86+ was 56.5 ± 4.8%. On the other hand, the percentage of loaded DCs that express CD11c + was 55.3 ± 1.4%, CD83+ was 56 ± 3.2%, and CD86+ was 56.8 ± 3.6%. Interestingly, mature DCs after being loaded with CSC lysate displayed almost identical phenotype as their unloaded DCs group. There were no significant differences in the expression levels of CD11c+, CD83+, and CD86+ between loaded and unloaded DCs groups. Our findings showed that loading DCs with CSC lysates didn't affect the expression pattern of mature DCs surface markers.

3.4. Effects of combined treatment with repeated low doses cisplatin and CSC loaded DCs on tumor growth and survival rate of SEC bearing mice

We examined the therapeutic efficacy of CSC based DC vaccine to prevent tumor growth and prolong the survival of SEC bearing mice. Groups treated with cisplatin (chemo), unloaded DC (DC), CSC-loaded DC (Vacc) and a combination of cisplatin and either unloaded

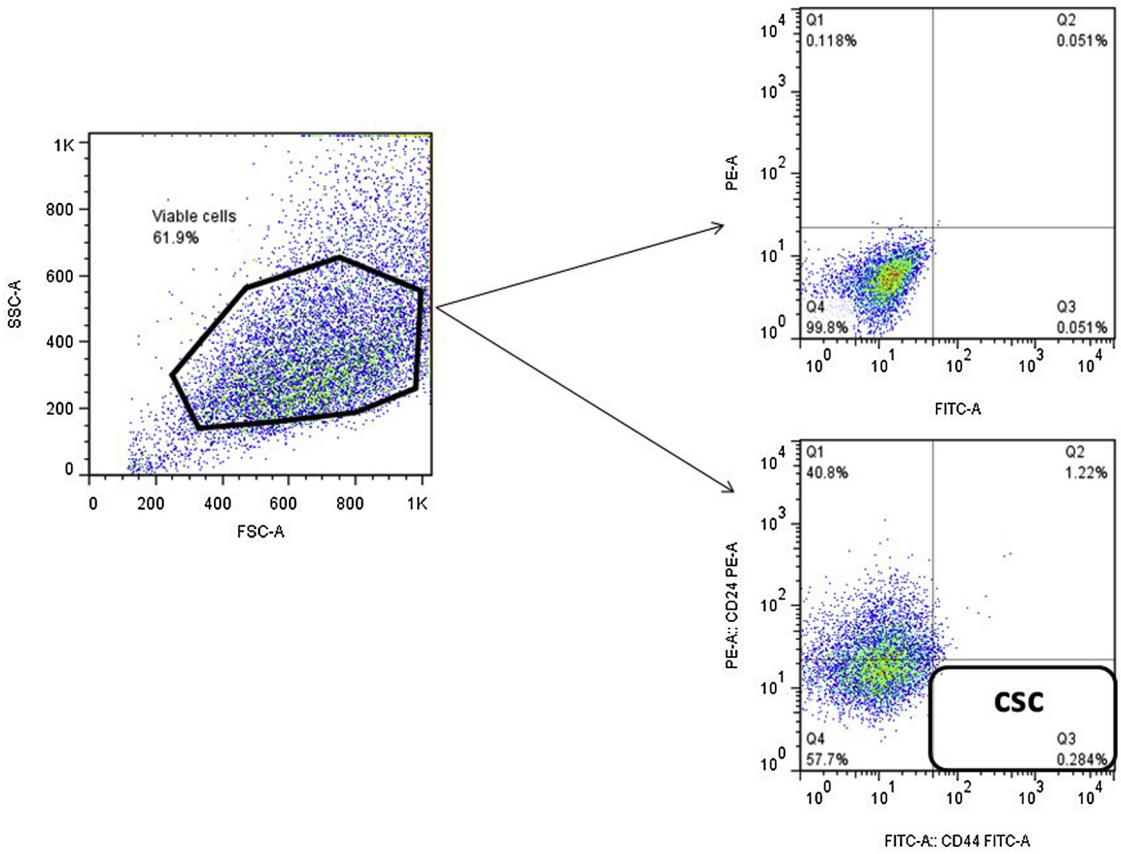

Fig. 3. The gating strategy of CD44/CD24 to determine the optimal phenotype for CSCs primed with different concentrations of chemotherapy. Expression patterns of CD24 and CD44 in EC cell line was analyzed by flow cytometry. Anti-CD44 antibody labeled with FITC and anti-CD24 antibody labeled with PE were applied to the analysis.

(DC + chemo) or loaded DC (Vacc + chemo) showed a significant decrease ($P < 0.001$) in tumor volume by 40.9%, 55.76%, 56.69%, 66.76% and 82% when compared to untreated control group respectively. Treatment with unloaded or loaded DC either alone or in combination with cisplatin showed a significant decrease in tumor volume on day 29 by 25% ($P < 0.05$), 43.75% ($P < 0.01$), 26.7% ($P < 0.05$), and 69.6% ($P < 0.001$) when compared to cisplatin - treated (chemo) group respectively **(**Fig. 6a**)**.

Growth of subcutaneous tumors in mice subjected to co-treatment with repeated low dose cisplatin and CSC - loaded DC (Vacc + chemo) was significantly decreased as compared with other treated groups. By day 29, the Vacc + chemo group significantly decreased tumor volume by 69.6% ($P < 0.001$), 59.35% ($P < 0.01$), 69.6% ($P < 0.01$), and 45.9% ($P < 0.05$) when compared to chemo, DC, DC + chemo, and vacc groups respectively **(**Fig. 6a**)**.

On day 30, only one out of six mice vaccinated with either unloaded DC alone or in combination with cisplatin was tumor free. In contrast, two out of six mice vaccinated with either CSC-loaded DC alone or in combination with cisplatin were tumor free. (Fig. 6b) shows comparing tumor volumes of studied groups from day 14 to day 30 post-tumor induction.

(Fig. 6c) illustrates that the induction of tumor in the untreated control group markedly decreased the percent survival rate by ↓ 66.66%. Whereas the survival rate was improved in all treated groups; chemo (83.33%), DC (83.33%), DC + chemo (83.33%), Vacc (100%), and Vacc + chemo (100%) groups. CSC loaded DC vaccinated groups showed the highest survival rate among the treated groups. These results demonstrated that vaccination with CSC loaded DCs significantly augmented the therapeutic efficacy of cisplatin as evidenced by the significant reduction of tumor volume as well as improved overall survival rate as compared with other treated groups in SEC tumor bearing mice.

