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Adjuvant effect of docetaxel on HPV16 L2E6E7 fusion protein vaccine in a mouse model

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ABSTRACT

We previously demonstrated that the antineoplastic agent docetaxel enhanced the immune response to an influenza vaccine. This study evaluated the adjuvant effect of docetaxel (DOC) on the therapeutic efficacy of HPV16 L2E6E7 fusion protein (HPV-LFP) in mice inoculated with TC-1 cells. The results demonstrated that docetaxel significantly enhanced the therapeutic effect of HPV-LFP on TC-1 cell-induced tumors in mice. The injection of HPV-LFP in combination with docetaxel in TC-1 tumor-bearing mice significantly reduced tumor volume and weight, and a greater percent survival was detected than mice treated with HPV-LFP alone. The inhibition of tumors was associated with significantly increased serum antigen-specific IgG and isotypes, activated CTLs, increased IFN-γ-secreting T cells, and decreased Treg cells and IL-10-secreting cells in spleen. In addition, down-regulation of IL-10, VEGF and STAT3, up-regulation of IFN-γ and decreased Treg cells in the tumor microenvironment may also important contributing factors to the antitumor effect. It may be valuable to use a DOC-containing water to dilute HPV-LFP powder before injection in patients because of its excellent adjuvant effect on HPV-LFP and solubility in water.

1. Introduction

Cervical cancer is a common cancer in females [1]. Approximately 400,000 women develop invasive cervical cancer, which results in 200,000 deaths annually worldwide [2].The annual cases in China are estimated at 62,000 with 30,000 deaths [3]. A close relationship was found between the infection of human papillomavirus (HPV) and cervical cancer and dysplasia [4–6]. It was reported that 5585 of 77,069 women were infected with HPV in an investigation that was performed in Pearl River Delta Region in Guangdong province of China [7].

HPV is a non-enveloped virus with double-stranded DNA and approximately 8000 base pairs [4]. Over 100 different HPV genotypes are recognized [8], and HPV16 and HPV18 are the most common types [9,10]. The papillomavirus genome encodes 6 early functional (E1 to E7) and 2 late structural proteins (L1 and L2) [11]. Vaccination with L2 induces widely cross-type neutralizing antibodies against HPV infection [12,13]. The viral antigens of E6 and E7 are constantly expressed throughout tumorigenesis, and these antigens are considered excellent target antigens as therapeutic vaccines [14]. Recently, a single fusion protein (TA-CIN) consisting of HPV16 L2, E6 and E7 exhibited potential to become a preventive and therapeutic HPV vaccine. TA-CIN elicited specific CTLs, T-helper cells and antibodies in mice [15].Vaccination with HPV-LFP and the subsequent administration of a boost of TA-HPV (recombinant vaccinia virus with E6 and E7 of HPV16 and HPV18) failed to stimulate improved lesion regression in phase II studies compared with the use of TA-HPV alone [16]. The poor responses to the therapeutic vaccination supported the formulation of a potent adjuvant to the HPV16 L2E6E7 fusion protein vaccine.

Docetaxel is a member of the taxane drug family, and it is a clinically well-established anti-tumor agent. Docetaxel is approved by the FDA to treat locally advanced or metastatic breast cancer, gastric cancer, head and neck cancer, non-small cell lung cancer and hormone-refractory prostate cancer [17]. Our recent investigation demonstrated that docetaxel acted as an adjuvant to influenza H1N1 vaccine via up-regulation of Th1/Th2 immune responses [18]. Fusion protein-based vaccines are generally presented as a lyophilized powder, and we dissolved the

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HPV16 L2E6E7 fusion protein powder in docetaxel solution prior to use. This study evaluated the potential effect of docetaxel on HPV16 L2E6E7 fusion protein in the treatment of mice inoculated with TC-1 cells. TC-1 cells are achieved by co-transforming HPV16 E6 and E7 and stimulated *ras* oncogene in lung epithelial cells of primary C57BL/6 mice [19]. The co-transformation produces a tumorigenic cell line that expresses E6 and E7. The cells mimic the natural sequence of tumor progression of cervical cancer and usually used to induce cancer in experimental animals [20].

2. Materials and methods

2.1. Vaccine formulations

Recombinant HPV16 L2E6E7 fusion protein (HPV-LFP) was isolated from solubilized *Escherichia coli* inclusion bodies under reducing conditions and purified chromatographically with purity of 99%. The protein was an 80-kD L2E6E7 monomer comprised of 725 amino acids, as predicted by the nucleotide sequence of the L2E6E7gene.The fusion protein was formulated in 5 mM glycine buffer (pH 8.0) containing 0.9 mM cysteine, freeze-dried and stored at 2–8 °C until use. Docetaxel (DOC) was the product of Jiangsu Aosaikang Pharmaceutical Co., Ltd. (Nanjing, China). Experimental HPV vaccine was formed by dissolving 6 mg of HPV-LFP in 10 ml of a sterile DOC solution at concentrations of 0.5, 1.0 and 2.0 mg/ml. The endotoxin level was <0.5 EU/ml according to a gel-clot Limulus lysate assay (Zhangjiang A & C Biological Ltd., Zhangjiang, China) when HPV-LFP was dissolved in the sterile DOC solution.

2.2. Tumor cell line

TC-1 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 (HyClone, USA) containing 10% fetal bovine serum (HyClone, USA).

2.3. Tumor model and vaccination

Female C57BL/6 (B6) mice were purchased from the Shanghai Laboratory Animal Center Co. Ltd. (Shanghai, China) and housed in polypropylene cages with sawdust bedding and hygienically controlled. For tumor therapeutic studies, animals were subcutaneously (s.c.) injected with 10^4 TC-1 cells (100 μl) into the right groin on day −1, and intramuscularly (i.m.) injected with 120 μg of HPV-LFP in 200 μl of a saline solution containing DOC (0, 100, 200 or 400 μg) in the left (100 μl) and right (100 μl) hind limbs on days 0, 3 and 7, as indicated in Fig. 1. Mouse survival was monitored daily until day 60. Blood was sampled on day 21 for determinations of IgG and isotypes responses. Splenocytes were harvested from sacrificed mice for analyses of CTL activity, IFN-γ- and IL-10-secreting cells (Elispot assay), and Treg cells (flow cytometry). Tumor nodules were collected for weight and volume measurements, which were calculated as length/3 × width × height (cm^3). All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China.

