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RESEARCH PAPER

Pleurotus ferulae polysaccharides improve the antitumor efficacy of therapeutic human papillomavirus dendritic cell-based vaccine

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ABSTRACT

We previously found that *Pleurotus ferulae* polysaccharides (PFPS) improved the maturation and function of dendritic cells (DCs). In this study, we investigated the effects of PFPS on the antitumor efficacy of therapeutic human papillomavirus (HPV) DC-based vaccine. PFPS stimulated DCs pulsed with HPV E6/E7 peptides were used to treat tumor mice on day 5 & 12 (HPV + PFPS-DCs early) and day 12 & 19 (HPV + PFPS-DCs late) after TC-1 cell injection. Compared to control group, both HPV + PFPS-DCs early and HPV + PFPS-DCs late strategies significantly inhibited tumor growth, which was significantly correlated with the increased activation status of both $CD4^+$ and $CD8^+$ T cells, the decreased frequencies of myeloid-derived suppressor cells, and the induction of HPV-specific $CD8^+$ T cell responses. The survival of tumor mice was also greatly improved by HPV + PFPS-DCs early. Moreover, HPV + PFPS-DCs early completely inhibited the growth of second challenged TC-1 cells in tumor free mice. The results showed that PFPS improved the antitumor efficacy of therapeutic HPV DC-based vaccine, suggesting that PFPS might be a potential adjuvant for DC-based vaccines. This study provides a potential strategy for developing the therapeutic DC-based vaccine against cervical cancer.

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Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells, which capture, process and present antigens to naïve T cells to generate antigen-specific immune responses. During antigen presentation, various signals including MHC-peptide complex, the interaction of co-stimulatory molecules between DCs and T cells and cytokines secreted by DCs were involved in the activation of antigen-specific T cells.[1] IL-12 facilitates the generation of $CD4^+$ Th1 cells and cytotoxic T lymphocytes,[2–4] which play pivotal roles in the treatment of tumors. DC-based vaccines are hopeful platform for the treatment of cancers due to the capacity of DCs. Antigen-specific immune responses induced by DC-based vaccines in clinical trials have been frequently observed.[5–9] However, the antitumor efficacy of DC-based vaccines needs to be improved due to the suboptimal maturation of DCs with low level of IL-12.[7,10]

Adjuvants such as toll-like receptor agonists have been used to improve the immunogenicity of various new-types of vaccines, such as peptide/protein vaccines, DNA vaccines and DC-based vaccines.[11,12] However, almost of adjuvants cannot be used for human vaccines due to their side effect. Only a few of adjuvants including aluminum salts, MF59, AS03, AF03 and AS04 have been licensed for human vaccines in the US and/or Europe.[11] Therefore, the development of safe and effective adjuvants for human use has been drawn the attentions from scientists.

Traditional Chinese medicine (TCM) has a long history in the treatment of human diseases. Accumulating evidence shows that TCM and its components have immunoregulatory effects, especially in the regulation of DC maturation.[13-15] Therefore, TCM is a good candidate pool to search adjuvants. *Pleurotus ferulae* is a kind of TCM and has various biological activities such as anti-oxidant, anti-tumor, and anti-microbial.[16-18] Our previous studies demonstrated that *P. ferulae* water extract promoted the maturation and IL-12 secretion of DCs and improved the antitumor effect of human papillomavirus (HPV) DC-based vaccine.[19,20] Recently, *P. ferulae* polysaccharides (PFPS) were purified and characterized, which promoted DC maturation and cytokine production through TLR-4 signaling pathway.[21] Here, we investigated the effects of PFPS on the antitumor efficacy of HPV therapeutic DC-based vaccine in tumor mouse model induced by TC-1 cells.

Results

PFPS treated DCs pulsed with HPV peptides (HPV + PFPS + DCs) inhibited tumor growth

TC-1 tumor mouse model was used to detect the antitumor effect of HPV + PFPS + DCs. Based on our previous studies,[20,22,23] tumor volumes can reach 2000 mm^3 in tumor mice around 30 days after TC-1 cell injection. Therefore, the one-week interval immunization was chosen. According to the time points of HPV + PFPS + DCs treatments, the strategies were designed as

