

In vitro and in vivo evaluations of human papillomavirus type 16 (HPV16)-derived peptide-loaded dendritic cells (DCs) with a CpG oligodeoxynucleotide (CpG-ODN) adjuvant as tumor vaccines for immunotherapy of cervical cancer

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Abstract

Purpose To evaluate the immunotherapeutic potentials for human dendritic cells (DCs) loaded with different HPV16-associated antigens, including HPV16E7 (E) protein, HPV16E7 polypeptide (P), as well as CpG-oligodeoxynucleotide (ODN) 2006 as a promising immune adjuvant for vaccination against cervical carcinoma.

Methods DCs derived from human peripheral blood and cord blood were isolated and loaded with HPV-derived protein or peptides, in combination with CpG-ODN2006 as a potential adjuvant. The IL-12 level, the allogeneic T cell-stimulatory capacity and the cytotoxicity of cytotoxic T lymphocytes (CTLs) were evaluated in vitro. Furthermore, an immune reconstitution model of human cervical carcinoma in SCID mice was used to assess the anti-tumor effects in vivo. The tumor sizes, the expression of IgG and IFN- γ , and the presence of the human CD3⁺, CD4⁺ and CD8⁺ T cells were measured in the mice inoculated with different DCs.

Results The antigen-loaded DCs displayed obvious anti-tumor activities in vitro and in vivo, and showed no toxicity to normal cells. The level of IL-12, an important cytokine for immune response, was up-regulated in all mice inoculated with antigen-loaded DCs. Stimulation and activity of CTLs were increased after treatment with antigen-loaded DCs. Significantly, DCs loaded with HPV 16E7 polypeptide (P) showed the most distinguished

immunotherapeutic activities, and such effect was further enhanced when HPV16E7 polypeptide (P) was used in combination with CpG-ODN2006. Interestingly, the same results were obtained in vivo: the tumor size was decreased, and IgG and IFN- γ levels were increased after the SCID mice were inoculated with the loaded DCs.

Conclusions HPV16E7 polypeptide combined with the immune adjuvant CpG-ODN2006 could be a suitable HPV16-associated tumor antigen. The research provides a new strategy for generating DCs vaccines for immunotherapy of cervical cancer.

Keywords Immunotherapy · Dendritic cells · HPV16E7 polypeptide · CpG-ODN2006

Introduction

Cervical cancer is the most common gynecologic malignant tumor causing high mortality in women worldwide [1]. Over the past two decades, epidemiological data have confirmed that human papillomavirus (HPV) infection, especially HPV type 16 (HPV16) and HPV18, is frequently detected in cervical neoplasia [2]. The HPV16 E6 and E7 transforming oncoproteins contribute to both the transformation and the maintenance of the malignant phenotype of these high-risk HPV genotypes [3]. Therefore, HPV16 E6 and E7 oncoproteins have become attractive tumor-specific antigen targets for immunotherapy of cervical cancer [4].

Preliminary clinical studies have shown that dendritic cells (DCs)-based vaccines against HPV16/18 E7 can induce broad T cell immune responses in cervical cancer patients [5, 6]. However, the HPV16E7 protein based vaccines can be rapidly degraded, resulting in poor processing and presentation by major histocompatibility

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complex (MHC) molecules on the cell membrane and accounting for the mild immunogenicity and immune system evasion of HPV [7, 8]. Recent studies have found that amino acids 49–57 from the E7 protein encode the murine H2-Db-binding epitope, which is the major tumor rejection epitope for HPV16 E7-transformed tumor cells [9–11]. For peptides poorly produced by the immunoproteasome, vaccination strategies based on peptides may provide more superior immunotherapy effect compared with strategies based on proteins or full-length genes [12].

In addition, strong immune adjuvants are required to induce robust immune responses. Recent studies have found that bacterial DNA or oligonucleotides containing unmethylated CpG dinucleotides (CpG-ODN) could activate various immune cells including macrophages, monocytes, natural killer (NK) cells and DCs as well as induce a Th1 cell-like pattern of cytokine production [13–15]. Co-administering CpG-ODN with protein antigens, polypeptide antigens or DNA vaccines may further enhance immunity [16].

In this study, we aimed to compare immunogenicity of DCs loaded with HPV16E7 protein, HPV16E7^{49–57} polypeptide, with or without the adjuvant CpG-ODN2006, by evaluating the anti-tumor activities of these DC vaccines both in vitro and in vivo.

Materials and methods

Cell cultures

Human cervical adenocarcinoma Hela cell (stored in our laboratory) and epidermoid cervical carcinoma CaSki cells (Cell Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) were cultured at 37 °C under a 5 % CO₂ humidified atmosphere in RPMI-1640 culture medium supplemented with 10 % fetal bovine serum (FBS).