2.4. CTL assay

Splenocytes (10^7) were incubated in RPMI1640 culture medium supplemented with 10% FBS, 500 ng/ml of interleukin-2 (IL-2; Cyagen Biosciences Inc., USA) and 10 μg/ml of $E7_{49–57}$-peptide (Sangon Co., Ltd., Shanghai, China) at 37 °C in 5% CO_2 for five days. Splenocytes were harvested and used as effector cells targeting the TC-1 cells. The cytolytic effect was measured using the Non-Radioactive Cytotoxicity Assay Kit (Promega Inc., USA) according to the manufacturer's protocol. Briefly, primed splenocytes were cultured with 10^4 target cells at the ratios (E/T) of 40:1, 20:1, 10:1 and 5:1 in plates at 37 °C in 5% CO_2 for 6 h. The supernatants (50 μl/well) were harvested and transferred to wells in another 96-well plate. Substrate Mix (50 μl per well) was added, and the plates were cultured for 30 min at room temperature. The reaction was stopped by the addition of 50 μl of a Stop Solution (1 M acetic acid), and OD values were measured at 490 nm wavelengths using an ELISA reader (Thermo Multi-scan MK3, USA). Spontaneously released lactate dehydrogenase (LDH) from target or effector cells was determined by culturing target cells in the absence of target or effector cells. Determination of the maximum LDH release was performed by incubating target cells in 1% Triton X-100 in assay medium. Specific lysis (percent) was estimated according to the following formula: [(experimental release − effector spontaneous release − target spontaneous release) / (target maximum release − target spontaneous release)] × 100. The same specimens were separately analyzed four times.

2.5. Elispot assay of IFN-γ- and IL-10-secreting cells

ELISPOT kits for IFN-γ and IL-10 assays (eBioscience, Inc., USA) were used to detect the existence of IFN-γ and IL-10 in splenocytes that were responsive to the $E7_{49–57}$ peptide. Splenocytes (5×10^5 cells/well) were cultured for IFN-γ (24 h) or IL-10 (48 h) at 37 °C in 5% CO_2 in the medium alone or in the presence of PHA (for IFN-γ), LPS (for IL-10) or $E7_{49–57}$ peptide. The wells were washed using PBS-Tween 20 (0.05%) and biotinylated antibodies were added and incubated overnight at 4 °C. The same washing steps were repeated, and an avidin-HRP reagent (100 μl/well) was added to the individual wells and cultured at room temperature for 45 min. Five washes with PBS-Tween 20 and two washes with PBS (no Tween-20) were performed. An aminoethyl carbazole staining solution (100 μl) was added to each well to develop the spots. Distilled water was added to stop the reaction after 10 min. A computer-assisted imaging system was used to count the spots (Cellular Technology Ltd., USA).

2.6. Flow cytometry for Treg cells

Separation of the tumor-infiltrating mononuclear cells was carried out by differential gradient centrifugation. The fresh TC-1 carcinoma mass (0.1 g) was harvested from euthanized mice on day 21 and homogenized in a ground glass homogenizer. Then cell suspensions

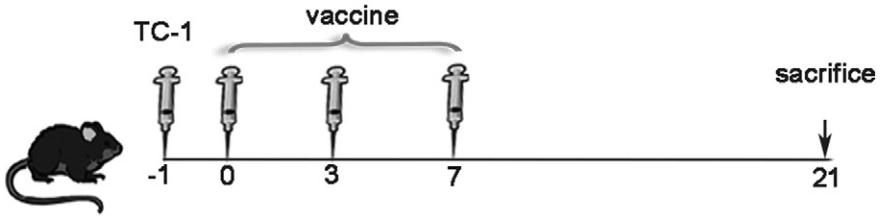

Fig. 1. Schedule for TC-1 cell challenge and HPV-LFP vaccination. Mice were s.c. challenged with 10^4 TC-1 cells on day −1, and then i.m. injected with HPV(+) or without HPV(−) 120 μg of HPV-LFP in 200 μl of saline solution containing DOC (0, 100, 200 or 400 μg) on days 0, 3 and 7.

were harvested and washed 3 times with PBS. The cells suspended in RPMI 1640 complete medium was layered on discontinuous gradients of 75 and 100% Ficoll-Hypaque and centrifuged at 400 ×*g* for 30 min. The tumor-infiltrating mononuclear cells were collected at the interface of 75 and 100% Ficoll-Hypaque. Flow cytometric analyses for Tregs were performed on a FACSCalibur (Becton Dickinson, USA) flow cytometer. Splenocytes or mononuclear cells isolated from the tumors (1×10^6) were cultured with anti-CD4-FITC (2 μl) in combination with anti-CD25-APC antibodies (5 μl) at 4 °C protected from light for 30 min. Cold PBS (2 ml) was added and centrifuged. The transcription factor buffer set (BD PharMingen, USA) was optimized for the fixing and permeabilization of cells prior to intracellular staining. A volume of 100 μl of 1 × Perm/Wash buffer (BD PharMingen, USA) and anti-Foxp3-PE antibodies (BD PharMingen, USA) was added to the cells to identify intracellular protein (Foxp3). PE rat IgG2bκ isotype control (BD PharMingen, USA) was added to each tube for nonspecific control staining for 40 min at 4 °C protected from light. The cells were washed with 2 ml of 1× Perm/Wash buffer and resuspended in 350 μl PBS. Cells were analyzed using a flow cytometer.

2.7. IgG and isotypes

Serum HPV-LFP-specific antibodies were identified using ELISA. Ninety-six-well plates were coated with 1 μg/ml HPV-LFP in 100 mM carbonate buffer, pH 9.6, overnight at 4 °C. Wells were washed using a 0.01 M phosphate buffer solution with 0.05% Tween-20 (v/v) (PBST) and blocked with 5% FBS in PBS for 1 h at 37 °C. Wells were washed using PBST, and 100 μl of serum samples was added. A 1:100 dilution was performed to analyze IgG subclass titers. Serum was serially diluted with an initial dilution of 1:50. Plates were incubated at 37 °C for 1 h. Each well was washed, and 100 μl of goat anti-mouse IgG (1:2000) (Santa Cruz Biotechnology Inc., USA) was added and incubated at 37 °C for 1 h. Wells were washed with PBST, and 100 μl of a TMB substrate solution (3,3′,5,5-tetramethylbenzidine solution in 100 μg/ml of 0.1 M citrate-phosphate, pH 5.0) (Sigma, USA) was added to each well and incubated for 5 min at 37 °C. The reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 to each well. The OD values of the wells were read at 450 nm using an automatic ELISA plate reader (Thermo Multiscan MK3, USA). Values above the cut-off background (mean of sera from unimmunized mice multiplied by 2.1) were regarded as positive. IgG titers are shown as reciprocal end-dilutions. To analyze IgG isotypes, 100 μl of HRP-conjugated goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (1:1000) (Santa Cruz Biotechnology, Inc., USA) was added and incubated for 45 min at 37 °C.

2.8. mRNA expression of IL-10, VEGF, STAT3 and IFN-γ in tumors

The total RNA was extracted from fresh TC-1 carcinoma mass and reverse transcription were performed according to the protocol of EASYspin Plus cell/tissue extraction kit (Aidlab biotechnologies Co., Ltd., China) and 5All-In-One RT MasterMix (Applied Biological Materials Inc., Canada).