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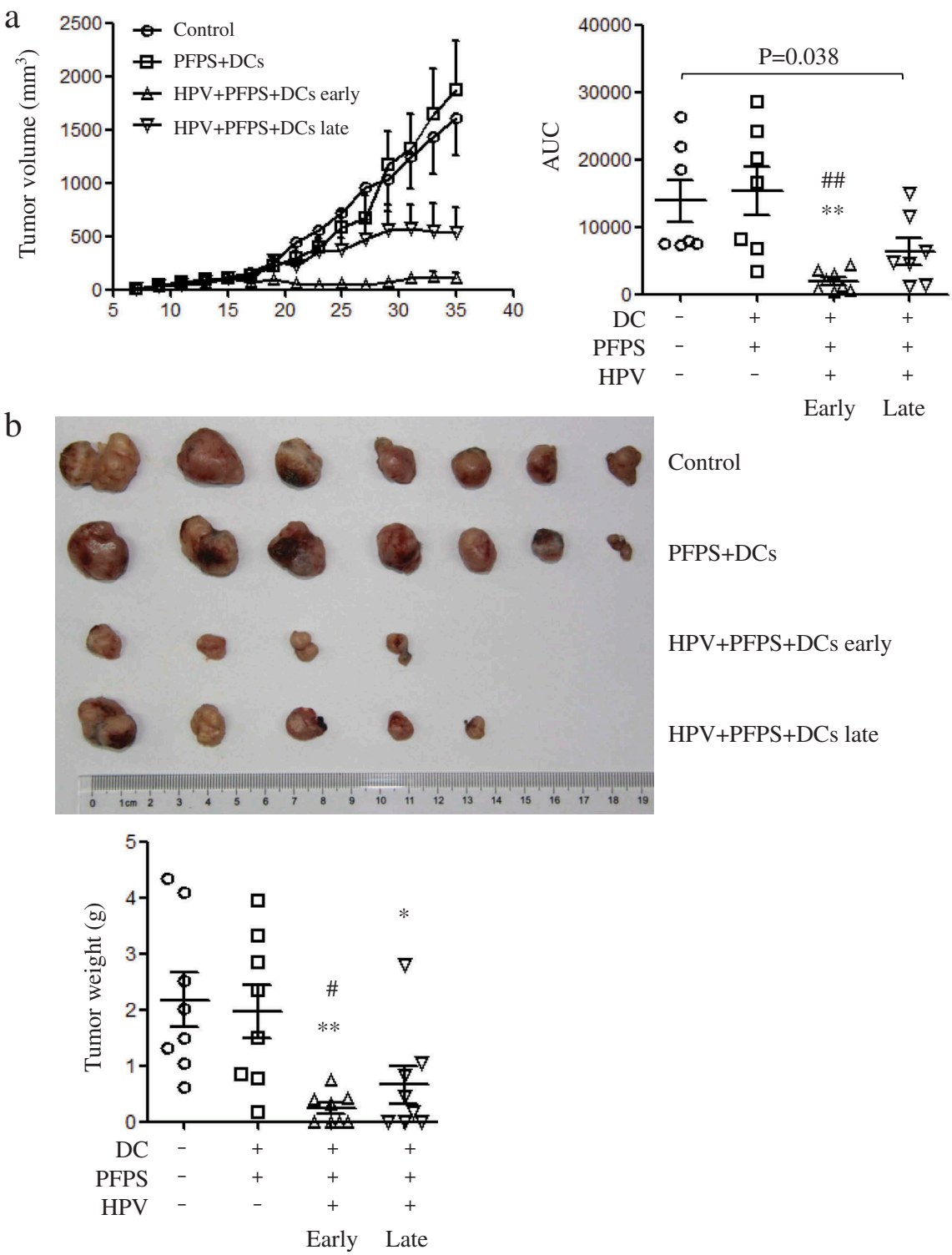

Figure 1. Tumor growth and tumor weight after HPV + PFPS + DCs early and late treatment.

After injection of TC-1 cells, tumor mice were immunized twice on days 5 and 12 in HPV + PFPS + DCs early and PFPS + DCs groups, or on days 12 and 19 in HPV + PFPS + DCs late group. (A) Tumor volumes (mean± SEM) were measured shown in the left panel. The area under curve (AUC) was calculated using Prism 5 and shown in right panel (mean± SEM). P value (Mann-Whitney test) is given. (B) Tumors were isolated and weighted at the end of this experiment. The tumor photo and weight (mean± SEM) are shown in upper and lower panels, respectively. *$P < 0.05$ and **$P < 0.01$ (ANOVA) compared to control group. #$P < 0.05$ and ##$P < 0.01$ (ANOVA) compared to PFPS + DCs group.

early therapy (HPV + PFPS+ DCs early) and late therapy (HPV + PFPS+ DCs late), in which tumor mice were treated with HPV + PFPS+ DCs on day 5 & 12 and day 12 & 19 after TC-1 cell injection, respectively. Tumor mice without treatment or injected with PFPS + DCs were named as control or PFPS + DCs, respectively. One mouse (1/8) with 3097 mm^3 tumor was dead on day 27 in control group. One mouse (1/8) with 242 mm^3 tumor was dead on day 17 in PFPS + DCs group. One mouse