Isolation of peripheral blood and cord blood mononuclear cells and induction of DCs maturation

Peripheral blood and cord blood mononuclear cells were isolated by Ficoll–Hypaque density centrifugation from fresh heparinized peripheral and cord blood obtained from healthy volunteers with informed consent. Peripheral blood and cord blood mononuclear cells (5×10^6 /ml) were suspended in RPMI1640 media and plated in 6-well culture plates. After incubation for 2 h at 37 °C, non-adherent cells were removed, and the adherent cells were further cultured at 37 °C in a humidified 5 % CO₂ atmosphere in RPMI-1640 medium supplemented with recombinant human granulocyte–macrophage colony stimulating factor (rhGM-

CSF, Beijing Medical University United Pharmaceutical Co., Ltd, 500 U/ml) and recombinant human Interleukin-4 (rhIL-4, R&D Systems, 250 U/ml) for three days. On day 4, various HPV16 E7-derived antigens, including HPV16E7 protein (E, prepared in the laboratory, 10 µg/ml) and HPV16E7^{49–57} polypeptide (P, Sangon Biotech, Shanghai, China, 10 µg/ml) [16–18], were added to the DCs cultures. Immune adjuvant CpG-ODN2006 (Sangon Biotech, Shanghai, China, 10 µg/ml) was also added depending on experimental designs (see “Results”). On day 6, rhTNF-α (10 ng/ml) was added to induce the maturation of DCs.

Immune reconstitution of human cervical carcinoma in SCID mice

Six-week old female C.B17/SCID mice (Silaike, Shanghai China) with serum IgG <5 µg/ml were intraperitoneally injected with cord blood mononuclear cells (3×10^7). After 24 h, the mice were inoculated with CaSki cells subcutaneously at axilla. One week later, 0.9 % saline (NS), DCs, DCs loaded with HPV16E7 (E) polypeptide and DCs loaded with HPV16E7 (E) polypeptide combined with CpG-ODN were injected to different treatment groups of the SCID mice. The same treatment was repeated once after 1 week to boost the immune reaction. The size of transplanted tumors and survival rate were recorded at different time points (see “Results”). All animal studies have been approved by China Ethics Committee and performed in accordance with the ethical standards.

Characterization of DCs with intracellular flow cytometry

The cell surface markers of isolated DCs were determined by flow cytometry. Briefly, DCs were harvested at the seventh day and washed twice with PBS, followed by immunofluorescence staining for 30 min with the following monoclonal antibodies: anti-CD14-FITC, anti-HLA-DR-PE, anti-CD4-PE, and anti-CD8-PE (Becton–Dickinson). The stained cells were washed twice with PBS and analyzed by flow cytometry (FACS Calibur, Becton–Dickinson, USA).

MTT assay

The cytotoxicity assay was performed with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] that was purchased from Sigma (Cat.# M-2128), according to the published literature with modifications [19]. Culturing medium containing 5 µg/ml of MTT (final concentration) was added to each well. The MTT solution was freshly prepared in warm 1 × PBS buffer at a concentration

of 5 mg/ml (stock). The plates were returned to 37 °C incubator for another 4 h. At the end of the incubation, the medium was carefully aspirated. The purple formazan was dissolved in 100 µl of 10 % SDS. Optical readings were performed with an automated spectrophotometric microplate reader at 570 nm.

Quantification of interleukin-12 (IL-12) and interferon- γ (IFN- γ) by ELISA

ELISA assays were performed according to the manufacturer's manuals (Shenzhen Jingmei Biology Engineering Co. Ltd., Shanghai, China) to quantify the production of IL-12 and IFN- γ in the supernatant of DC-cell line co-cultures or in the serum of the SCID mouse model of human cervical carcinoma. Samples (the cell culture supernatant or the serum) were added to each well in a 48-well plate pre-coated with either anti-human IL-12 monoclonal antibody (for the cell culture supernatant) or anti-IFN- γ monoclonal antibody (for the serum), and incubated at 37 °C for 90 min. At the end of incubation, the plate was washed for four times, followed by incubation with the secondary antibody (horseradish peroxidase (HRP)-conjugated anti-mouse IgG) for 30 min. Following washing the plate for 4 times, chromogenic agent was added, and the absorbance was measured at 450 nm. Quantification of IL-12 and IFN- γ in the samples was performed based on the standard curves established by the standards supplied by the kits.

Mixed lymphocyte reaction (MLR)

Purified T cells (the responder) were isolated from PBMCs of a separate donor and plated in a 96-well, flat-bottom tissue culture plate (Becton–Dickinson) at different concentrations in 100 µl of RPMI1640. DCs (the stimulator) were treated with mitomycin-C at 37 °C for 45 min, before being added to the appropriate wells such that the ratios of stimulator:responder (DC:T cell) were 1:10, 1:100 and 1:1,000, respectively, and allowed for incubation for 4 days. A control containing responder cells only was also included. All treatments were performed in triplicate for each group. MTT assay was performed to measure the proliferation of the responder T cells. The stimulation index (SI) was calculated as the following: $SI = A_{570} \text{ of experimental cells} / A_{570} \text{ of control cells}$.

Measurement of immunologic activities

Human immunoglobulin G (IgG) was quantified in the mouse blood according to the manufacturer's manual (PIERCE, USA). Briefly, the serum was added to a 96-well plate pre-coated with anti-IgG magnetic beads, followed by

the addition of confining solutions. The absorbance value was read at 405 nm using a microplate reader.

For quantifying the human CD3⁺, CD4⁺, and CD8⁺ T cells in mouse blood, anti-human CD4/CD8/CD3 antibodies labeled with FITC/PE/PE-Cy5 were incubated with the blood samples for 20 min at room temperature and measured by flow cytometry.