Table 1
Primers and their parameters used for RT-PCR.

Gene	Primer sequence
IL-10	Forward GAGAGCGCTCATCTCGATTT
	Reverse GGGTCTCCCAAGGAAAGGTA
IFN-γ	Forward GGATGCATTCATGAGTATTGC
	Reverse GCTTCCTGAGGCTGGATTC
VEGF	Forward TTACTGCTGTACCTCCACC
	Reverse ACAGGACGGCTTGAAGATG
STAT-3	Forward GAAGCCGACCCAGGTGC
	Reverse GTCACGTCTCTGCAGCTTCT
β-actin	Forward CCTTCTGACCCATTCCCACC
	Reverse GCTTCTTTGCAGCTCCTTCG

Relative quantitation of IL-10, IFN-γ, VEGF and STAT-3 mRNA were conducted on ABI 7300 (PE Applied Biosystems, USA) using EvaGreen 2× qPCR Mastermix (Applied Biological Materials Inc., Canada). Primers were described in Table 1. Amplification was carried out in a total volume of 20 μl containing 10 μl of EvaGreen 2× qPCR Master, 5 μl of cDNA template (80 ng/μl), 0.6 μl of form primer (10 μM), 0.6 μl of reverse primer (10 μM), 3.8 μl of nuclease-free water. Reaction was performed for pre-denaturation at 95 °C for 10 min, denaturation step of 15 s at 95 °C, annealing and elongation at 60 °C for 60 s with 40 PCR cycles, and then the dissociation curve was collected with one cycle of 95 °C 15 s, 60 °C 1 min, 95 °C 15 s and 60 °C 15 s. The relative quantification between samples was achieved by the $2^{-\Delta\Delta Ct}$ method, and calculated by the software REST 2005 (provided by Eppendorf Company, German). It is reported as the n- fold difference relative to the target gene mRNA expression of the calibrator group (group of mice immunized with saline).

2.9. Statistical analysis

Kaplan-Meier analyses were used to analyze survival, and the log-rank test was used for comparisons. Independent *t*-tests were performed to analyze tumor sizes and weights 21 days post-TC-1 cells injection, Elispot data and specific lysis date. Treg date was analyzed using one-way ANOVA and Duncan post-hoc tests or LSD multiple comparison. All graphical data are expressed as the means ± S.D. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Docetaxel and HPV-LFP synergistically suppress tumor growth

Mice (n = 6) were challenged with TC-1 cells and injected with DOC, HPV-LFP alone or HPV-LFP in combination with different DOC doses to determine whether docetaxel and HPV vaccine synergistically suppressed tumor growth. Tumor nodules were isolated from mice after treatment (Fig. 2A), and tumor volumes and weights were analyzed. Fig. 2B and C show that tumors from mice injected with HPV-LFP and/or DOC exhibited significantly smaller volumes and weights than tumors from mice without treatment ($P < 0.05$). Injection of HPV-LFP or DOC resulted in tumor inhibitory rates of 69.2% and 43.2%, respectively ($P < 0.05$). The tumor inhibitory rate reached 92.4% when 120 μg HPV-LFP and 200 μl of DOC were injected together, which was significantly higher than HPV-LFP or DOC alone ($P < 0.05$).

3.2. Docetaxel and HPV-LFP synergistically increase the survival of mice inoculated with TC-1 cells

Mice (n = 6) were inoculated and treated in the same manner described above to determine whether docetaxel and HPV vaccine synergistically increased the survival of TC-1-injected mice. Mouse survival after TC-1 cell injection was monitored daily for 60 days. Fig. 3 shows that animals in the control group or mice treated with DOC all died within 42 to 50 days. A total of 17% of mice treated with HPV-LFP survived for 60 days. The addition of 200 μg of DOC in the HPV vaccine prolonged the life span of mice, with a percent survival up to 50% ($P < 0.05$).

3.3. Docetaxel and HPV-LFP synergistically activate CTL cells

Mice (n = 6) were inoculated and treated as described above to investigate whether DOC and HPV vaccine synergistically affected CTL cell activation. Splenocytes were isolated from euthanized mice after treatment for analyses of specific CTL cytotoxicity induced by the $E7_{49-56}$ peptide using a non-radioactive cytotoxicity assay. Fig. 4 shows that the effector cells from mice treated with saline or DOC exhibited low cytotoxicity against TC-1 cells. Injection of HPV-LFP increased cytotoxicity, and the addition of DOC in the vaccine significantly enhanced cytolysis of CTLs.