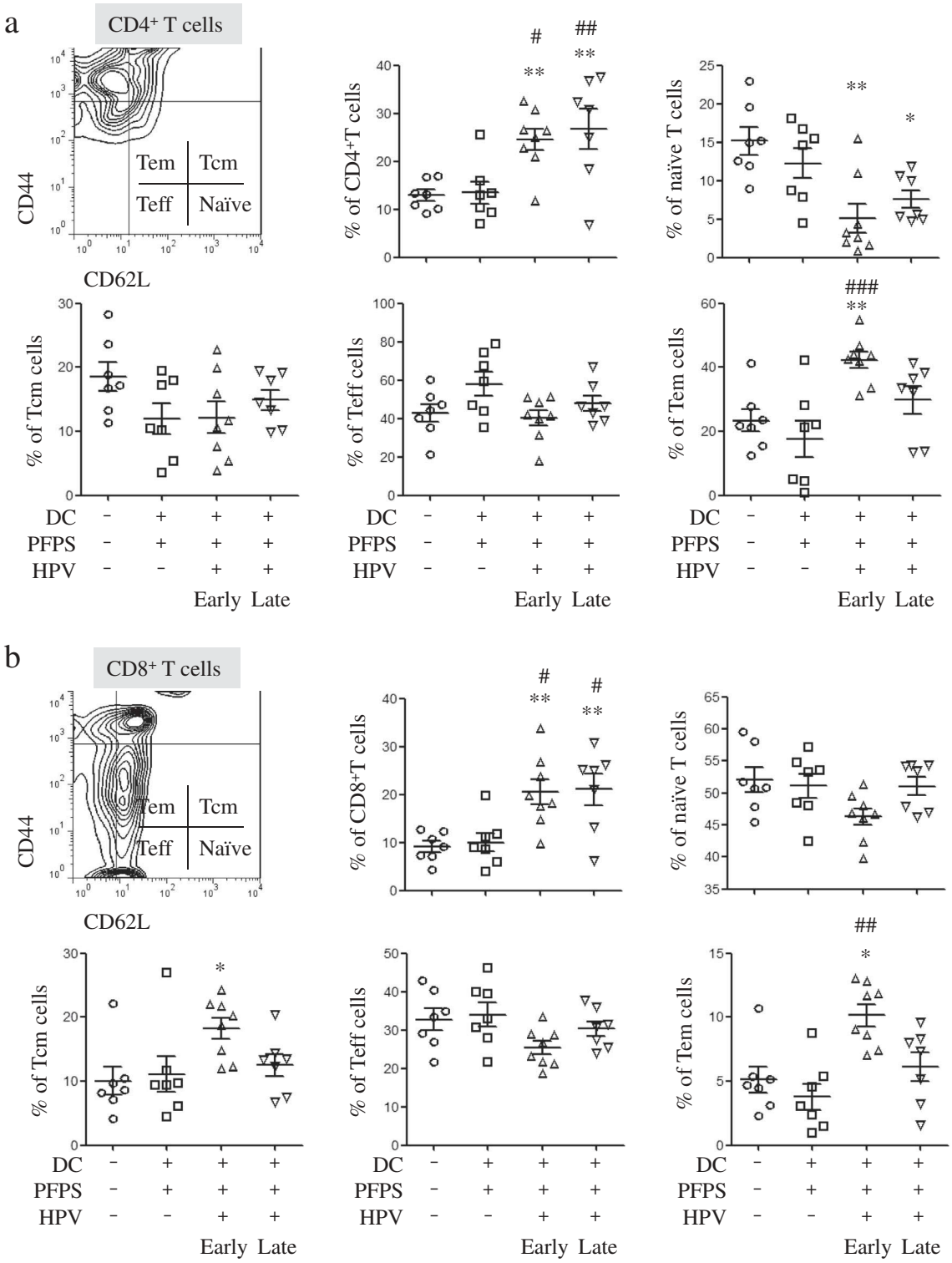

Figure 2. The frequencies of $CD4^+$ and $CD8^+$ T cells and their subsets in spleens of tumor mice.

Splenocytes were isolated from tumor mice at the end of this experiment to detect the frequencies (mean± SEM) of $CD4^+$ (A) and $CD8^+$ (B) T cells and their subsets by flow cytometry. The contour panels show the gating strategy. *P < 0.05 and **P < 0.01 (ANOVA) compared to control group. #P < 0.05, ##P < 0.01 and ###P < 0.001 (ANOVA) compared to PFPS + DCs group.

(1/8) with 1742 mm^3 tumor was dead on day 31, one mouse was free of tumor and another one had very small tumor (6.8 mm^3) in HPV + PFPS + DCs late group at the end of this experiment. The tumors of the three dead mice were isolated and weighted before the end of this experiment. All mice (8/8) were live and 4 mice were free of tumor in HPV + PFPS+ DCs early group at the end of this experiment. Compared to control and PFPS + DCs groups, HPV + PFPS + DCs early greatly inhibited tumor growth. HPV + PFPS+ DCs late also significantly suppressed tumor growth (Figure 1A). At the end of the experiment, tumor mice were sacrificed and tumors were isolated to take photo and weight (Figure 1B). Tumors were obtained from 7 mice in

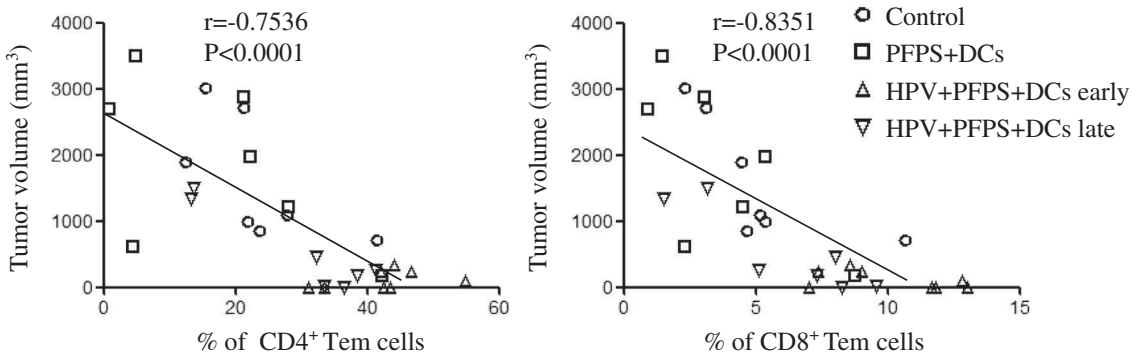

Figure 3. The correlation of $CD4^+$ and $CD8^+$ Tem cells with tumor volumes. The nonparametric correlation was calculated by GraphPad Prism 5.