3.5. Beneficial immune responses after vaccination with DCs loaded with CSC enriched lysate

(Fig. 7a) illustrates the immunomodulatory effects induced by vaccination with unloaded DCs and DCs loaded with the lysate of CSC-enriched population either as single treatment or in combination with cisplatin in comparison with untreated control group. Compared with tumor control group, the serum IFN-γ level in mice treated with chemo, DC, DC + chemo, vacc or vacc + chemo was significantly increased ($P < 0.05$, ↑42.6%), ($P < 0.001$, ↑188%), ($P < 0.001$, ↑224%), ($P < 0.001$, ↑245%) and ($P < 0.001$, ↑339%) respectively.

A significant increase in serum IFN-γ level was also observed in DC + chemo and vacc groups ($P < 0.05$, ↑12.5%) and ($P < 0.01$, ↑ 19.7%) respectively, when compared to DC group. In contrast, there was no significant difference in serum IFN-γ levels between DC + chemo and vacc groups. The combined treatment with CSC-loaded DC and cisplatin showed a significant increase in serum IFN-γ level when compared to groups treated with either unloaded DC ($P < 0.001$, ↑52%), DC + chemo ($P < 0.001$, ↑35%) or single treatment with CSC-loaded DC ($P < 0.001$, ↑27%).

3.6. Upregulation of p53 relative gene expression in co-treated group with repeated low doses cisplatin and CSC loaded DCs

We examined the expression of p53 gene at the mRNA level by real-time PCR. As shown in Fig. (7 b), no statistically significant difference in p53 gene expression levels were found between treatment of SEC

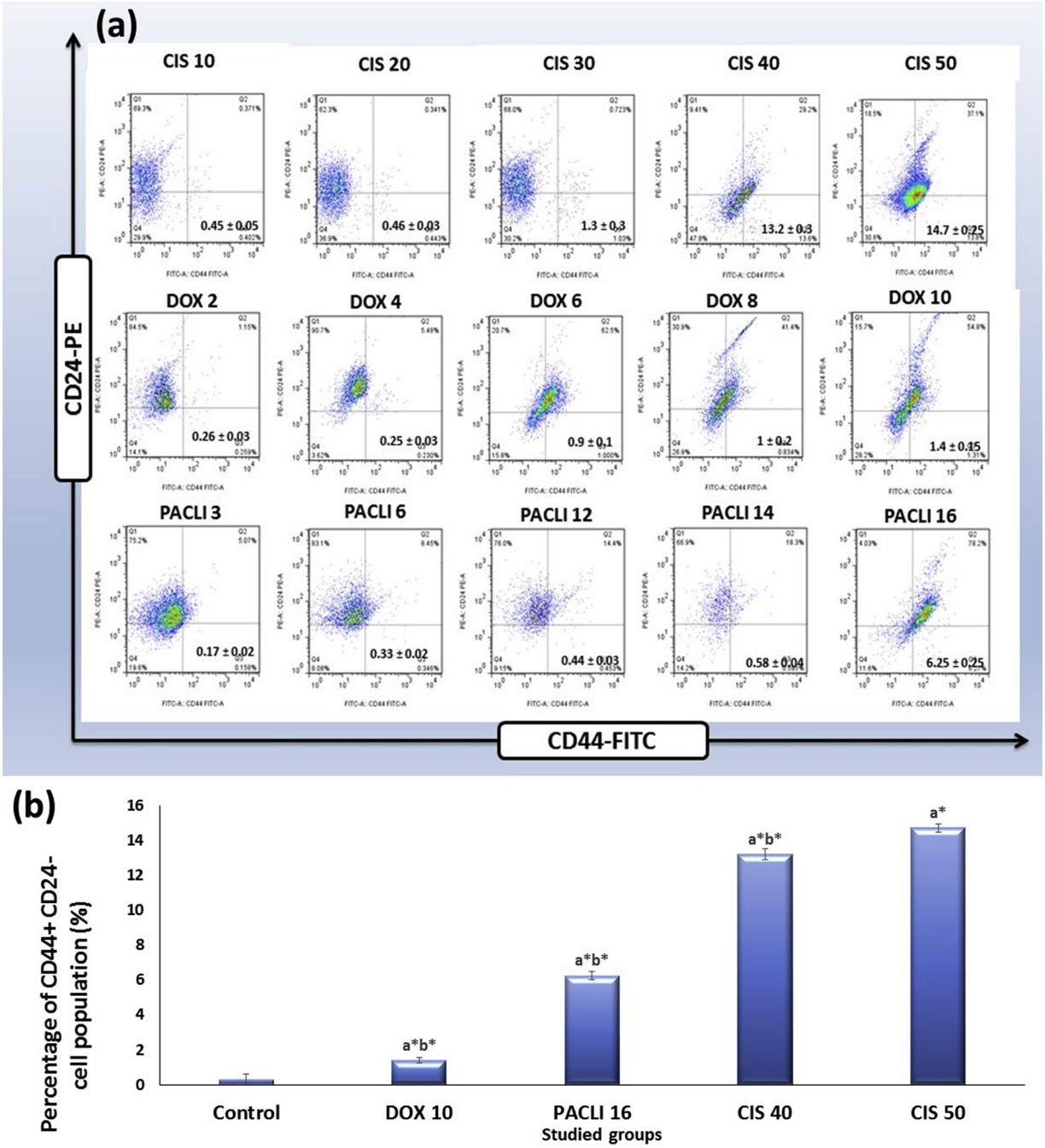

Fig. 4. (a) Enrichment of tumor cells bearing CSC markers identified as CD44 + /CD24- by flow cytometry based on their chemotherapeutic drug resistance. Percentages of CD44 + /CD24 − cell population in parental EC and drug-treated cell cultures were assessed by flow cytometry. Chemotherapeutic drugs were applied with the indicated concentrations for 72 h after primary EC cell cultures. **(b) Comparison between the drug-treated cell cultures with the highest expression levels of CD44 + /CD24- cells.** The percentage of CD44 + /CD24- cells is indicated in the dot plots. Data are presented as mean ± SD. **a:** significant increase *versus* control group; **b:** significant decrease *versus* CIS 50 group. Triplicate experiments were performed. * $P < 0.01$. CIS, cisplatin; DOX, doxorubicin; PACLI, paclitaxel; CD, cluster of differentiation. Concentrations are expressed as μg/mL.

bearing mice with either cisplatin at the selected dose (chemo group) or unloaded DC (DC group) when compared to untreated control group.