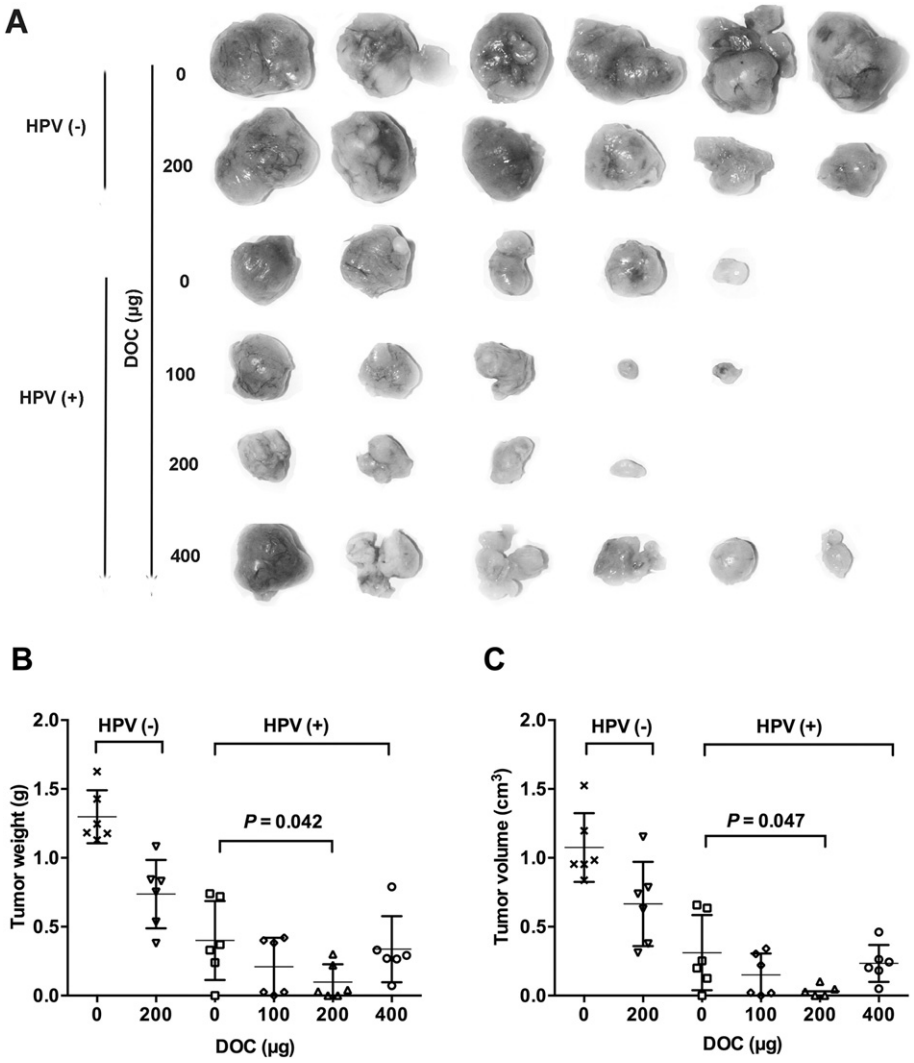

Fig. 2. Combined inhibitory effect of docetaxel and HPV-LFP on tumor growth. Mice (n = 6/group) were s.c. inoculated with 10^4 of TC-1 cells on the day before i.m. injection of 200 μl saline solution with or without DOC (200 μg) (HPV(−)), or 120 μg of HPV-LFP in 200 μl saline solution containing DOC (0, 100, 200, 400 μg) (HPV(+)) on days 0, 3 and 7. A, tumor nodules were isolated from euthanized mice on day 21. B, tumor weight was measured and expressed as mean ± S.D. (g); C, tumor volume was evaluated by measuring the three dimensions with a venier caliper and expressed as mean ± S.D. (cm^3). Data with *P* value ≤ 0.05 were significantly different.

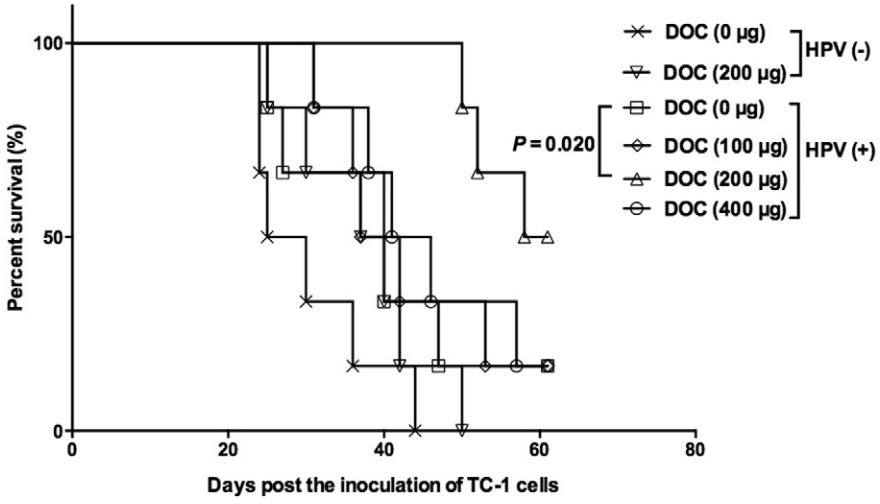

Fig. 3. Percent survival curve plotted with the method of Kaplan-Meier. Mice (n = 6/group) were treated as described in the legend of Fig. 1. The survivals of animals were monitored every day. Data with *P* value ≤ 0.05 were significantly different.

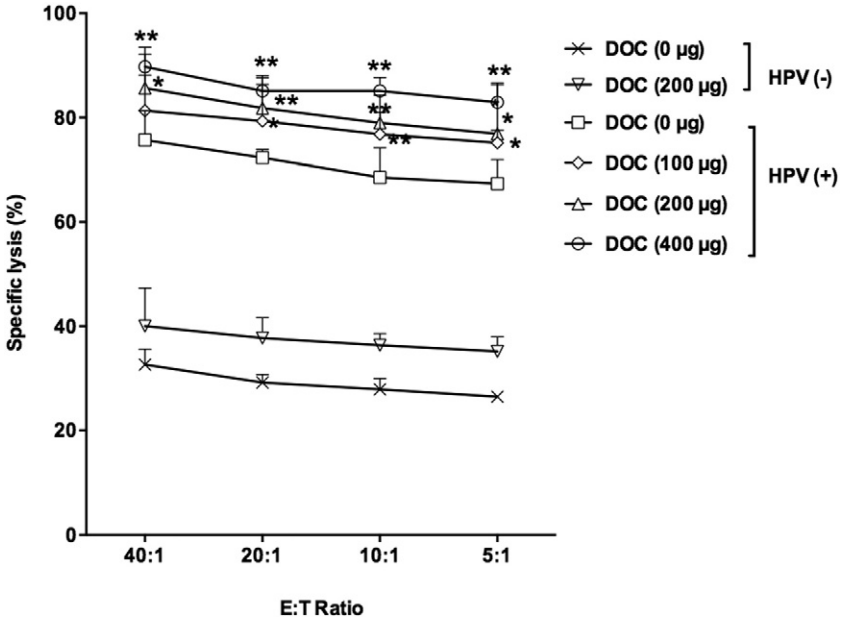

Fig. 4. CTL activity against TC-1 cells. Mice (n = 6/group) were treated as described in the legend of Fig. 1. Splenocytes were harvested from the euthanized mice on day 21. Effector cells were obtained by incubating the splenocytes (2×10^7) with HPV16 $E7_{49-57}$ (10 μg/mL) in the presence of 500 ng/mL of IL-2 for 5 days, and then cultured with target cells (TC-1 cells) for 6 h at the ratios of effector to target cells (E/T) of 40:1, 20:1, 10:1 and 5:1, respectively. The LDH released from the target cells was assessed as the specific cytolytic activity of effector T cells. The same specimens were separately analyzed four times and data shown were mean ± S.D. * and ** indicated statistical difference when compared with the data from mice immunized with HPV-LFP without DOC at $P \leq 0.05$ and $P \leq 0.01$, respectively.