control group, 7 mice in PFPS + DCs group, 4 mice in HPV + PFPS + DCs early group and 5 mice in HPV + PFPS + DCs late group. Tumors in both HPV + PFPS + DCs early and late groups were smaller than that in control and PFPS + DCs groups. Tumor weight was also significantly decreased in both HPV + PFPS + DCs early and late groups.

HPV + PFPS + DCs changed the profile of T cells, induced HPV-specific cellular responses and decreased myeloid-derived suppressor cells (MDSCs)

To investigate how HPV + PFPS + DCs suppressed tumor growth *in vivo*, the profile of T cells, HPV-specific cellular responses and the frequencies of MDSCs in spleens of tumor mice were analyzed by flow cytometry. We found that the frequencies of $CD4^+$ and $CD8^+$ T cells were significantly increased in both HPV + PFPS+ DCs early and late groups compared with control and PFPS + DCs groups (Figure 2A,B). The activation status of $CD4^+$ and $CD8^+$ T cells was further detected using CD44 and CD62L as markers to distinguish naïve ($CD62L^+CD44^{low}$), effector (Teff: $CD62L^-CD44^{low}$), effector memory (Tem: $CD62L^-CD44^{hi}$) and central memory (Tcm: $CD62L^+CD44^{hi}$) subsets.[24-26] Compared to control group, the frequencies of $CD4^+$ naïve T cells were significantly decreased in both HPV + PFPS + DCs early and late groups but the frequencies of $CD4^+$ Tem cells in HPV + PFPS + DCs early group were significantly increased (Figure 2A). As shown in Figure 2B, the frequencies of $CD8^+$ Tem and Tcm cells in HPV + PFPS + DCs early group were significantly increased compared to control group. We analyzed the correlations among the frequencies of $CD4^+$ and $CD8^+$ Tem cells with tumor volumes and found that the frequencies of $CD4^+$ and $CD8^+$ Tem cells were negatively correlated with tumor volumes (Figure 3). There are no significant changes in the frequencies of $CD4^+$ Teff and Tcm cells, and $CD8^+$ naïve and Teff cells among all groups.

Next, we detected HPV-specific cellular responses in spleen of tumor mice upon HPV peptide treatment. HPV-specific $CD4^+$ T cell responses were slightly increased in both HPV + PFPS + DCs early and late groups, whereas HPV-specific $CD8^+$ T cell responses were significantly induced by HPV + PFPS+ DCs regardless of early or late treatment, which characterized by the increased frequencies of $CD8^+IFN\text{-}\gamma^+$ T cells (Figure 4A). The frequencies of HPV-specific $CD8^+IFN\text{-}\gamma^+$ T cells were negatively correlated with tumor volumes (Figure 5). MDSCs are expended during cancer and inhibit antitumor immune responses.[27,28] The frequencies of MDSCs ($CD11b^+Gr\text{-}1^+$) and macrophages ($CD11b^+Gr\text{-}1^-$) in spleens of tumor mice were analyzed by flow cytometry. HPV + PFPS + DCs early treatment significantly decreased the frequencies of MDSCs compared with control group but did not change the frequencies of $CD11b^+Gr\text{-}1^-$ macrophages (Figure 4B). PFPS + DCs increased the frequencies of MDSCs and $CD11b^+Gr\text{-}1^-$ macrophages, whereas HPV + PFPS + DCs late treatment did not change them. We observed that the frequencies of MDSCs were positively correlated with tumor volumes (Figure 5). These results suggested that HPV + PFPS + DCs enhanced the activation status of $CD4^+$ and $CD8^+$ T cells, induced HPV-specific cellular responses and decreased the frequencies of MDSCs, which might mediate the inhibition of tumor growth.

HPV + PFPS + DCs improved the survival of tumor mice and suppressed tumor recurrences

The above results showed that HPV + PFPS + DCs significantly inhibited the growth of tumors in TC-1 mouse model, especially the early treatment. Therefore, the survival of tumor mice was further measured after early treatment with HPV + PFPS + DCs. The strategy of treatment was shown in Figure 6A. Tumor volumes were measured from 9 days to 31 days due to tumor mice began to die on day 31 in control group. The growth of tumors was significantly suppressed in HPV + PFPS + DCs group compared with control group, which was consistent with the above experiment. At the end of this experiment (on day 63), all mice (8/8) died in control group and 3 mice (3/8) died in HPV + PFPS + DCs group (Figure 6B), suggesting that the survival of tumor mice was greatly improved by HPV + PFPS + DCs treatment. For the 5 survived mice in HPV + PFPS + DCs group, 2 mice had small tumors with the volumes of 903 and 1037 mm^3, and 3 mice were free of tumor.