Statistical analysis

The data are presented as mean $\pm$ standard deviation (SD) and compared with the use of Student's *t* test or one-way ANOVA followed by Student–Newman–Keuls post hoc tests with SAS statistical software (version 8.0, Cary, NC, USA). $P < 0.05$ was considered statistically significant. The survival data were analyzed using the Kaplan–Meier test.

Results

Monocyte-derived DCs are predominant in the purified cell populations

The phenotypes of mature DCs were determined by HLA-DR, CD86, and CD209 expression with flow cytometry analysis (Fig. 1). More than 90 % of the isolated DCs were positive for HLA-DR and CD86. A majority of DCs that isolated from peripheral blood or from cord blood was positive for CD209 (87.44 and 83.14 %, respectively). On the other hand, the fraction of the cells that were positive for CD14, a marker of mononuclear cells, was less than 5 %. It was evident that the matured DCs were predominant in the purified cell populations.

IL-12 levels are up-regulated in DCs loaded with different HPV16 E7 antigens

The expression of IL-12 on DCs (DC), HPV16E7 -loaded DCs (DC + E), HPV16E7 polypeptide -loaded DCs (DC + P), HPV16E7(E)-combined-with-CpG-ODN-loaded DCs (DC + E + O) and HPV16E7 polypeptide(P)-combined-with CpG-ODN-loaded DC (DC + P + O) groups was measured with ELISA (Fig. 2). Compared with unloaded DCs, the levels of IL-12 were significantly up-regulated in all of the antigen-loaded DCs. Compared with DC + E group, IL-12 level was higher in DC + P group. The expression of IL-12 was further enhanced by adding immune adjuvant CpG-ODN2006 into the antigen-loaded DCs (Fig. 2). There was no significant difference between peripheral blood- and cord blood-derived DCs ($P > 0.05$).

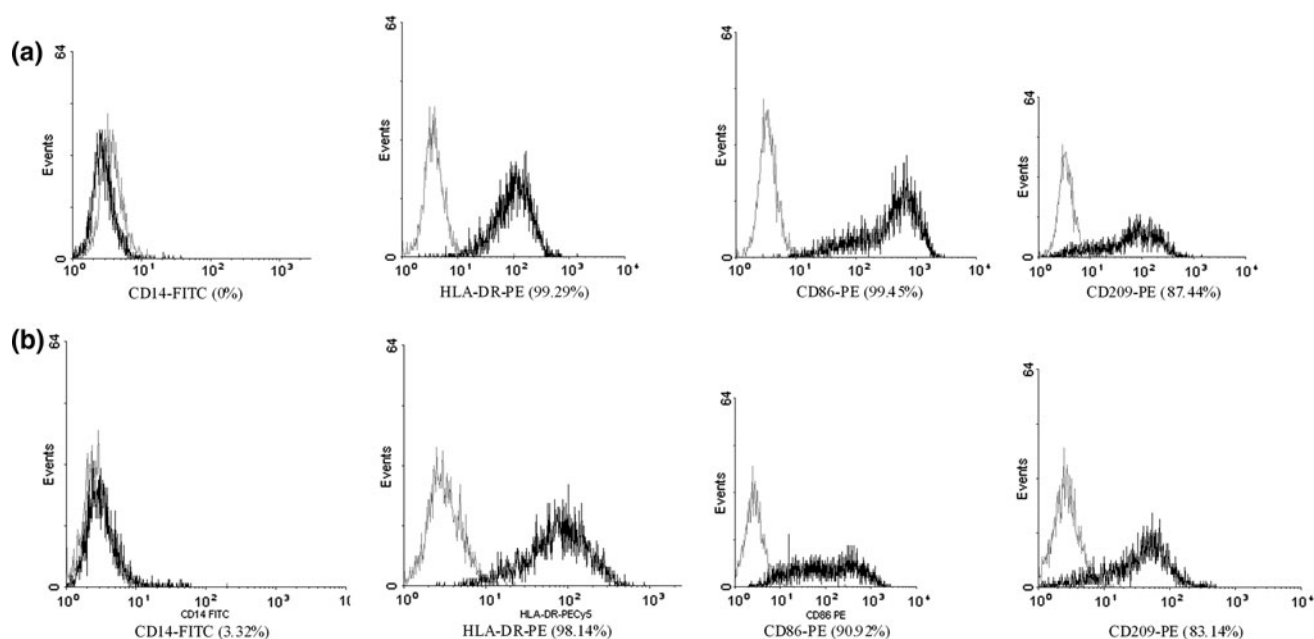


Fig. 1 Monocyte-derived DCs were predominant in the purified cell populations. The phenotypes of mature DCs were determined by expression of the cell surface markers HLA-DR, CD86, and CD209 with flow cytometry analysis. The immunostaining of the cells was

performed using corresponding monoclonal antibodies that are indicated in the graph. The dendritic cells were isolated from peripheral blood (a) and cord blood (b)