In contrary, DC + chemo, vacc or vacc + chemo groups significantly increased p53 relative gene expression levels by ($P < 0.01$, ↑ 1.34 ± 0.1 fold), ($P < 0.001$, ↑2.08 ± 0.26 fold), and ($P < 0.001$, ↑ 5.95 ± 0.6 fold) respectively compared to tumor control group. Furthermore, the mRNA gene expression of p53 was significantly increased in DC + chemo group ($P < 0.05$), vacc group ($P < 0.01$), and vacc + chemo group ($P < 0.001$) when compared to chemo group (Fig. 7b).

A significant increase in p53 gene expression levels was also observed in CSC-loaded DC treated group ($P < 0.05$) when compared to unloaded DC treated group. Interestingly, p53 gene expression level was significantly up-regulated in co-treated vacc + chemo group ($P < 0.001$) when compared to other treated groups (Fig. 7b).

4. Discussion

Cancer stem cells are a subset of tumorigenic cells that were found to be rare representing a small proportion of the tumor mass. It was

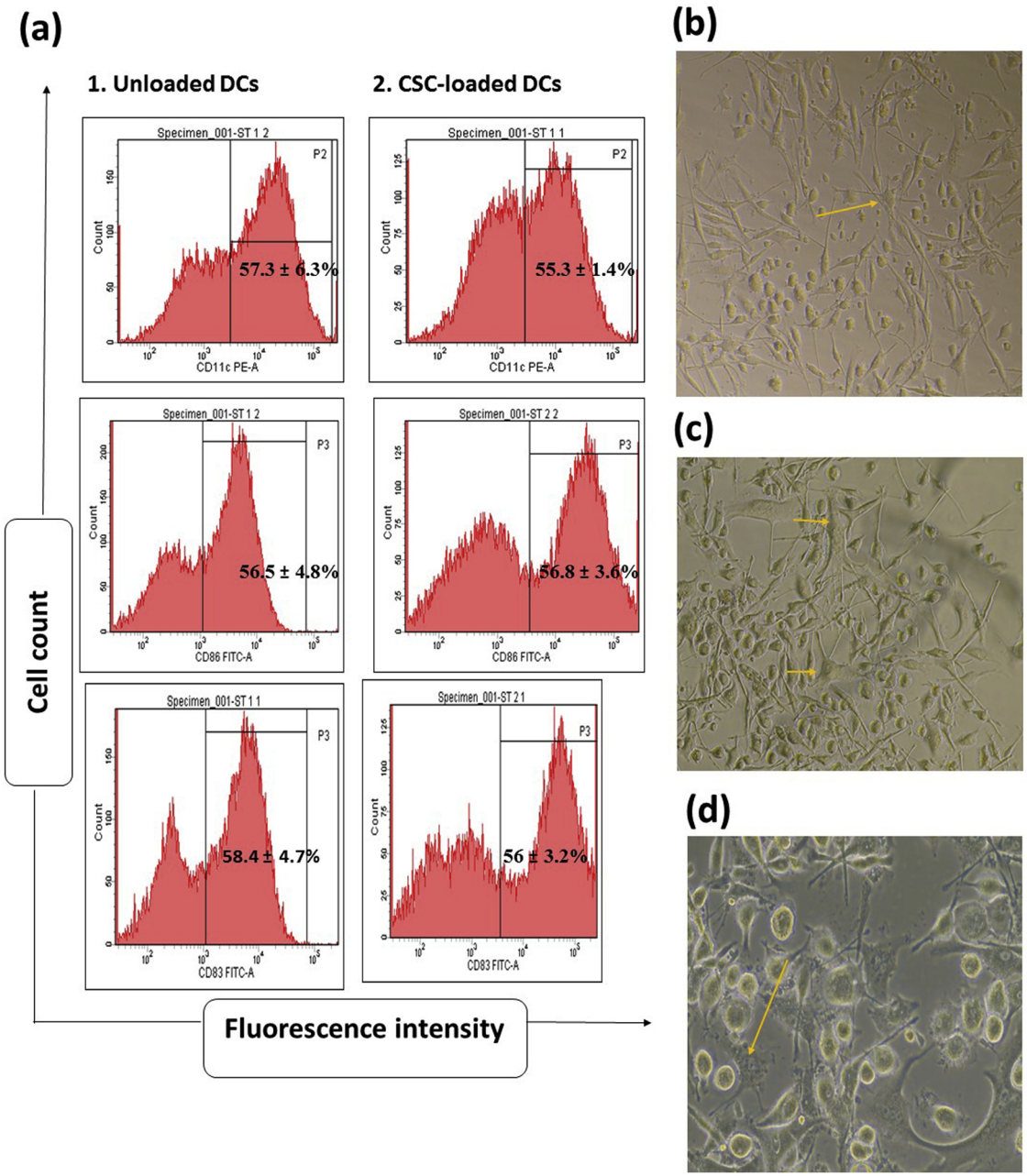

Fig. 5. Mature bone-marrow derived dendritic cells (BMDCs) generated from naïve mice either loaded or unloaded with enriched CSCs lysate harvested on day 9 of culture in presence of GM-CSF and IL-4 and maturation factor TNF-α. Mature loaded and unloaded DCs expressed typical mature DCs surface markers CD11c and co-stimulatory molecules CD83 and CD86. **(a)** Flow cytometric analysis showed that the expression pattern of mature CSC-loaded DCs exhibited almost identical phenotype as unloaded DCs group. Representative histogram plots for mature DCs surface markers stained with fluorochrome-conjugated monoclonal antibodies including anti-CD11c, CD83, and CD86 as indicated. The percentages of positive cells are indicated in the histograms. Triplicate experiments were performed. Data are presented as mean ± SD. **(b)** Immature loaded DCs on day 6 exhibited typical morphological changes and appeared as loosely adherent cell with branched and extended morphology (arrow) (original magnification ×20), **(c)** Mature unloaded DCs on day 9 (original magnification ×20), and (**d)** Mature CSC-loaded DCs on day 9 (original magnification ×40) by digital inverted microscope.

reported that CSCs are responsible for tumorigenesis, tumor maintenance, metastasis and relapse. Moreover, these cells are characterized by their high resistance to chemotherapeutic drugs, radiotherapy and apoptosis and are considered as major factors of treatment failure (Luo et al., 2014).