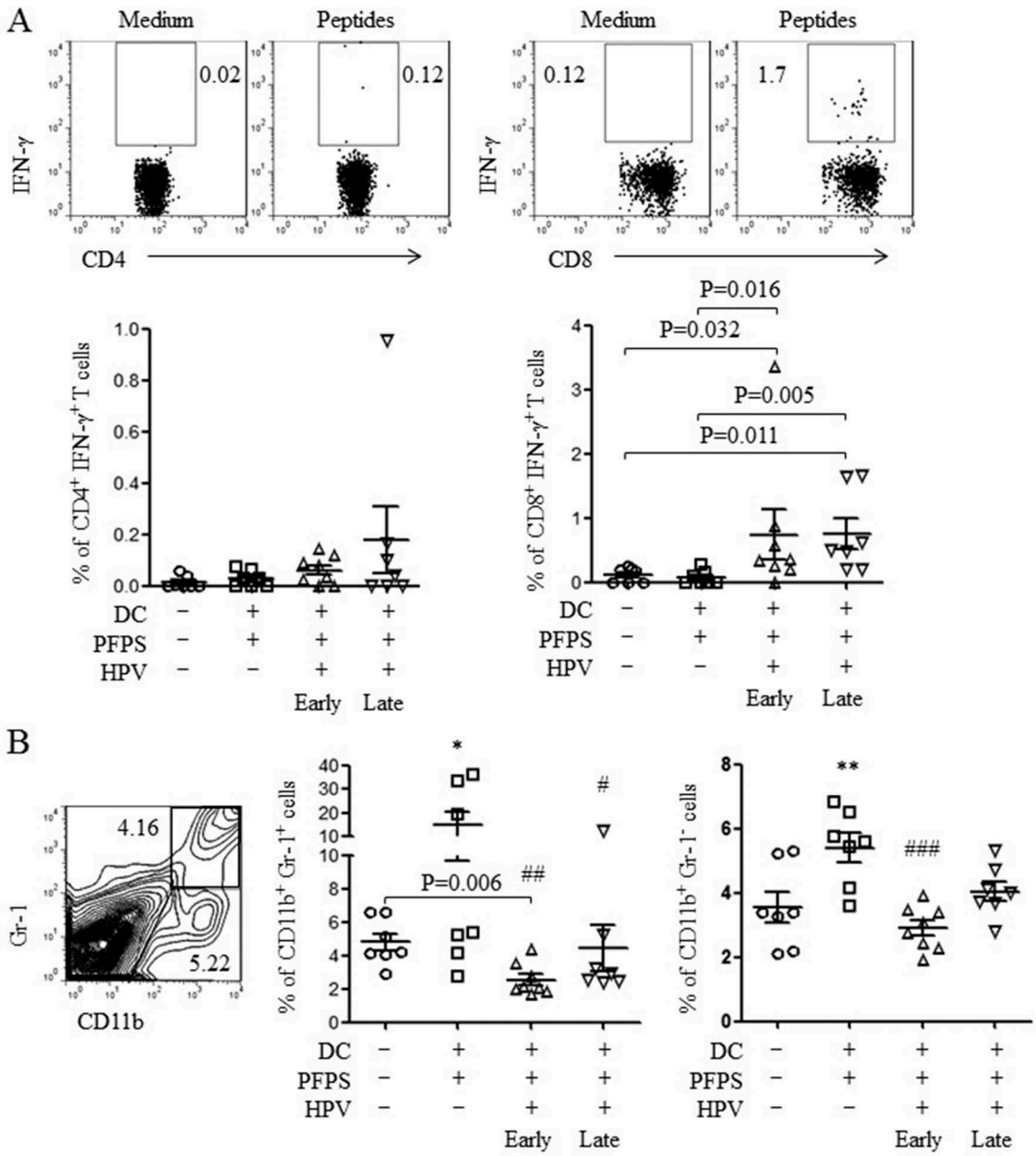

Figure 4. HPV-specific cellular responses and the frequencies of MDSCs and macrophages.

Splenocytes were isolated from tumor mice at the end of this experiment. (A) Splenocytes were stimulated with HPV-16 E6 and E7 peptides overnight. HPV-specific cellular responses were analyzed by flow cytometry. The representative dot plots are shown in upper panels and the summary data (mean± SEM) of HPV-specific $CD4^+$ and $CD8^+$ T cells are shown in lower panels. P values (Mann-Whitney test) are given. (B) The frequencies (mean± SEM) of MDSCs ($CD11b^+Gr\text{-}1^+$) and macrophages ($CD11b^+Gr\text{-}1^-$) in spleens of tumor mice were detected by flow cytometry. The contour panel shows the gating strategy. P value (Mann-Whitney test) is given. *$p < 0.05$ and **$p < 0.01$ (ANOVA) compared to control group. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ (ANOVA) compared to PFPS + DCs group.

The re-challenge of tumor cells has been used to evaluate the effect of tumor vaccine on tumor recurrences.[29] To investigate whether HPV + PFPS + DCs can suppress the tumor recurrences, the 3 mice free of tumor were chosen from the 5 survived mice in HPV + PFPS + DCs group and re-challenged with TC-1 cells on day 69. At the same time, 2 naïve mice were challenged with TC-1 cells and served as control group. Tumors grew fast in control group but the tumor growth was completely suppressed in re-challenged tumor free mice (Figure 6C), suggesting that HPV + PFPS + DCs might induce memory immune responses. According to the above results (Figures 4 and 5), we speculated that the memory immune responses might be the HPV-specific $CD8^+$ T cell responses.