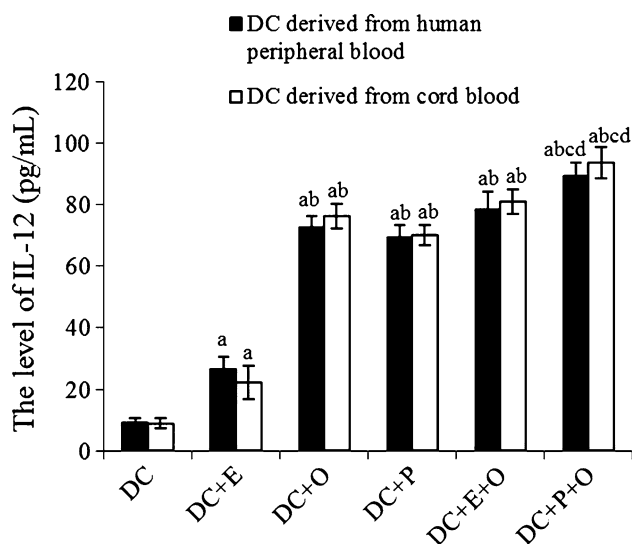


Fig. 2 IL-12 levels were up-regulated in DCs loaded with different HPV16 E7 antigens. The levels of IL-12 in DCs of different origins were quantified by ELISA. DC dendritic cells only, DC + E HPV16 E7 loaded DCs, DC + O DCs primed by ODN2006 only, DC + P HPV16 E7 polypeptide-loaded DCs, DC + E + O HPV16E7(E)-combined-with-CpG-ODN-loaded DCs, DC + P + O HPV16E7 polypeptide(P)-combined-with CpG-ODN-loaded DCs. ^a $P < 0.05$, compared with DC group; ^b $P < 0.05$, compared with DC + E group; ^c $P < 0.05$, compared with DC + O group; ^d $P < 0.05$, compared with DC + P group

Antigen-loaded DCs stimulate proliferation of allogeneic T cells

The functionality of DCs and antigen-loaded DCs was evaluated by their ability to induce the proliferation of allogeneic T cells with MLR and the SI values were calculated. As shown in Fig. 3, the SI values of antigen-loaded DCs were higher than unloaded DCs. At the ratio of 1:10 [i.e. stimulator:responder (DC:T cell)], antigen-loaded DCs showed significantly higher stimulation indexes than unloaded DCs. MLR assays with peripheral blood- and cord blood-derived DCs showed similar results (Fig. 3a, b), confirming the ability to stimulate allogeneic T-cell proliferation due to functionality of the purified DCs, regardless of the origins.

CTLs activated by antigen-loaded DCs show cytotoxicity to cervical cancer cells

We performed MTT assays to measure the cytotoxicity of the CTLs activated by antigen-loaded DCs to different cervical cancer cells, including the HeLa (Fig. 4a, c) and CaSki cells (Fig. 4b, d). We also compared the cytotoxicity to the cancer cells of these CTLs activated by DCs of different origins (i.e. DCs from peripheral blood (Fig. 4a,

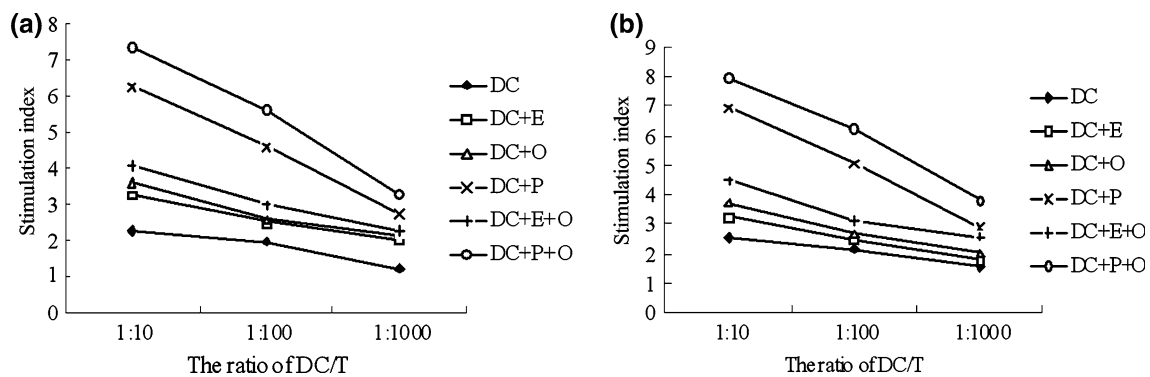


Fig. 3 Allogeneic T cell-stimulatory capacity of dendritic cells and antigen-loaded dendritic cells. Stimulator:responder cells [DC:T cell] were mixed at *different ratios* as indicated in the graph. The

stimulation index (SI) values were calculated based on MTT assays for proliferation of the T cells. **a** DCs derived from peripheral blood; **b** DCs derived from cord blood