Most of the current immune strategies are mainly directed to target differentiated tumor antigens. However, CSC population may lack these differentiated antigens and therefore they may not be targeted by these traditional immune strategies. Strategies specifically target drug – resistant CSCs may represent an effective way to prevent tumor relapse and metastasis (Nguyen et al., 2015).

Li ; et al.; (2014) previously reported that CSCs could be used as an effective antigen source to load DCs than traditional bulk tumor cells producing much more protective immune responses against tumorigenesis (Li et al., 2014). There are four common methods for the separation or enrichment of CSCs: a) separation by detecting side population phenotype by Hoeschst dye; b) isolation using flow cytometry or magnetic beads with CSC specific cell surface markers; c) ability of the CSCs to grow and float in serum-free medium; and d) selection based on the drug resistance property of CSCs, which increases the purity of CSCs by inducing the apoptosis of other tumor cells achieved by *in vitro* or *in vivo* treatment with therapeutic agents (Zhang et al., 2015).

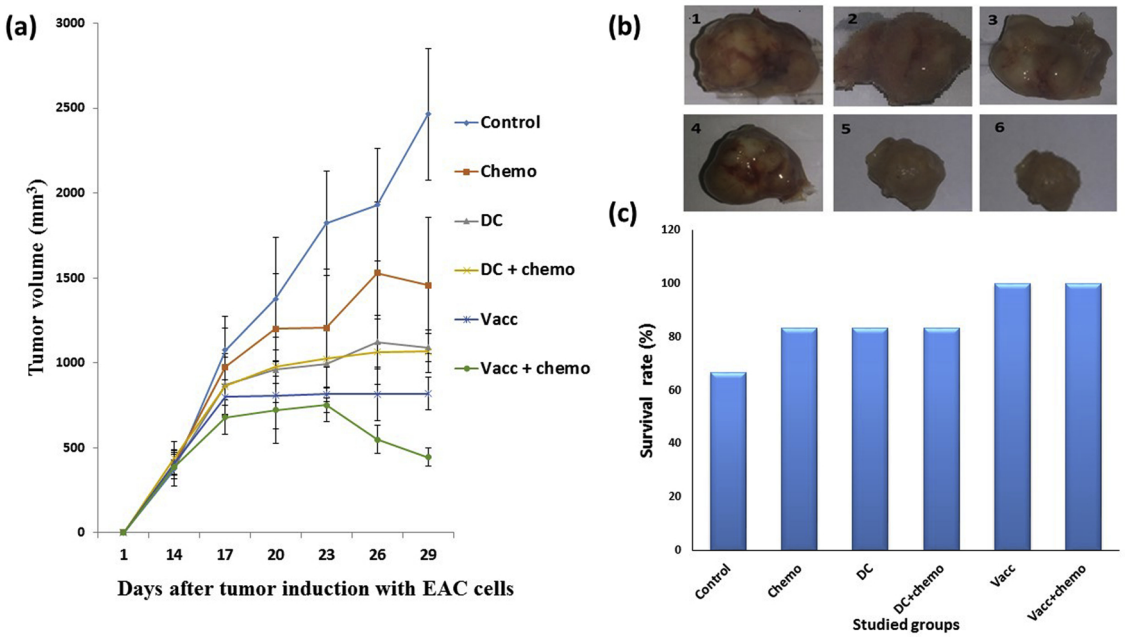

Fig. 6. Beneficial effects of CSC – loaded DCs either alone or in combination with repeated low doses of cisplatin on tumor volume and survival rate. (a) Tumor volume (mm^3). (b) Tumor masses excised at the end of the study from different studied groups. (c) Survival rate of tumor bearing mice in different studied groups. Data are presented as mean ± SD. Tumor volume $(mm)^3$ = [Length (mm) × $width^2$ (mm^2)] / 2. Tumor formation and growth was monitored twice a week. Photographs in panel (B) are tumor masses excised from: (1) control, (2) chemo, (3) DC, (4) DC + chemo, (5) Vacc, (6) Vacc + chemo groups; EAC: Ehrlich ascites carcinoma, chemo: chemotherapy (cisplatin) treated group, DC: unloaded dendritic cells treated - group, Vacc: vaccine group treated with CSC – loaded dendritic cells, DC + chemo: combined treatment with DC + cisplatin, Vacc + chemo: combined treatment with CSC-loaded DC + cisplatin.

In our study, we used transplantable EC cells to establish our tumor cell line. EC cell line is an aggressive tumor cell line consisting of a heterogeneous pool of malignant cells which are originally derived from a spontaneous mouse breast carcinoma (Salem et al., 2016b). It was reported by Goltsev ; et al.; (2017) the existence of CSCs as a part of EC cells that were identified by their cell surface phenotypic markers as $CD44^+/CD24^-$ and were found to be responsible for maintenance of the tumor growth (Goltsev et al., 2017).

Additionally, Goltsev and his co-workers demonstrated that all the signs of CSCs in EC cell line (capability for self-renewal, high tumorigenicity, metastatsis and chemo-resistance) were characteristics of cells with high expression levels of CD44+ marker and the absence of CD24 expression (Goltsev et al., 2015).

We therefore hypothesized that CSCs can be enriched from EC tumor cell population following chemotherapeutic drug treatment based on their high resistance to chemotherapeutic agents. Isolation of CSCs based on chemotherapeutic drug resistance is a functional assay that has been applied to enrich cancer stem cells as reported previously (Nguyen et al., 2015). In the present study, drug surviving cells that have been selected from EC cell line treated *in vitro* with increasing doses of cisplatin, doxorubicin, or paclitaxel possessed drug resistant phenotype as well as CSC-like phenotype. This results indicate that $CD44^+/CD24^-$ cells were highly enriched using isolation based on drug-resistance isolation method as previously reported (Calcagno et al., 2010).

Considering that the undifferentiated CSCs are more resistant to chemotherapy and apoptosis than differentiated cancer cells, the present study initially used flow cytometry to detect the percentage of $CD44^+/CD24^-$ cells in DSCs following treatment with different chemotherapies. Subsequently, the isolated drug surviving cells populations that exhibited the highest expression of CSC surface markers identified as $CD44^+$ /CD24- were selected. Our results showed that treatment with 50 µg/mL cisplatin resulted in the highest expression of $CD44^+/CD24^-$ compared to other treated groups and this was the maximum concentration of cisplatin in which EC cells could survive and grow.