Discussion

DC-based vaccines are potent vaccine platform against cancer due to the important role of DCs in immune system, but the clinical efficacy need to be improved. Lots of efforts have been focused on how to enhance DC maturation and IL-12 production.[30-32] Our previous studies showed that the crude

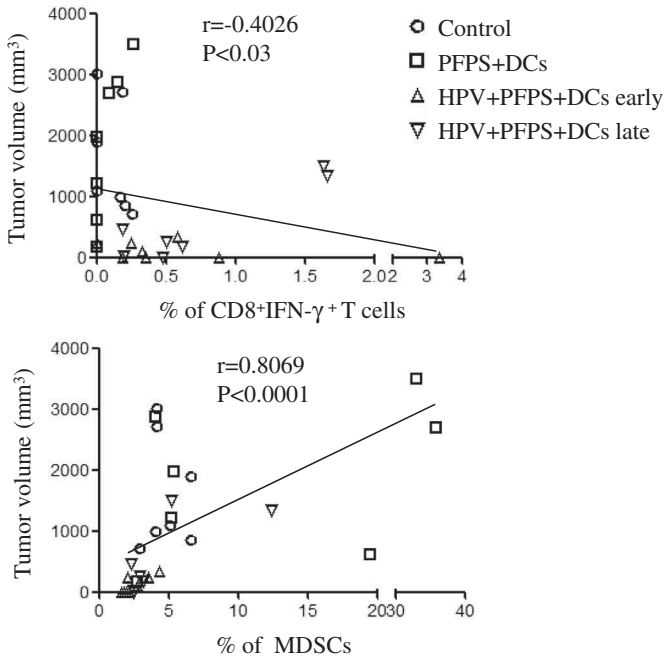

Figure 5. The correlation of $CD8^+IFN\text{-}\gamma^+$ T cells and MDSCs with tumor volumes. The nonparametric correlation was calculated by GraphPad Prism 5

and purified polysaccharides from *P. ferulae* enhanced DC maturation and IL-12 production.[19,21] In this study, we found that PFPS treated HPV DC-based vaccine inhibited tumor growth and recurrences, induced antigen-specific cellular responses, and improved the survival of tumor mice.

The survival rates of cancer patients are closely correlated with disease stages.[33] In developing countries, the survival rates were low due to the lack of early screening strategies, the poor treatment and clinical follow-up care.[34] Cervical cancers were mainly caused by HPV infection, especially high-risk types of HPV including HPV-16 and HPV-18,[35-37] which were usually diagnosed at high disease stages in developing countries. Therefore, the antitumor effects of PFPS treated DCs pulsed with HPV peptides were investigated in both early and late therapeutic strategies. We found that HPV + PFPS + DCs showed powerfully antitumor effect in TC-1 tumor mouse model, which significantly suppressed tumor growth and reduced tumor weight in both early and late treatment groups compared with control group. Both HPV + PFPS + DCs early and late induced strong HPV-specific $CD8^+$ T cell responses and increased the frequencies of $CD4^+$ and $CD8^+$ T cells in spleens of tumor mice. These results are similar with our previous study.[20] There are lack of effective strategies for the treatment of advanced cervical cancer. The HPV + PFPS + DCs showed the therapeutic effect on TC-1 tumor mice at late stage, which might provide a potential strategy for developing the therapeutic DC-based vaccine against cervical cancer.

Using CD44 and CD62L as markers, the activation status of $CD4^+$ and $CD8^+$ T cells was determined.[24-26] We found the significantly negative correlations among the frequencies of $CD4^+$ and $CD8^+$ Tem cells with tumor volumes, indicating that the increased frequencies of activated T cells contributed the inhibition of tumor growth. We also found that HPV-specific $CD8^+$ T cell responses were negatively correlated with tumor volumes, suggesting that HPV + PFPS + DCs induced antigen-specific cytotoxic responses promoted the control of tumor growth.

MDSCs and regulatory T cells (Tregs) were involved in the suppressive microenvironment induced by tumors, which facilitated tumor growth and inhibited immune responses.[27,28,38-40] Consistently, we observed that the frequencies of MDSCs positively correlated with tumor volumes. Interestingly, the frequencies of MDSCs were significantly decreased in HPV + PFPS + DCs early group compared to control group, indicating that the decreased frequencies of MDSCs contributed the control of tumor growth and the generation of antitumor immune responses. Rossowska, et al.[41] reported that IL-12-transduced DCs enhanced the antitumor activity of DC-based vaccine through the reduction of MDSCs. PFPS treated DCs also produced high level of IL-12 that might be caused the decrease of MDSCs. We also observed that PFPS + DCs increased the frequencies of both MDSCs and macrophages ($CD11b^+Gr\text{-}1^-$) compared with control group. The tumor growth is similar between control and PFPS + DCs group, suggesting that the stimulatory and suppressive effects on tumor growth might be counteracted. In our previous study, we found that HPV DC-based vaccines significantly decreased the frequencies of Tregs.[20] However, the frequencies of Tregs were not significantly decreased by HPV + PFPS + DCs (data not shown). The different doses of DCs (1×10^6 VS 0.5×10^6) in these two studies might be caused the discrepancy. These results indicated that the promoted activation status of T cells, the induction of HPV-specific $CD8^+$ T cell responses and the decreased frequencies of MDSCs were together contributed to the inhibition of tumor growth in HPV + PFPS + DCs early and late groups.