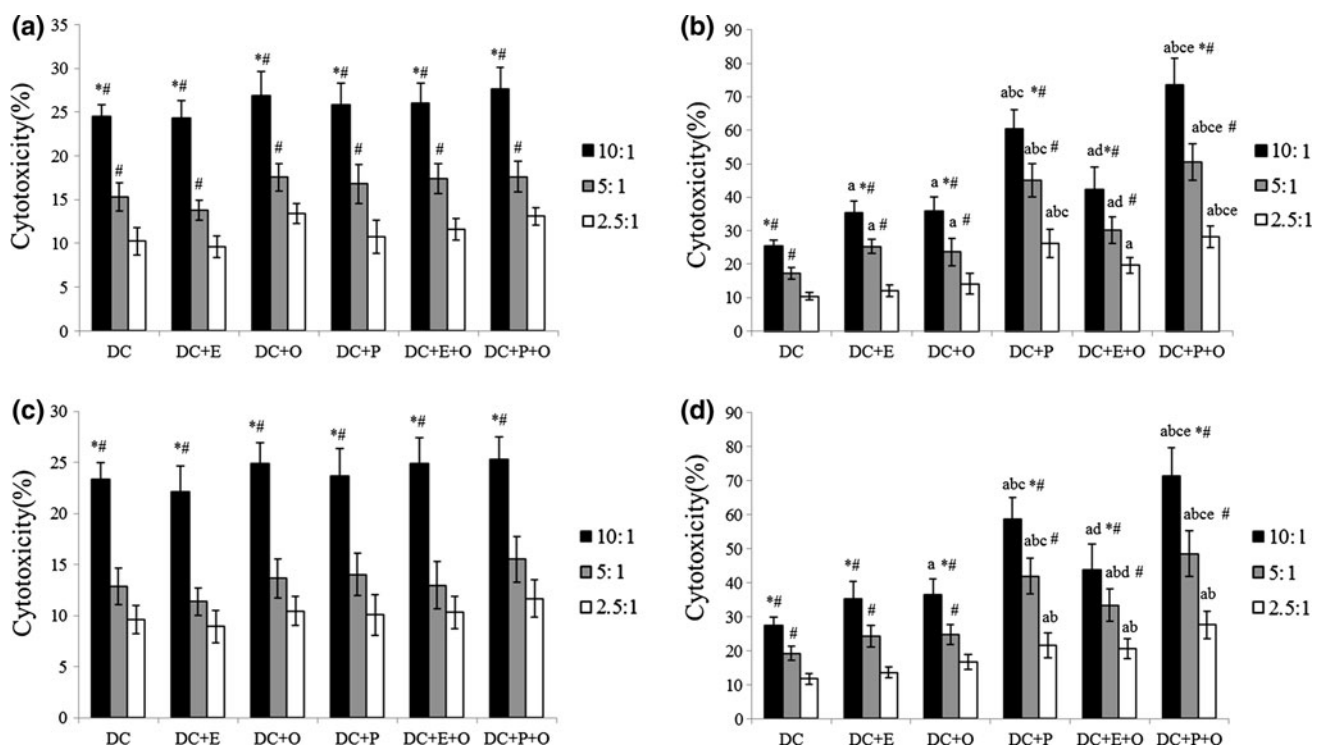


Fig. 4 CTLs activated by antigen-loaded DCs showed cytotoxicity to cervical cancer cells. The cytotoxicity effects of CTLs activated by DCs of different origins (**a** and **b** DCs from peripheral blood vs. **c** and **d** DCs from cord blood) on HeLa cells (**a** and **c**) and Caski cells (**b** and **d**) were evaluated based on MTT assays. The *different ratios* indicate the effector-to-target ratios (DCs-to-T-cell ratios). *Error bars* represent SE of triplicates for each treatment group. DC dendritic cells only, DC + E HPV16 E7 loaded DCs, DC + O DCs primed by

ODN2006 only, DC + P HPV16 E7 polypeptide-loaded DCs, DC + E + O HPV16E7(E)-combined-with-CpG-ODN-loaded DCs, DC + P + O HPV16E7 polypeptide(P)-combined-with CpG-ODN-loaded DCs * $P < 0.05$, compared with 5:1; #compared with 2.5:1; ^a $P < 0.05$, compared with DC group; ^b $P < 0.05$, compared with DC + E group; ^c $P < 0.05$, compared with DC + O group; ^d $P < 0.05$, compared with DC + P group; ^e $P < 0.05$, compared with DC + E + O group

b) vs. DCs from cord blood (Fig. 4c, d). It was evident that CTLs that had been activated by the antigen-loaded DCs, especially by the DCs loaded with a combination of HPV16E7 polypeptide and CpG-ODN2006, presented obvious cytotoxicity to all cancer cells tested. It is interesting that the cytotoxicity to CaSki cells was generally greater than that to HeLa. On the other hand, the

cytotoxicity of the CTLs was not dependent on the origin of the DCs that were used to activate them, since CTLs activated by either DCs derived from peripheral blood or from cord blood showed similar cytotoxicity to the cancer cells (Fig. 4a and b vs. c and d). It is notable that the effector-to-target ratio (i.e. DCs-to-T-cell ratio) could affect the cytotoxicity of the activated CTLs. The