Elimination of drug sensitive differentiated tumor cells and enrichment of CSCs following drug treatment observed in our *in vitro* model suggest that similar selection of drug resistant CSCs could be observed in clinical practice during chemotherapeutic treatment. It was previously reported that breast tumors from patients treated with chemotherapy contained higher percentage of $CD44^+$ $CD24^{low}$ cells with CSC features than from untreated patients (Yu et al., 2007). The initial therapy of established tumors may result in destruction of non-CSCs which in turn lead to an increase in the relative percentage of CSCs which is consistent with our findings (Lu et al., 2015).

We developed a vaccination strategy using *ex vivo* generated DCs loaded with cell lysates of chemo-resistant cancer stem – like enriched cells population to be used for inducing effective anti-tumor immune responses in SEC bearing mice. Our results showed that DCs isolated from bone marrow of healthy mice - which were loaded *in vitro* using resistant CSCs enriched population lysate - showed typical mature DCs phenotype characteristics with high expression of CD 11c + and co-stimulatory molecules CD83 and CD86 suggesting that loading immature DCs with CSC enriched antigens could successfully generate DCs displaying typical mature DC surface markers (Salem et al., 2016a).

Moreover, the expression patterns of co-stimulatory molecules of loaded DCs and unloaded DCs after maturation were almost identical. These findings indicate that loading of DCs with CSC lysate didn't change the phenotypic characteristics of mature DCs as shown by flow cytometric analysis. These findings were supported by the study of Lee et al. (2008).

It was reported previously that repetitive administration of chemotherapeutic agents at low doses (so-called, metronomic chemotherapy) induce strong antitumor effects with the advantage of the absence of toxicity (Tagliamonte et al., 2016). Additionally, a prior study showed that very low concentrations of different chemotherapeutic agents increase the ability of DC vaccine to induce T cell

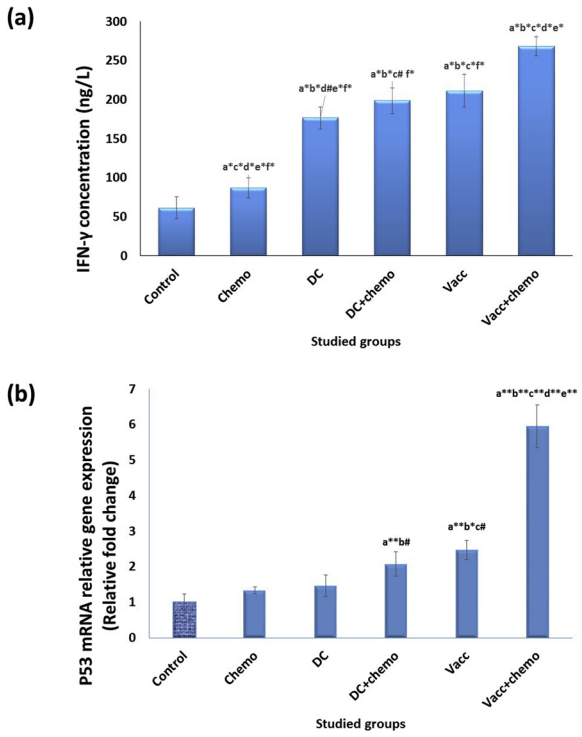

Fig. 7. Effect of vaccination with either loaded or unloaded DCs with or without cisplatin on (a) IFN-γ serum levels in SEC bearing mice using ELISA assay. (b) p53 mRNA relative gene expression in tumor tissues excised from SEC bearing mice using real time PCR. The expression of p53 was significantly up-regulated in vacc + chemo group *versus* all other groups. Data are expressed as relative fold change. Results were calculated and normalized to reference GAPDH gene. The relative gene expression levels were calculated by using $2^{-\Delta\Delta CT}$ method. The data represents the average of three replicates for each group. Data shown in (a) and (b) are presented as mean ± SD. # $P < 0.05$, *$P < 0.01$, **$P < 0.001$. **a:** Significant increase *versus* tumor control group, **b:** Significant increase *versus* chemo - treated group, **c:** Significant increase *versus* DC - treated group, **d:** Significant increase *versus* DC + chemo treated group, **e:** Significant increase *versus* Vacc - treated group. **SEC:** solid Ehrlich carcinoma.

proliferation (Lu et al., 2015). In the current study, we evaluated the potential therapeutic efficacy of using CSC-loaded DC vaccine either alone or in combination with repeated low doses of cisplatin on SEC bearing mice. We hypothesized that CSC-based vaccines might be able to enhance the antitumor efficiency of chemotherapy by targeting resistant CSCs.

The present findings showed that vaccination with CSC-loaded DC combined with repeated cycles of low doses of cisplatin resulted in a significant inhibition of tumor growth compared with untreated control group as well as all other treated groups. These findings were supported by the work of Lu et al. (2015). Furthermore, vaccination with loaded DC either alone or in combination with cisplatin prolonged the survival of tumor bearing mice as demonstrated by our findings. These data indicate that co-treatment with CSC loaded DC and cisplatin resulted in enhanced anti-tumor outcomes compared to single treatment with either chemotherapy or loaded DCs as indicated by inhibition of subcutaneous tumor growth and prolonged survival of SEC bearing mice which were in line with the study of Hu et al. (2016).

In the present study, we examined the ability of CSC-DC vaccine to induce cellular immune responses *via* induction of significant IFN- γ production. A significant increase in IFN- γ serum levels was observed in DC + chemo, Vacc, and Vacc + chemo groups as compared to untreated control, DC, and chemo groups. Interestingly, the combination of CSC-loaded DC and cisplatin significantly augmented the antitumor immune response of cisplatin as evidenced by the significant increase in IFN- γ serum levels as compared to either single treatment with CSC-loaded DC or combined treatment with unloaded DC and cisplatin. The higher IFN- γ serum levels in the Vacc + chemo group is consistent with the higher survival rate and reduction of tumor volume observed in the same group as reported by previous studies (Yu et al., 2003; Xu et al., 2009).

Dendritic cells must present tumor antigens, express co-stimulatory signals, and produce inflammatory mediators such as IFNs in order to activate naïve T cells into a functional state (Mac Keon et al., 2015). IFN-γ produced by immune cells affects other distinct immune cells within the tumor microenvironment. It plays a major role in activating anticancer immunity by promoting the activity of T-cells, NK cells, and DCs. Moreover, it was reported that IFN-γ inhibits regulatory T (Treg) cells, suggesting that IFN-γ plays an important role in tumor cell elimination (Ni and Lu, 2018).