Although the tumor growth was significantly inhibited in HPV + PFPS + DCs late group, there was the room for the further improvement. The maturation of DCs is suppressed in tumor microenvironment,[42-44] which dampened antitumor immune responses. Therefore, it is very important to enhance DC maturation *in vivo* using adjuvant to improve antigen presentation. Recently, Wang et al.[45] have reported that alum adjuvant only can suppress the established hepatocarcinoma in mouse model. In the future studies, the strategy of HPV + PFPS + DCs combined with PFPS as adjuvant *in vivo* will be investigated for the tumors with late stage.

In conclusion, PFPS treated HPV DC-based vaccine showed powerful antitumor effect in both early and late therapeutic strategies, suggesting that PFPS might be a good candidate adjuvant for DC-based vaccines.

Materials and methods

Animals

6–8 weeks old C57BL/6 female mice were bought from the Beijing laboratory animal research center (Beijing, China). Mice were housed in a temperature-controlled, light-cycled

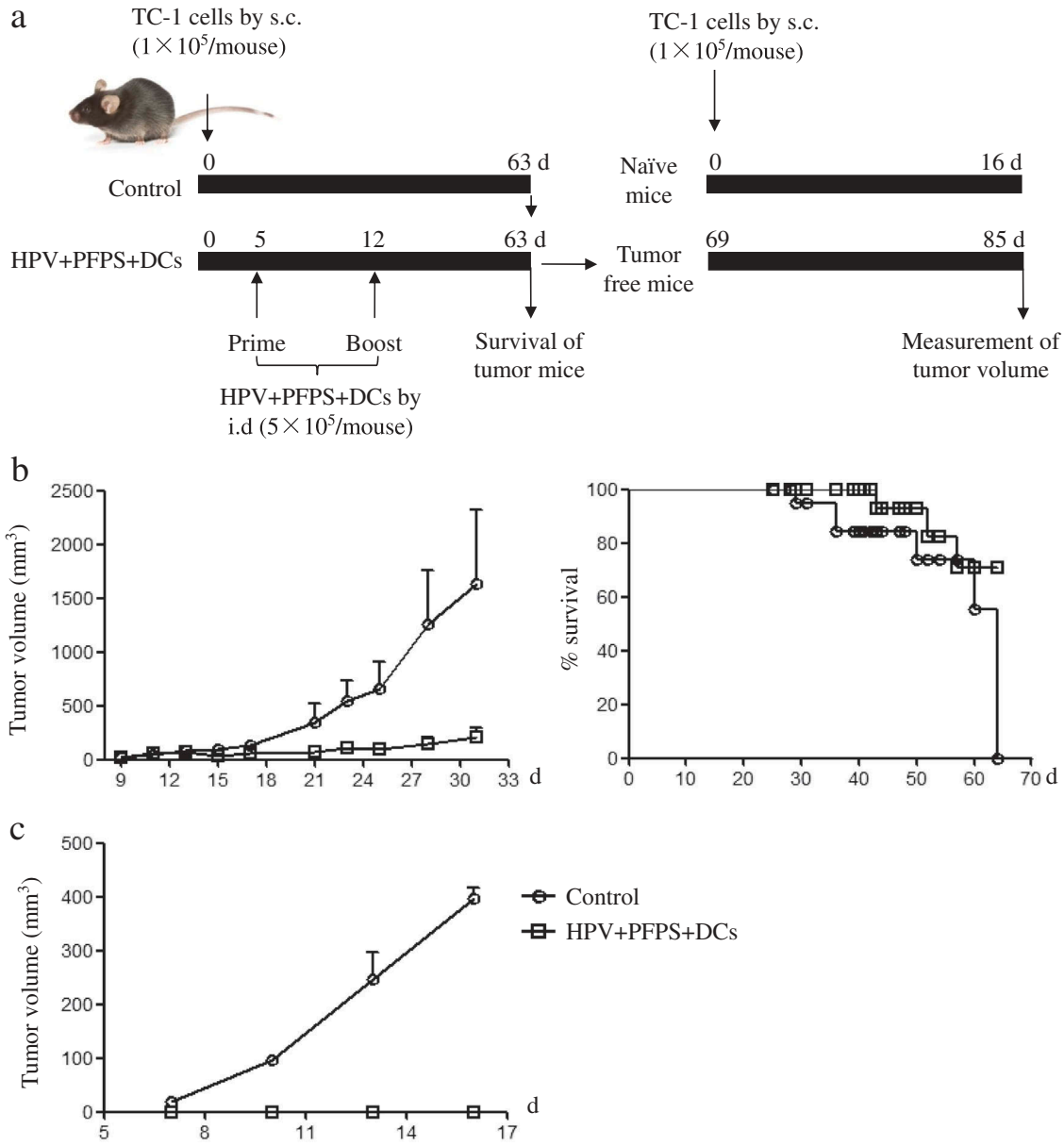

Figure 6. The survival of tumor mice and inhibition of tumor recurrence.