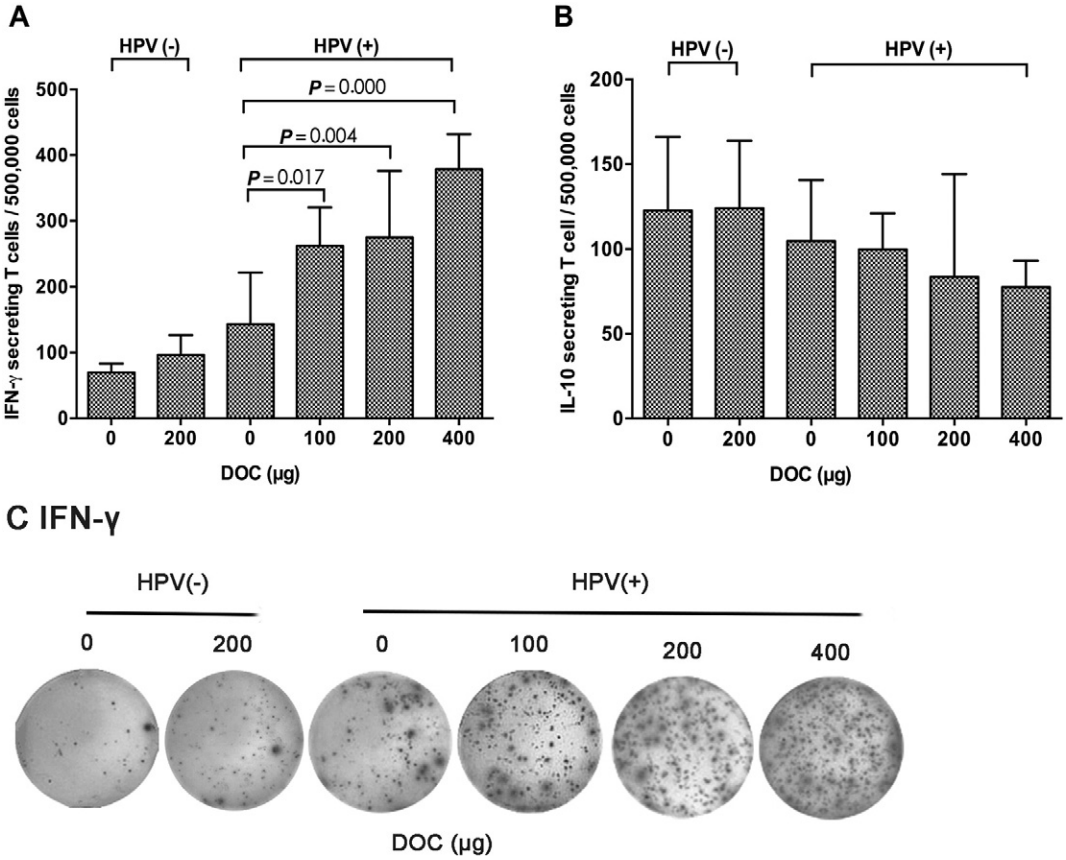

Fig. 5. IFN-γ and IL-10 secreting T cells. Mice (n = 6/group) were treated as described in the legend of Fig. 1. Splenocytes were harvested from the euthanized mice on day 21. Cells (5×10^5/well) were cultured with HPV16 $E7_{49-57}$in presence of PHA (for IFN-γ) or LPS (for IL-10) for 24 (for IFN-γ) or 48 h (for IL-10) at 37 °C in 5% CO_2. The number of IFN-γ (A) or IL-10 (B) secreting T cells was counted with Elisport assay and expressed as mean ± S.D. (T cells/500,000 cells). Data with *P* value ≤ 0.05 were significantly different.

3.4. Docetaxel and HPV-LFP synergistically increase IFN-γ and decrease IL-10 secreting cells

Mice (n = 6) were inoculated and treated as described above to determine the synergistic effect of DOC and HPV vaccine on IFN-γ- and IL-10-secreting cells. Splenocytes were isolated from euthanized mice after treatment to determine the proportion of IFN-γ- and IL-10-secreting T cells responding to $E7_{49–57}$ peptide stimulation. The results indicated that the proportion of IFN-γ-secreting T cells significantly increased (Fig. 5A and C), and IL-10-secreting T cells numerically decreased (Fig. 5B) in the spleens of mice immunized with HPV vaccine containing DOC compared with mice injected with HPV vaccine alone.

3.5. Docetaxel enhances HPV-LFP-elicited antibody responses

Mice (n = 6) were inoculated and treated as described above to determine whether docetaxel enhances the humoral responses elicited by HPV vaccine. Blood samples were collected after treatment for determinations of HPV-specific IgG titers and IgG isotypes. Fig. 6 indicates that DOC significantly enhanced IgG (Fig. 6A) and IgG isotypes (Fig. 6B) responses in a dose-dependent manner, and the highest antibody response was detected in mice injected with the vaccine supplemented with 200 μg DOC; but when the dose of supplemented DOC reached 400 μg, no significantly increased antibody response was detected.

3.6. Docetaxel decreases Treg cells in $CD4^+$ splenocytes

Mice (n = 6) were inoculated and treated as described above to determine the synergistic effect of DOC and HPV vaccine on Treg cells in $CD4^+$ splenocytes. Control mice were not inoculated or treated. Splenocytes were isolated from euthanized mice to determine the population of Tregs using FACS. Fig. 7 shows that inoculation of TC-1 cells significantly increased Tregs in $CD4^+$ cells. Injection of HPV-LFP decreased Tregs in mice with TC-1-induced tumors. Treg cells declined further when HPV-LFP was supplemented with DOC. The number of Treg cells depended on the dose of DOC that was added to HPV-LFP, and a significant decrease in Treg cells was found with 200 μg ($P = 0.003$) and 400 μg ($P = 0.007$) DOC.

3.7. Docetaxel decreases Treg cells in tumors

To determine the effect of DOC and HPV-LFP on Treg cells in the tumors, mononuclear cells were isolated from fresh TC-1 carcinoma mass and analyzed by FACS. Fig. 8 shows that the numbers of Treg cells in the tumors were significantly less in DOC and HPV groups than in the control group and the lowest Treg cells were found in the tumors of mice treated with HPV-LFP in combination with DOC.

3.8. Docetaxel and HPV-LFP synergistically up-regulate IFN-γ and down-regulate IL-10, VEGF and STAT-3 mRNA expression in tumors

To determine the synergistic effect of DOC and HPV vaccine on the production of IFN-γ, IL-10, VEGF and STAT-3 in the tumors, total RNA was isolated from fresh TC-1 carcinoma mass and mRNA expression of IFN-γ, IL-10, VEGF and STAT-3 was quantified by real-time PCR. Fig. 9 showed that injection of HPV-LFP in combination with DOC significantly up-regulated IFN-γ and down-regulated IL-10, VEGF and STAT-3 mRNA expression in the tumors when compared to only injection of HPV-LFP or DOC.

4. Discussion

The present study demonstrated that docetaxel significantly enhanced the therapeutic effect of HPV16 L2E6E7 fusion protein (HPV-LFP) on TC-1 cell-induced tumors in mice. The injection of HPV-LFP in a solution containing docetaxel into TC-1 tumor-bearing mice significantly reduced tumor volume and weight and increased the percent survival compared with mice treated with HPV-LFP alone. Tumor inhibition was associated with significantly activated CTLs, increased IFN-γ-secreting T cells, serum antigen-specific IgG and IgG isotypes and the suppression of Treg cells and IL-10-secreting cells.