Wang and his co-workers showed that plasma IFN-γ levels were significantly decreased in lung cancer patients (Wang et al., 2013). It was reported by Higgs et al. that increased IFN-γ is correlated with higher response and longer survival in patients with non-small cell lung carcinoma or urothelial cancerwhich is in line with our findings (Higgs et al., 2018).

In addition to the direct activation of specific antitumor immune response, recent approaches are directed toward combining DC-based vaccines with agents that modulate the tumor microenvironment such as chemotherapy to improve the immunogenicity and overcome the immunosuppression within the tumor microenvironment (Huber et al., 2018).

Previous studies have shown that p53 mutation/inactivation promote tumorigenesis either directly or indirectly which in turn induce tumor pro-inflammatory responses and acts as a driver of tumorigenesis through changing the immunological tumor microenvironment leading to immunosuppression. On contrary, the activation/reactivation of p53 expression contribute in reversing the immunosuppression and enhancing antitumor immunity (Ventura et al., 2007; Menendez et al., 2013; Cui and Guo, 2016; Guo et al., 2017). In this context, we examined the relative gene expression of p53 in tumor bearing mice which plays a significant role in the inhibition of tumor cell proliferation and induction of apoptosis (Bassiony et al., 2014).

Our results illustrated that Vacc + chemo combined treatment showed a more profound significant increase ($P < 0.001$) in p53 relative gene expression levels in comparison to its levels in other treated groups. The present findings were in accordance with previous study which illustrated that enhanced p53 expression directs the tumor cells to induce apoptosis of Ehrlich solid carcinoma cells thus inhibiting tumorigenesis (Bassiony et al., 2014).

The main limitation of this study is that we didn't use a pure population of CSCs but instead we used residual cisplatin resistant cancer cells with CSCs like phenotype ($CD44^+$ $CD24^-$) as an antigen source in the preparation of DC-based vaccine.

To date, the mechanisms that are involved in CSC-DC vaccination and detailed immunological analysis data on their apparent superior outcomes regarding the development of enhanced antitumor immunity have not been fully defined in addition to the limited experimental evidence. In addition, the identification of CSC antigen(s) requires further investigation. It was stated that immunotherapeutic strategies that engage different effector mechanisms is better to overcome the immunosuppressive mechanisms of cancer (Apetoh et al., 2015).

5. Conclusion

Our study opens the field for the development of effective chemo-immunotherapeutic strategies for higher antitumor efficacy and enhanced immune responses compared to currently available chemotherapies. We used drug selection method based on incubation of EC cell line with cisplatin (50 μg/mL) for 72 h, which not only selected drug-resistant cells, but also the resistant cells were highly enriched with CSC-like phenotype (CD44 + /CD24 −) population. The study

revealed an enhancement of the *in vivo* chemotherapeutic efficacy of repeated low doses of cisplatin when combined with DCs loaded with antigens enriched in drug - resistant cancer stem – like cell lysates. This proposed combined treatment was associated with suppression of tumor growth and potentiated antitumor immune responses compared to either treatment alone. Thus, targeting resistant and tumor cells bearing stem cell characteristics could be beneficial for improving the therapeutic efficacy of conventional chemotherapy taking the advantage of using low doses of cisplatin rather than high doses.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest statement

The author(s) declare no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

Apetoh, L., Ladoire, S., Coukos, G., Ghiringhelli, F., 2015. Combining immunotherapy and anticancer agents: the right path to achieve cancer cure? Ann. Oncol. 26 (9), 1813–1823. https://doi.org/10.1093/annonc/mdv209.

Arican, G., Arican, E., 2006. Evaluation of the apoptotic and antiproliferative activities of paclitaxel in ehrlich ascites tumor cells. Biotechnol. Biotechnol. Equip. 20, 69–75. https://doi.org/10.1080/13102818.2006.10817382.

Aysan, E., Bayrak, O.F., Aydemir, E., Telci, D., Sahin, F., Yardimci, C., Muslumanoglu, M., 2013. Cancer preventive effects of whole cell type immunization against mice Ehrlich tumors. Asian Pac. J. Cancer Prev. 14 (6), 3515–3519.

Bassiony, H., Sabet, S., Salah El-Din, T.A., Mohamed, M.M., El-Ghor, A.A., 2014. Magnetite nanoparticles inhibit tumor growth and upregulate the expression of p53/p16 in Ehrlich solid carcinoma bearing mice. PLoS One 9 (11), e111960. https://doi.org/10.1371/journal.pone.0111960.

Calcagno, A.M., Salcido, C.D., Gillet, J.P., Wu, C.P., Fostel, J.M., Mumau, M.D., Gottesman, M.M., Varticovski, L., Ambudkar, S.V., 2010. Prolonged drug selection of breast cancer cells and enrichment of cancer stem cell characteristics. J. Natl. Cancer Inst. 102 (21), 1637–1652. https://doi.org/10.1093/jnci/djq361.

Cui, Y., Guo, G., 2016. Immunomodulatory function of the tumor suppressor p53 in host immune response and the tumor microenvironment. Int. J. Mol. Sci. 17 (11), E1942.

Dallas, N.A., Xia, L., Fan, F., Gray, M.J., Gaur, P., van Buren 2nd, G., Samuel, S., Kim, M.P., Lim, S.J., Ellis, L.M., 2009. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. Cancer Res. 69 (5), 1951–1957. https://doi.org/10.1158/0008-5472.

El-Ashmawy, N.E., Khedr, N.F., El-Bahrawy, H.A., 2017. Abo Mansour HE. Metformin augments doxorubicin cytotoxicity in mammary carcinoma through activation of adenosine monophosphate protein kinase pathway. J. Immunother. Emphasis Tumor Immun. Tumuor Biol. 39 (5), 1010428317692235. https://doi.org/10.1177/1010428317692235.

Elbialy, N.S., Mady, M.M., 2015. Ehrlich tumor inhibition using doxorubicin containing liposomes. Saudi Pharm. J. 23 (2), 182–187. https://doi.org/10.1016/j.jsps.2014.07.003.

El-Zamarany, E.A., El-Ashmawy, N.E., Salem, M.L., El-Bahrawy, H.A., Al-Ashmawy, G.M., 2015. Effect of dexamethasone on phenotyping of dendritic cells: in vitro and in vivo study. Sci. Technol. Dev. 34 (2), 74–81.