(A) The strategy of treatment. (B) The growth of tumors and survival of tumor mice. After HVP + PFPS+ DCs treatment, tumor volumes (mean± SEM) were measured and shown in left panel, the survival of tumor mice were monitored and shown in right panel. (C) The protective effect of HVP + PFPS+ DCs on tumor recurrences. 3 tumor free mice in HVP + PFPS + DCs group were re-challenged and 2 naïve mice were inoculated with TC-1 cells, then tumor growth was measured. The mean ± SEM of tumor volumes was shown.

animal facility of Xinjiang University. All animal experiments were done under the guidelines of the Animal Care and Use Committee of College of Life Science and Technology, Xinjiang University.

Preparation of HPV DC-based vaccine

Bone marrow cells were collected from C57BL/6 mice and immature DCs were induced in the presence of GM-CSF (Peprotech) according to our previous description.[19] On day 7, immature DCs were collected and stimulated with 100 μg/ml of PFPS for 12 h, then 2 × 10^6/ml of PFPS treated-DCs were pulsed with HPV-16 E6 and E7 peptides (10 μg/ml for each peptide) including $E6_{43-57}$ (QLLRREVYDFAFRDL), $E6_{53-62}$ (AFRDLCIVYR), $E7_{11-20}$ (YMLDLQPETT), $E7_{44-62}$ (QAEPDRAHYNIVTFCCKCD) and $E7_{81-94}$ (DLLMGTLG IVCPIC). After 2 h, DCs were washed twice with PBS and resuspended in PBS at the concentration of 5 × 10^5 DCs/50 μl, named as HPV + PFPS + DCs. PFPS + DCs without HPV peptides were used as DC control.

Treatment of tumor model

TC-1 tumor cell line constitutively expressed HPV-16 E6 and E7,[46] was used to establish tumor mouse model to detect the antitumor effect of HPV + PFPS + DCs. TC-1 cells at log-phase growth were collected, washed and resuspended in PBS at a density of 1 × 10^6/ml. 1 × 10^5

TC-1 cells were subcutaneously injected into the right flank of C57BL/6 mice that were randomly divided into 4 groups (8 mice/group). Tumor mice were intradermally immunized twice with 5×10^5 HPV + PFPS + DCs at peri-tumoral sites at a one-week interval because the tumor mice without treatment will die around 30 days. For early therapy, tumor mice were immunized on day 5 & 12 (HPV + PFPS + DCs early). For late therapy, tumor mice were immunized on day 12 & 19 (HPV + PFPS + DCs late). Untreated group and PFPS + DCs treated group (on day 5 & 12) were named as control and PFPS + DCs, respectively. Tumors were measured every other day using calipers and tumor volumes were calculated according to the formula: tumor volume (mm^3) = (length× $width^2$)/2. After 35 days, mice were sacrificed and tumors were isolated to weight. Splenocytes were used to analyze the frequencies of macrophages, $CD4^+$ and $CD8^+$ T cells, and HPV-specific cellular responses by flow cytometry.

Flow cytometry

The splenocytes were used to analyze the frequencies of macrophages and T cells, and the subtypes of T cells. After washing with PBS, cells were stained with CD11b-PE and Gr-1-APC (Elabscience Biotechnology Co., Ltd, China) or CD4-FITC and CD8-PE (BD Biosciences) to analyze MDSCs, macrophages and T cells, respectively. For analysis of T cell subtypes, splenocytes were stained with CD4-FITC, CD44-PE and CD62L-APC or CD8-FITC, CD44-PE and CD62L-APC (BD Biosciences).

HPV-specific cellular responses were detected upon the treatment of HPV-16 E6 and E7 peptides in the presence of Golgi stop (monensin) (BD Biosciences) according to our previous description.[20] Briefly, IFN-γ production was detected by intracellular staining with IFN-γ-APC antibody (BD Biosciences) after surface staining with CD4-FITC and CD8-PE antibodies. After washing, all samples were collected on FACSCalibur (BD Biosciences) and analyzed by FlowJo platform (Tree Star, Inc., Ashland, OR).

The survival of tumor mice and tumor cell re-challenge

TC-1 tumor mice were randomly divided into 2 groups (8 mice/group). Tumor mice treated with HPV + PFPS + DCs twice on day 5 & 12 were also named as HPV + PFPS + DCs early group. The untreated tumor mice were served as control group. Tumors were measured every other day until day 31 due to tumor mice in control group began to die. The survival of tumor mice were monitored every day until all mice died in control group. To detect the effect of HPV + PFPS + DCs on tumor recurrences, tumor free mice (n = 3) in HPV + PFPS + DCs early group were re-challenged with 1×10^5 TC-1 cells on day 69. At the same time, 2 naïve mice were inoculated 1×10^5 TC-1 cells. Tumors were measured every other day.

Statistical analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) or Mann-Whitney test. $P < 0.05$ was considered to be statistically significant.

Disclosure of potential conflicts of interest

The authors declare no competing financial interests.

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