The HPV genome codes two late proteins (L1 and L2) and six early proteins including E1 and E7. The early proteins E6 and E7 are the primary transforming proteins [5]. L1 and L2 are viral capsid proteins. E6 exerts its effects via interaction with the cellular tumor-suppressor gene product p53, which results in the dysfunction of p53. E7 binds to retinoblastoma protein (pRb) and triggers the expression of genes that are responsible for tissue propagation, which interrupts cell-cycle control in normal chicks [21]. This interruption allows occasional mitotic actions to occur and errors to run unchecked, which favors the malignant transformation of the cells [11].

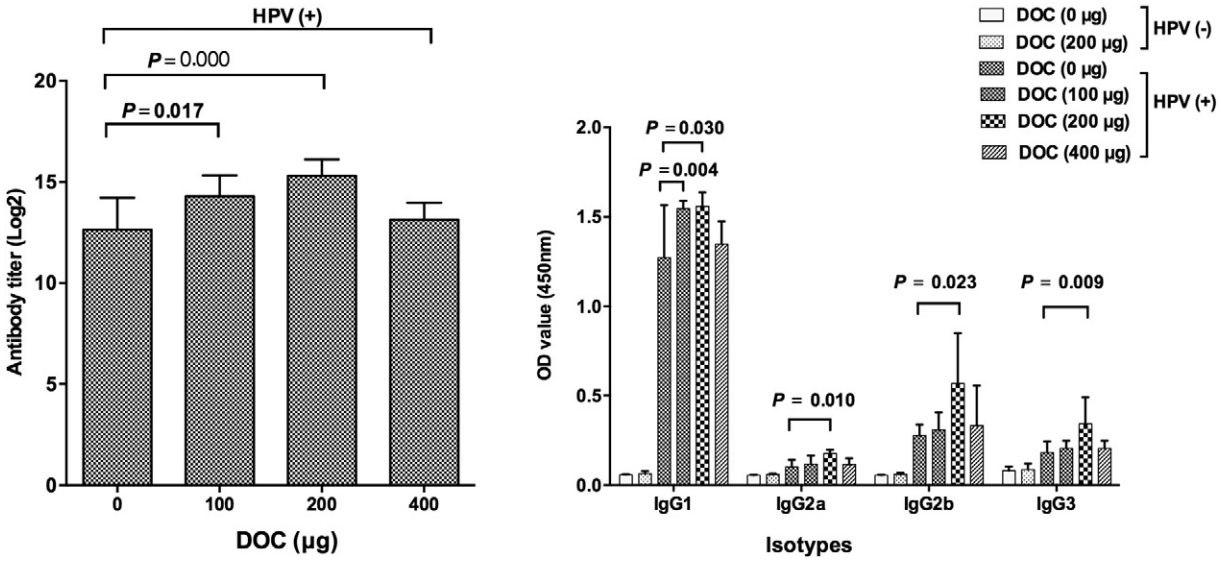

Fig. 6. Antibody responses. Mice (n = 6/group) were treated as described in the legend of Fig. 1. Serum was collected on day 21 for measurement of IgG titer (A) and the isotype levels (B). Data shown were mean ± S.D. *P* values ≤ 0.05 were significantly different.