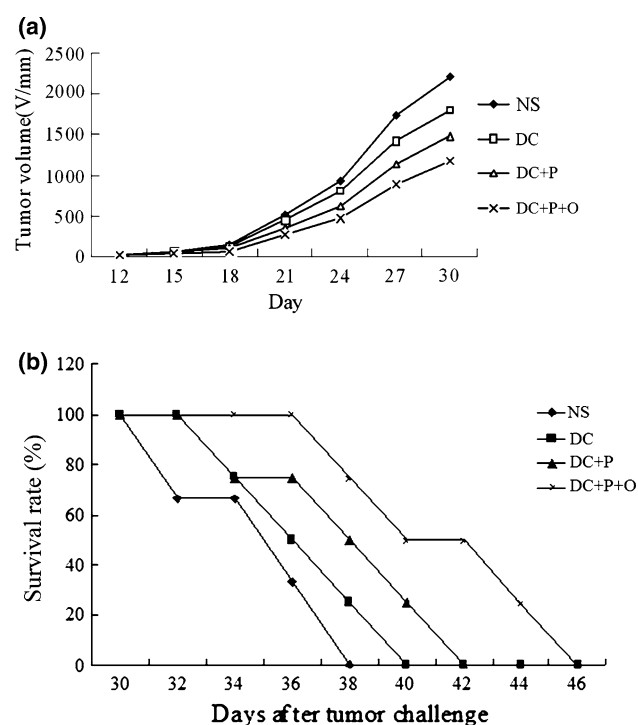


Fig. 5 Loaded DCs induced regression of tumor size and survival rate in SCID mice of human cervical carcinoma model. The tumor size (a) and survival rate (b) were recorded at different days in SCIP mice inoculated with saline (NS), dendritic cells only (DC), HPV16 E7 polypeptide-loaded DCs (DC + P), and HPV16E7 polypeptide (P)-combined-with CpG-ODN-loaded DCs (DC + P + O)

Table 1 Human CD3⁺, CD4⁺ and CD8⁺ T cell in blood of different groups of SCID (%)

Group	CD3 ⁺	CD4 ⁺	CD8 ⁺
NS	4.72 ± 1.39	2.39 ± 1.01	1.49 ± 0.56
DC	4.84 ± 1.55	2.48 ± 0.97	1.35 ± 0.47
DC + P	4.46 ± 1.83	2.27 ± 1.05	1.31 ± 0.58
DC + P + O	4.69 ± 1.74	2.36 ± 1.08	1.42 ± 0.50

cytotoxicity of the induced CTLs was positively related to the amount of DCs that activate them (high cytotoxicity, 1:10).

Loaded DCs induce regression of tumor size in SCID mice of human cervical carcinoma model

To assess the immunogenicity of the antigen-loaded DCs, we reconstructed a human cervical cancer model in SCID mice, followed by inoculation of the DC vaccines to evaluate the tumor growth in these mice. The rate of tumor transplantation was 100 % (data not shown). We tracked the tumor size with the progression of cervical tumorigenesis in the mice. Generally, the size of tumor was increased accompanied with the prolongation of

tumorigenesis (Fig. 5a). Compared with the control group which received saline injection only, the SCID mice treated with DCs or antigen-loaded DCs showed regression of tumor size, confirming the anti-tumor activity of the DC vaccines in vivo. Furthermore, mice injected with the HPV16E7 polypeptide (P)-loaded DCs and HPV16E7 polypeptide (P)-and-CpG-ODN-loaded DCs showed significant tumor regression. As expected, the survival rate of P + O group was significantly prolonged compared with NS group. No difference was observed between DC and DC + P group (Fig. 5b). Meanwhile, the Human CD3⁺, CD4⁺ and CD8⁺ T cells could be detected in the vaccinated mice, as evidenced by flow cytometry analysis for the cell surface markers (see “Materials and methods”). However, there was no significant difference between groups (Table 1).

The levels of IgG and IFN- γ in SCID mice of human cervical carcinoma model

To evaluate the immune reconstruction of the human cervical carcinoma in the SCID mice, we measured the levels of human IgG in the serum. There was no significant difference in the human IgG levels between the four groups at day 14 ($P = 0.9691$), and the same was true for the four groups at day 28 ($P = 0.3626$) (Fig. 6a). However, the levels of human IgG in all groups at day 28 were higher than those at day 14, suggesting the human IgG did attain a higher level with the progression of immune-reconstruction. Most interestingly, the IFN- γ level was elevated in mice treated with DCs (loaded or unloaded), compared to saline-treated controls. Furthermore, the IFN- γ production was enhanced in mice treated with HPV16E7 polypeptide (P)-loaded or HPV16E7 polypeptide (P)-and-CpG-ODN2006-loaded DCs (Fig. 6b), suggesting that the DC vaccines had induced T cells-mediated immunity against tumor in vivo.

Discussion

Dendritic cells are rare but extremely potent antigen-presenting cells (APCs) that can function in vitro and in vivo to initiate T-lymphocyte responses to tumor-associated antigens. After microbial challenge, DCs can secrete IL-12 and IL-12 induces T-helper type 1 (Th1) type immune responses through inducing the maturation of type 1 Th cells from the uncommitted T-cell pool and promotes natural killer (NK) to produce interferon- γ (IFN- γ) and CTL maturation [20, 21]. Exogenous administration of IL-12 into tumor-bearing mice can delay, reduce, and, in some cases, completely inhibit tumor development [22]. Based on these theories, we aimed to investigate the antitumour