Goltsev, A.N., Babenko, N.N., Gayevskaya, Yu A., Bondarovich, N.A., Ostankov, M.V., Chelombitko, O.V., Dubrava, T.G., Klochkov, V.K., Kavok, N.S., Malyukin, Yu V., 2013. Capability of othovanadate-based nanoparticles to in vitro identification and in vivo inhibition of cancer stem cells. Nanosyst. Nanometer. Nanotechnol. 11 (4), 729–739.

Goltsev, A.N., Babenko, N.N., Gaevskaya, Yu A., Chelombitko, O.V., Dubrava, T.G., Bondarovich, N.A., Ostankov, M.V., Klochkov, V.K., Kavok, N.S., Malyukin, Yu., 2015. Application of nanoparticles based on rare earth orthovanadates to inactivate Ehrlich carcinoma growth. Biotechnol. Acta 8 (4), 113–121.

Goltsev, A.N., Babenko, N.N., Gaevskaya, Y.A., Bondarovich, N.A., Dubrava, T.G., Ostankov, M.V., Chelombitko, O.V., Malyukin, Y.V., Klochkov, V.K., Kavok, N.S., 2017. Nanotechniques inactivate cancer stem cells. Nanoscale Res. Lett. 12 (1), 415. https://doi.org/10.1186/s11671-017-2175-9.

Gorbach, O., Khranovska, N., Skachkova, O., Sydor, R., Svergun, N., Pozur, V., 2014. Low doses of cisplatin can help dendritic cell vaccines to reduce immunosuppression in tumor microenvironment. Ann. Oncol. 25 (Supplement 6), vi3–vi7. https://doi.org/10.1093/annonc/mdu467.5.

Guo, G., Yu, M., Xiao, W., Celis, E., Cui, Y., 2017. Local activation of p53 in the tumor microenvironment overcomes immune suppression and enhances antitumor immunity. Cancer Res. 77 (9), 2292–2305. https://doi.org/10.1158/0008-5472.

Higgs, B.W., Morehouse, C.A., Streicher, K., Brohawn, P.Z., Pilataxi, F., Gupta, A., Ranade, K., 2018. Interferon gamma messenger RNA signature in tumor biopsies predicts outcomes in patientswith non-small cell lung carcinoma or urothelial Cancer Treated with durvalumab. Clin. Cancer Res. 24 (16), 3857–3866. https://doi.org/10.1158/1078-0432.CCR-17-3451. Epub 2018 May 1.

Hu, Y., Lu, L., Xia, Y., Chen, X., Chang, A.E., Hollingsworth, R.E., Hurt, E., Owen, J., Moyer, J.S., Prince, M.E., Dai, F., Bao, Y., Wang, Y., Whitfield, J., Xia, J.C., Huang, S., Wicha, M.S., Li, Q., 2016. Therapeutic efficacy of cancer stem cell vaccines in the adjuvant setting. Cancer Res. 76 (16), 4661–4672. https://doi.org/10.1158/0008-5472.CAN-15-2664.

Huber, A., Dammeijer, F., JGJV, Aerts, Vroman, H., 2018. Current State of Dendritic Cell-Based Immunotherapy: Opportunities for in vitro Antigen Loading of Different DC Subsets? Front. Immunol. 9 (2804). https://doi.org/10.3389/fimmu.2018.02804. eCollection 2018.

Khedr, N.F., Khalil, R.M., 2015. Effect of hesperidin on mice bearing Ehrlich solid carcinoma maintained on doxorubicin. J. Immunother. Emphasis Tumor Immun. Tumuor Biol. 36 (12), 9267–9275. https://doi.org/10.1007/s13277-015-3655-0.

Konduri, V., Li, D., Halpert, M.M., Liang, D., Liang, Z., Chen, Y., Fisher, W.E., Paust, S., Levitt, J.M., Yao, Q.C., Decker, W.K., 2016. Chemo-immunotherapy mediates durable cure of orthotopic KrasG12D/p53-/- pancreatic ductal adenocarcinoma. Oncoimmunology 5 (9), e1213933.

Lee, J.J., Foon, K.A., Mailliard, R.B., Muthuswamy, R., Kalinski, P., 2008. Type 1-polarized dendritic cells loaded with autologous tumor are a potent immunogen against chronic lymphocytic leukemia. J. Leukoc. Biol. 84 (1), 319–325. https://doi.org/10.1189/jlb.1107737.

Levina, V., Marrangoni, A.M., DeMarco, R., Gorelik, E., Lokshin, A.E., 2008. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. PLoS One 3 (8), e3077. https://doi.org/10.1371/journal.pone.0003077.

Li, Q., Lu, L., Tao, H., Xue, C., Teitz-Tennenbaum, S., Owen, J.H., Moyer, J.S., Prince, M.E., Chang, A.E., Wicha, M.S., 2014. Generation of a novel dendritic-cell vaccine using melanoma and squamous cancer stem cells. J. Vis. Exp.(83), e50561. https://doi.org/10.3791/50561.

Lopez-Ayllon, B.D., Moncho-Amor, V., Abarrategi, A., Ibañez de Cáceres, I., Castro-Carpeño, J., Belda-Iniesta, C., Perona, R., Sastre, L., 2014. Cancer stem cells and cisplatin-resistant cells isolated from non-small-lung cancer cell lines constitute related cell populations. Cancer Med. 3 (5), 1099–1111. https://doi.org/10.1002/cam4.291.

Lu, L., Tao, H., Chang, A.E., Hu, Y., Shu, G., Chen, Q., Egenti, M., Owen, J., Moyer, J.S., Prince, M.E., Huang, S., Wicha, M.S., Xia, J.C., Li, Q., 2015. Cancer stem cell vaccine inhibits metastases of primary tumors and induces humoral immune responses against cancer stem cells. OncoImmunology 4 (3), e990767. https://doi.org/10.4161/2162402X.2014.990767. 4:3.

Luo, H., Zeng, C., Fang, C., Seeruttun, S.R., Lv, L., et al., 2014. A new strategy using ALDHhigh-CD8+T cells to inhibit tumorigenesis. PLoS One 9 (8), e103193. https://doi.org/10.1371/journal.pone.0103193.

Ma, L., Lai, D., Liu, T., Cheng, W., Guo, L., 2010. Cancer stem-like cells can be isolated with drug selection in human ovarian cancer cell line SKOV3. Acta Biochim. Biophys. Sin. (Shanghai). 42 (9), 593–602. https://doi.org/10.1093/abbs/gmq067.