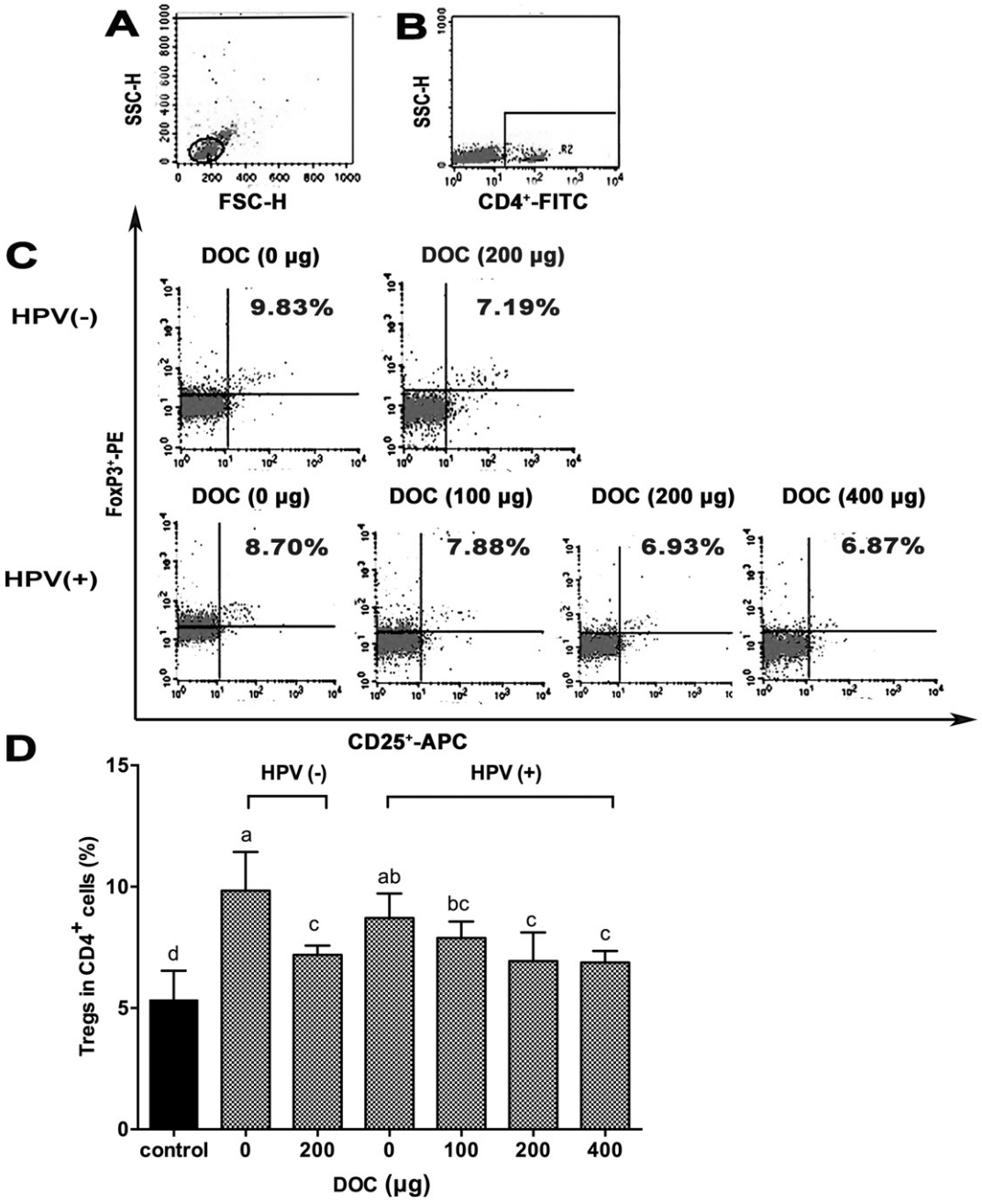

Fig. 7. Percent Tregs in splenocytes determined by flow cytometry. Mice (n = 6/group) were treated as described in the legend of Fig. 1. In addition, the control mice were not inoculated and treated. Splenocytes were harvested from the euthanized mice on day 21 and incubated with anti-CD4-FITC, anti-CD25-APC, anti-Foxp3-PE for Tregs ($CD4^+CD25^+FoxP3^+$). A, Flow cytometric gating strategy involved identification of lymphocyte population by size (SSC-H)/granularity (FSC-H), and the smaller cells were discriminated as lymphocytes. B, $CD4^+$ splenocytes were distinguished from $CD4^-$ cells. C, CD25 (PE)- and FoxP3 (APC)-positive $CD4^+$ population were identified as Treg cells in mice of six groups. Representative dotplots of six individual experiments in each group were shown. D, Treg cells in $CD4^+$ splenocytes (%) in the indicated groups were shown. The values were presented as mean ± S.D. *P* was determined by independent *t*-test when compared to the mice immunized with HPV-LFP without DOC.

The single fusion protein that includes HPV16 L2, E6 and E7 was used as a candidate prophylactic and therapeutic vaccine against HPV. The fusion protein may control HPV16-related malignancies by stimulating a patient's E6, E7 and L2-specific cellular and humoral immune responses to identify and kill cancer cells that express these oncoproteins [22–24]. However, a placebo-controlled and double-blinded phase I study found that the fusion protein induced low L2-specific cross-neutralizing antibodies, weak E6/E7-specific IFN-γ and a poor proliferative T cell response [25,26]. Vaccination with the fusion protein followed by a boost administration of TA-HPV (recombinant vaccinia virus with E6 and E7 of HPV16 and HPV18) failed to improve lesion regression [27]. Therefore, the identification of appropriate adjuvants may be useful to improve the therapeutic efficacy of current HPV fusion protein vaccines.

Co-administration of HPV-LFP and DOC in the TC-1 cell-induced cancer model produced a significantly higher tumor inhibitory rate (92.4%) than the use of either of these therapies alone (69.2% and 43.2%, respectively) (Fig. 2). Co-administration also significantly extended the life span of mice inoculated with TC-1 cells (Fig. 3). These results indicated that DOC had adjuvant effects on HPV-LFP in the treatment of HPV-induced cancer.

DOC improved the tumor inhibitory effect of HPV-LFP in a dose-dependent manner. Fig. 2 showed that 200 μg of DOC stimulated higher tumor inhibitory activity than 400 μg of DOC, when DOC was

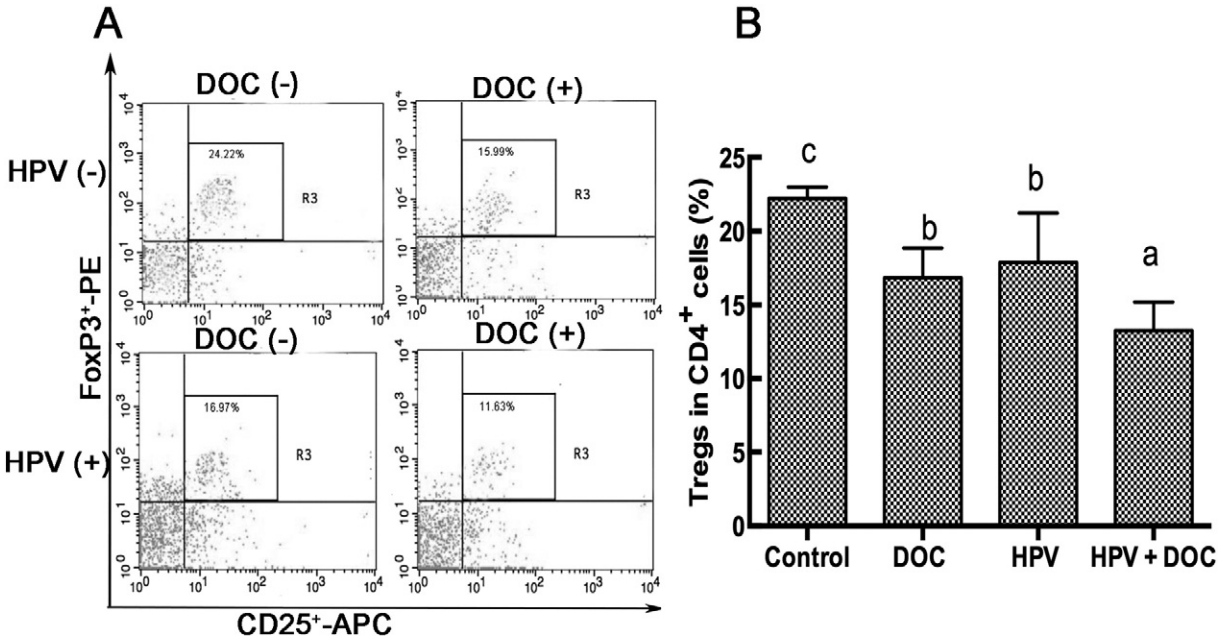

Fig. 8. Percent Tregs in $CD4^+$ cells in tumors determined by flow cytometry. Mice (n = 8/group) were treated as described in the legend of Fig. 1. The fresh TC-1 carcinoma mass were harvested from euthanized mice on day 21 and homogenized in a ground glass homogenizer. Separation of the tumor-infiltrating mononuclear cells was carried out by differential gradient centrifugation and the tumor-infiltrating mononuclear cells were found at the interface of 75 and 100% Ficoll-Hypaque. Then the cells incubated with anti-CD4-FITC, anti-CD25-APC, anti-Foxp3-PE for Tregs ($CD4^+CD25^+FoxP3^+$). A, $CD4^+$ lymphocyte were distinguished from $CD4^-$ cells. B, CD25 (PE)- and FoxP3 (APC)-positive $CD4^+$ population were identified as Treg cells in mice of four groups. Representative dotplots of eight individual experiments in each group were shown. C, Treg cells in $CD4^+$ T cell (%) in the indicated groups were shown. The values were presented as mean ± S.D. *P* was determined by one-way ANOVA and LSD multiple comparison when compared to the mice immunized with HPV-LFP without DOC.

used together with HPV-LFP. The exact reason for that is unclear. It could be associated with decreased antibody responses at a higher dose of DOC as seen in Fig. 6. Diminished antibody responses were also observed in our previous studies when DOC or paclitaxel was used with antigens [18,28].