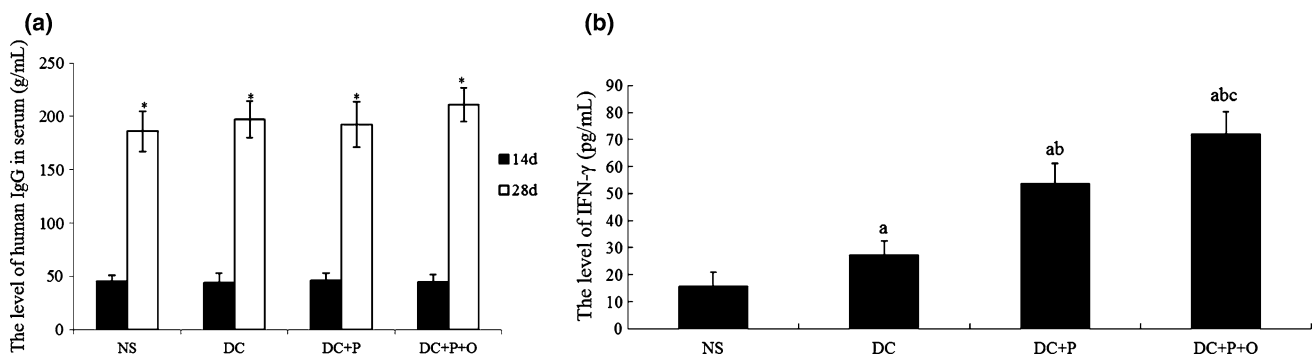


Fig. 6 The levels of IgG and IFN- γ in SCID mice of human cervical carcinoma model. The human IgG level (a) and IFN- γ (b) were measured by ELISA in the mice inoculated with saline (NS), dendritic cells only (DC), HPV16 E7 polypeptide-loaded DCs (DC + P), and

HPV16E7 polypeptide (P)-combined-with CpG-ODN-loaded DCs (DC + P + O). * $P < 0.05$, compared with 14 days; ^a $P < 0.05$, compared with NS group; ^b $P < 0.05$, compared with DC group; ^c $P < 0.05$, compared with DC + P group

immunity of antigen-loaded DCs against cervical carcinoma by measuring the expressions of IL-12 and IFN- γ and CTL activity. As expected, our results showed that IL-12 was significantly increased after antigen-loaded DCs treatment, especially the DC + P + O group [16]. ODN may interact with Toll-like receptor (TLR 9) and activate APC including DC through the Toll/interleukin (IL) receptor signaling pathway, leading to secretion of cytokines, such as IL-12 and IL-6 [23–25]. Furthermore, CTLs induced by the antigen-loaded DCs showed cytotoxicity to cervical cancer cell lines like HeLa and CaSki. However, cytotoxicity to CaSki (more than 70 % killing activity, 1:10) was obviously greater to that of HeLa (lower than 30 % killing activity, 1:10). We speculate that this may be attributable to the fact that p53 in CaSki cell may strengthen the cytotoxicity of drug in tumor cells with interruption of mitosis by depleting cyclin B1 [26]. It appeared that the cytotoxicity of the induced CTLs was positively related to the amount of DCs that activate them. We speculate that loaded DCs may possess “multivalent” effects in initiating CTL activities.

To further confirm the anti-tumor activity of the DC vaccines in vivo, we reconstructed human cervical carcinoma in SCID mice. In line with our in vitro study, we found that IFN- γ level was significantly elevated in mice treated with the loaded DCs, especially DC + P + O group. The enhanced immune response resulted in reduced tumor size and high survival rate [27]. Antibody and CD3⁺, CD4⁺ and CD8⁺ T cells were detected after mice were treated with different antigen-loaded DCs vaccine, indicating that T cellular immunity has been activated [28]. However, our results indicated no significant increase in antibody and CD3⁺, CD4⁺ and CD8⁺ T cells was observed between groups unfortunately, which is in contrast to previous results [29]. Thus, further studies are still needed.

In conclusion, it was observed that covaccination with a HPV16 E7^{49–57} polypeptide and CpG-ODN2006 may be a

suitable HPV16-associated tumor antigen to induce significant antitumor immunity for cervical cancer patients.

Conflict of interest None.

References

- Pagliusi S, Organization WH (2006) Human papillomavirus infection and cervical cancer. World Health Organization Web site [http://www.who.int/vaccine_research/diseases/hpv/en/\(Slide14\)](http://www.who.int/vaccine_research/diseases/hpv/en/(Slide14))
- de Sanjose S, Quint WG, Alemany L, Geraets DT, Klaustermeier JE et al (2010) Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study. *Lancet Oncol* 11:1048–1056
- Moody CA, Laimins LA (2010) Human papillomavirus oncoproteins: pathways to transformation. *Nature Rev Cancer* 10:550–560
- Stoler MH (2000) Human papillomaviruses and cervical neoplasia: a model for carcinogenesis. *Int J Gynecol Pathol* 19:16–28
- Nonn M, Schinz M, Zumbach K, Pawlita M, Schneider A et al (2003) Dendritic cell-based tumor vaccine for cervical cancer I: in vitro stimulation with recombinant protein-pulsed dendritic cells induces specific T cells to HPV16 E7 or HPV18 E7. *J Cancer Res Clin Oncol* 129:511–520
- Santin AD, Bellone S, Palmieri M, Zanolini A, Ravaggi A et al (2008) Human papillomavirus type 16 and 18 E7-pulsed dendritic cell vaccination of stage IB or IIA cervical cancer patients: a phase I escalating-dose trial. *J Virol* 82:1968–1979
- Rock KL, York IA, Saric T, Goldberg AL (2002) Protein degradation and the generation of MHC class I-presented peptides. *Adv Immunol* 80:1–70
- Zhou L, Zhu T, Ye X, Yang L, Wang B et al (2009) Long-term protection against human papillomavirus E7-positive tumor by a single vaccination of adeno-associated virus vectors encoding a fusion protein of inactivated E7 of human papillomavirus 16/18 and heat shock protein 70. *Hum Gene Ther* 21:109–119
- van Driel WJ, Rensing ME, Kenter GG, Brandt RM, Krul EJ et al (1999) Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I-II trial. *Eur J Cancer* 35:946–952
- Vierboom MP, Feltkamp MC, Neisig A, Drijfhout JW, ter Schegget J et al (1998) Peptide vaccination with an anchor-replaced CTL epitope protects against human papillomavirus