Mac Keon, S., Ruiz, M.S., Gazzaniga, S., Wainstok, R., 2015. Dendritic cell-based vaccination in cancer: therapeutic implications emerging from murinemodels. Front. Immunol. 6 (243). https://doi.org/10.3389/fimmu.2015.00243. eCollection 2015.

Menendez, D., Shatz, M., Resnick, M.A., 2013. Interactions between the tumor suppressor p53 and immune responses. Curr. Opin. Oncol. 25 (1), 85–92. https://doi.org/10.1097/CCO.0b013e32835b6386.

Nandi, S., Ulasov, I.V., Tyler, M.A., Sugihara, A.Q., Molinero, L., Han, Y., Zhu, Z.B., Lesniak, M.S., 2008. Low-dose radiation enhances survivin-mediated virotherapy against malignant glioma stem cells. Cancer Res. 68, 5778–5784.

Nguyen, S.T., Nguyen, H.L., Pham, V.Q., Nguyen, G.T., Tran, C.D., Phan, N.K., Pham, P.V., 2015. Targeting specificity of dendritic cells on breast cancer stem cells: in vitro and in vivo evaluations. Onco. Ther. 8, 323–334. https://doi.org/10.2147/OTT.S77554.

Ni, L., Lu, J., 2018. Interferon gamma in cancer immunotherapy. Cancer Med. 7 (9), 4509–4516. https://doi.org/10.1002/cam4.1700. Epub 2018 Jul 23.

Osman, A.M., Alqahtani, A.A., Damanhouri, Z.A., Al-Harthy, S.E., ElShal, M.F., Ramadan, W.S., Kamel, F., Osman, M.A., Khan, L.M., 2015. Dimethylsulfoxide excerbates cisplatin-induced cytotoxicity in Ehrlich ascites carcinoma cells. Cancer Cell Int. 29 (15), 104. https://doi.org/10.1186/s12935-015-0258-1.

Peng, S., Wang, J.W., Karanam, B., Wang, C., Huh, W.K., Alvarez, R.D., Pai, S.I., Hung, C.F., Wu, T.C., Roden, R.B., 2015. Sequential cisplatin therapy and vaccination with HPV16 E6E7L2 fusion protein in saponin adjuvant GPI-0100 for the treatment of a model HPV16+ cancer. PLoS One 10 (1), e116389. https://doi.org/10.1371/journal.pone.0116389.

Sabado, R.L., Balan, S., Bhardwaj, N., 2017. Dendritic cell-based immunotherapy. Cell Res. 27 (1), 74–95. https://doi.org/10.1038/cr.2016.157.

Salem, M.L., Abdel Salam, S.G., Nassef, M., Hammad, S., El Adl, R., 2015. Immunoenhancing properties of the anti-tumor effects of adoptively transferred T cells with chemotherapeutic cyclophosphamide by co-administration of bone marrow cells. J. Basic Appl. Zool. 72, 96–103.

Salem, M.L., Eissa, I.R., Mohamed, T.M., 2016a. Dendritic cells generated from naïve and tumor-bearing mice uniquely restores different leukocyte subpopulations in chemotherapy-treated tumor-bearing mice. Clin. Cancer Investig. J. 5, 1–10.

Salem, M.L., Shoukry, N.M., Teleb, W.K., Abdel-Daim, M.M., Abdel-Rahman, M.A., 2016b. In vitro and in vivo antitumor effects of the Egyptian scorpion Androctonus amoreuxi venom in an Ehrlich ascites tumor model. Springerplus 5, 570. https://doi.

org/10.1186/s40064-016-2269-3.
Tagliamonte, M., Petrizzo, A., Napolitano, M., Luciano, A., Rea, D., Barbieri, A., Arra, C., Maiolino, P., Tornesello, M., Ciliberto, G., Buonaguro, F.M., Buonaguro, L., 2016. A novel multidrug metronomic chemotherapy significantly delays tumor growth in mice. J. Transl. Med. 14, 58. https://doi.org/10.1186/s12967-016-0812-1.
Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R., Jacks, T., 2007. Restoration of p53 function leads to tumour regression in vivo. Nature 445 (7128), 661–665.
Verma, A.K., Prasad, S.B., 2012. Bioactive component, cantharidin from Mylabris cichorii and its antitumor activity against Ehrlich ascites carcinoma. Cell Biol. Toxicol. 28 (3), 133–147. https://doi.org/10.1007/s10565-011-9206-6.
Wang, F., Xu, J., Zhu, Q., Qin, X., Cao, Y., Lou, J., Xu, Y., Ke, X., Li, Q., Xie, E., Zhang, L., Sun, R., Chen, L., Fang, B., Pan, S., 2013. Downregulation of IFNG in CD4(+) T cells in lung cancer through hypermethylation: a possible mechanism of tumor-induced immunosuppression. PLoS One 8 (11), e79064. https://doi.org/10.1371/journal.pone.0079064. eCollection 2013.
Weng, D., Song, B., Koido, S., Calderwood, S.K., Gong, J., 2013. Immunotherapy of radioresistant mammary tumors with early metastasis using molecular chaperone vaccines combined with ionizing radiation. J. Immunol. 191 (2), 755–763. https://doi.org/10.4049/jimmunol.1203286.
Xu, Q., Liu, G., Yuan, X., Xu, M., Wang, H., Ji, J., Konda, B., Black, K.L., Yu, J.S., 2009. Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens. Stem Cells 27 (8), 1734–1740. https://doi.org/10.1002/stem.102.
Yu, B., Kusmartsev, S., Cheng, F., Paolini, M., Nefedova, Y., Sotomayor, E., Gabrilovich, D., 2003. Effective combination of chemotherapy and dendritic cell administration for the treatment of advanced-stage experimental breast cancer. Clin. Cancer Res. 9 (1), 285–294.
Yu, F., Yao, H., Zhu, P., Zhang, X., Pan, Q., Gong, C., Huang, Y., Hu, X., Su, F., Lieberman, J., Song, E., 2007. let-7 regulates self-renewal and tumorigenicity of breast cancer cells. Cell 131 (6), 1109–1123.
Zhang, D.G., Jiang, A.G., Lu, H.Y., Zhang, L.X., Gao, X.Y., 2015. Isolation, cultivation and identification of human lung adenocarcinoma stem cells. Oncol. Lett. 9 (1), 47–54.