Fig. 6 shows significant increases in serum IgG and IgG isotype responses of mice immunized with HPV-LFP + DOC compared with mice immunized with HPV-LFP alone. A similar result was found when DOC was formulated in an H1N1 influenza virus vaccine in our previous study [18]. In that study, progressively increased antibody response was detected when H1N1 influenza virus antigen was injected together with DOC at a dose range from 50 μg to 200 μg. In the present study, 200 μg of DOC was found optimal to enhance antibody response when 100, 200 or 400 μg of DOC was used as an adjuvant for HPV-LFP vaccine. Humoral responses do not effectively eliminate established tumors, but our result suggested that DOC might be useful in prophylactic vaccines in which the antibody response plays a significant role. Another advantage of DOC use as an adjuvant is that it promoted significantly lower IgE responses than the conventional adjuvant aluminum hydroxide. The frequent use of alum-adjuvanted vaccines may be one reason for IgE-mediated allergies due to activated Th2 immune response [29,30].

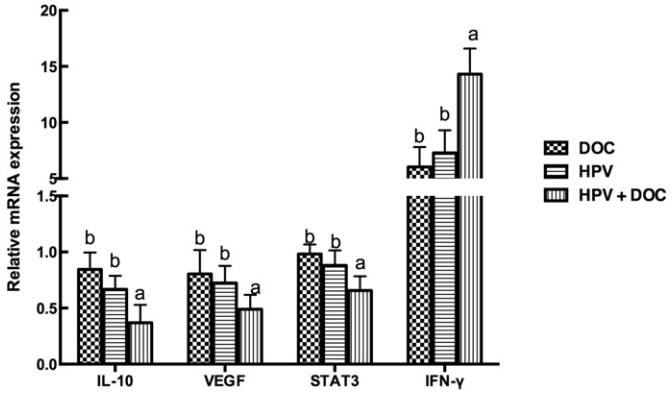

Fig. 9. mRNA expression of IL-10, IFN-γ, VEGF and STAT-3 in tumors. Mice (n = 8/group) were treated as described in the legend of Fig. 1. RNA was isolated from fresh TC-1 carcinoma mass from euthanized mice and reverse transcription were performed according to the manufacture protocol. mRNA expression of IL-10, IFN-γ, VEGF and STAT-3 was quantified by real-time PCR. Housekeeping gene of beta-actin (β-actin) mRNA was measured as an internal standard. The mRNA quantification was normalized to the β-actin. The values were presented as mean ± S.D. *P* was determined by one-way ANOVA and LSD multiple comparison when compared to the mice immunized with HPV-LFP without DOC.

The DOC-potentiated therapeutic effect of HPV-LFP may be associated with increased cellular and humoral immune responses. $CD8^+$ T cells are important for the protection against HPV infection and the associated cancers [31]. Tumor cells are killed as MHC I/peptide complexes on tumor cell surfaces are recognized by antigen-specific class I-restricted CTLs. Fig. 4 shows that CTLs from mice treated with HPV-LFP in combination with DOC exhibited significantly higher cytolysis than mice treated with HPV-LFP alone, which suggests that DOC up-regulated CTLs. CTL activation by IFN-γ is well documented. Mice lacking lymphocytes and/or the IFN-γ signaling pathway exhibit a higher incidence of carcinogen-induced sarcomas, spontaneous epithelial tumors and lymphoma [32]. Fig. 5A and C show that DOC significantly elevated the number of antigen-specific IFN-γ-secreting T cells. The increase in IFN-γ-secreting T cells paralleled the enhanced activities of CTLs in the HPV-LFP + DOC group.

Treg cells naturally exist at low numbers, but tend to increase in patients with malignancies. These cells suppress co-stimulatory molecules that are expressed by dendritic cells (DCs) and transform the DCs into weak CTL activators [33]. Enhanced reactivity to a tumor-associated antigen may be induced by Treg cell depletion with the injection of an antibody that blocks CTLA-4 (cytotoxic T lymphocyte antigen 4) [34]. Figs. 7 and 8 show that DOC significantly decreased the number of Treg cells in spleen and tumors, which may be associated with the increased cytotoxicity of CTLs shown in Fig. 4.

Neutralizing IL-10 may enhance CTL responses to therapeutic vaccines in vitro and in vivo [20]. IL-10 blockade also improves DNA vaccine-induced T cell responses [35]. Neutralizing IL-10 also reduces chronic viral infections in a mouse model of lymphocytic choriomeningitis infection [35–37]. Elevated IL-10 is observed in individuals with chronic HPV infection [38]. Although we did not find significantly decreased IL-10-secreting T cells in mice injected with HPV-LFP in combination with DOC, these cells progressively declined with increasing dose of DOC (Fig. 5B).

Pathological interactions between cancer cells and host immune cells create the tumor microenvironment. In tumor cells, STAT-3 is frequently activated, which promotes the expression of immunosuppressive factors including VEGF and IL-10 [39]. Treg cells suppressed IFN-γ production of antigen-specific $CD8^+$ T cells [28]. These factors are important for sustaining the immunosuppressive environment and maintain tumor growth [40]. In the present study, increased antitumor effect may be associated with up-regulated IFN-γ, down-regulated IL-10, VEGF and STAT3, and decreased Treg cells in the tumor microenvironment when HPV-LFP was injected together with DOC.

Safety should be considered in the search for adjuvant candidates. Side effects, such as short-lasting neutropenia and hypersensitivity reactions, were reported when docetaxel was used as an antineoplastic agent [41], but the suggested dose of docetaxel for adjuvant purposes was 200 μg/mice, which is much lower than the recommended dose for cancer treatment (47 mg/kg). Throughout this study no side local reaction at the injection site and abnormal organs was observed.

In conclusion, our study revealed that DOC significantly enhanced the therapeutic effect of HPV-LFP in a mouse model. The injection of HPV-LFP in combination with DOC into TC-1 tumor-bearing mice significantly suppressed tumor growth and extended the life span of mice compared with mice treated with HPV-LFP alone. Tumor inhibition was associated with significantly activated CTLs, increased IFN-γ-secreting T cells and serum antigen-specific IgG and IgG isotypes, and the suppression of Treg cells and IL-10-secreting cells. In addition, decreased Treg cells, down-regulation of IL-10, VEGF and STAT3 and up-regulation of IFN-γ in the tumor microenvironment may contribute the antitumor effect. The excellent adjuvant effect of DOC on HPV-LFP and the solubility of DOC in water suggest that it may be valuable to use DOC-containing water to dilute HPV-LFP powder before injection in cervical cancer patients.

Authors' contributions

Conception and design: SH Hu; XY Su.

Acquisition of data: XY Su; W Xu; YH Wang; R Guan; J Wu; G Chen.

Analysis and interpretation of data: SH Hu; XY Su; R Guan.

Writing, review, and/or revision of the manuscript: SH Hu; XY Su; W Xu; R Guan.

Study supervision: SH Hu.

Conflict of interest

None.

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