- type 16-induced tumors expressing the wild-type epitope. *J Immunother* 21:399–408
11. Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT et al (1996) Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 56:21–26
 12. Pvd Bruggen, Eynde BJ (2006) Processing and presentation of tumor antigens and vaccination strategies. *Cur opin immunol* 18:98–104
 13. Bauer M, Redecke V, Ellwart JW, Scherer B, Kremer JP et al (2001) Bacterial CpG-DNA triggers activation and maturation of human CD11c⁺, CD123⁺ dendritic cells. *J Immunol* 166: 5000–5007
 14. Hacker G, Redecke V, Hacker H (2002) Activation of the immune system by bacterial CpG-DNA. *Immunology* 105:245–251
 15. Mutwiri G, Pontarollo R, Babiuk S, Griebel P, van Drunen Littel-van den Hurk S et al (2003) Biological activity of immunostimulatory CpG DNA motifs in domestic animals. *Vet Immunol Immunopathol* 91: 89–103
 16. Kim TG, Kim CH, Won EH, Bae SM, Ahn WS et al (2004) **CpG-ODN-stimulated dendritic cells act as a potent adjuvant for E7 protein delivery to induce antigen-specific antitumour immunity in a HPV 16 E7-associated animal tumour model. *Immunology* 112:117–125**
 17. Krieg AM (2001) From bugs to drugs: therapeutic immunomodulation with oligodeoxynucleotides containing CpG sequences from bacterial DNA. *Antisense Nucleic Acid Drug Dev* 11:181–188
 18. O'Neill DW, Adams S, Bhardwaj N (2004) Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood* 104:2235–2246
 19. Edmondson JM, Armstrong LS, Martinez AO (1988) A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *J Tissue Culture Methods* 11:15–17
 20. Alli RS, Khar A (2004) Interleukin-12 secreted by mature dendritic cells mediates activation of NK cell function. *FEBS Lett* 559:71–76
 21. Xu M, Mizoguchi I, Morishima N, Chiba Y, Mizuguchi J et al (2010) Regulation of antitumor immune responses by the IL-12 family cytokines, IL-12, IL-23, and IL-27. *Clin Develop Immunol* 2010. [10.1155/2010/832454](https://doi.org/10.1155/2010/832454)
 22. Ahn W-S, Bae S-M, Kim T-Y, Kim T-G, Lee J-M et al (2003) A therapy modality using recombinant IL-12 adenovirus plus E7 protein in a human papillomavirus 16 E6/E7-associated cervical cancer animal model. *Human Gene Ther* 14:1389–1399
 23. Hemmi H, Kaisho T, Takeda K, Akira S (2003) The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets. *J Immunol* 170:3059–3064
 24. Kline JN, Krieg AM (2010) Toll-like receptor 9 activation with CpG oligodeoxynucleotides for asthma therapy. *Prog Respir Res* 39:95–99
 25. Ma C, Muranyi M, Chu CH, Zhang J, Chu W-M (2013) Involvement of DNA-PKcs in the IL-6 and IL-12 response to CpG-ODN is mediated by its interaction with TRAF6 in dendritic cells. *PLoS ONE* 8:e58072
 26. Kreis N, Sanhaji M, Krämer A, Sommer K, Rödel F et al (2010) Restoration of the tumor suppressor p53 by downregulating cyclin B1 in human papillomavirus 16/18-infected cancer cells. *Oncogene* 29:5591–5603
 27. Woo SJ, Kim CH, Park MY, Kim HS, Sohn HJ et al (2008) Co-administration of carcinoembryonic antigen and HIV TAT fusion protein with CpG-oligodeoxynucleotide induces potent antitumor immunity. *Cancer Sci* 99:1034–1039
 28. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Löwik MJ et al (2008) Induction of tumor-specific CD4⁺ and CD8⁺ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin Cancer Res* 14:178–187
 29. Kim T-Y, Myoung H-J, Kim J-H, Moon I-S, Kim T-G et al (2002) Both E7 and CpG-oligodeoxynucleotide are required for protective immunity against challenge with human papillomavirus 16 (E6/E7) immortalized tumor cells involvement of CD4⁺ and CD8⁺ T Cells in protection. *Cancer Res* 62:7234–7240