Vaccine Research

ISSN: 2423-4923
eISSN: 2383-2819

Potent Therapeutic Efficacy of a Novel HPV16E7-HBcAg-Hsp65 Fusion Protein Vaccine

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A R T I C L E I N F O

Research Article

VacRes, 2021
Vol. 8, No.1, 60- 66
Received: November 24, 2021
Accepted: December 11, 2021
Pasteur Institute of Iran

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KEYWORDS: Human papillomaviruses, Therapeutic vaccine, Cervical caner, T-cell response, Combination therapy

A B S T R A C T

Introduction: Cervical cancer is the fourth leading cause of cancer death in women worldwide. Nearly all cervical cancers are resulted from high-risk Human Papillomavirus (HPV) infection. Currently, there is no available HPV-specific therapy. Cancer therapeutic vaccines have shown anti-tumor efficacy in preclinical animal models as well as clinical patients. **Methods:** Here, we used a previously-reported therapeutic vaccine candidate (VR_{111}) based on HPV16E7-HBcAg-Hsp65 fusion protein (with aluminum hydroxide adjuvant) and injected mice with 2 doses of VR_{111} at a two-week interval 2 days after TC-1 tumor cell implantation. Tumor growth and animal survival rates were monitored and the vaccine-associated immune responses were evaluated by cytotoxic T lymphocytes assay, T-cell proliferation assay and $CD4^+/CD8^+$ T-cell depletion. **Results:** In TC-1 tumor murine model, VR_{111} vaccine showed potent dose dependent therapeutic efficacy against tumor growth and improved survival rates in the medium (10 µg) and high doses (30 µg). The three fusion components of VR_{111} were all necessary to induce the best anti-tumor activity, CTL response and T cell proliferation. The tumor growth inhibition and a higher mouse survival rate were among the beneficial effects of cisplatin-based combination treatment. Moreover, the anti-tumor potency of VR_{111} vaccine was proved to be significantly associated with E7 specific $CD8^+$ T cell immune response and the adoptive lymphocyte transfer therapy also showed tumor growth inhibition. **Conclusion:** The results confirmed VR_{111} as a potent therapeutic HPV vaccine candidate with superior anti-tumor efficacy in a murine model of HPV-induced cancer which its potentials could be considered for combination therapies against cervical cancer.

Citation:

INTRODUCTION

High-risk human papillomavirus (HPV) infection has been firmly proved to be responsible for nearly all invasive cervical cancer, cervical pre-cancerous lesions and several other anogenital malignancies [1, 2]. According to the GLOBOCAN 2020 report, cervical cancer has dropped to the fourth most common cancer and the fourth most common cause of death from cancer in women due to the increasing coverage of prophylactic vaccination against HPV [3]. However, there is still no HPV-specific therapy and most used therapies involve removal or physical destruction of the lesions [4]. Recently, the WHO proposed to eliminate cervical cancer, and the third target is to reduce mortality from cervical cancer which needs more efficient therapies as well as preventing the relapses. The great successes of cancer immunotherapies using anti-PD1/PDL1 and CAR-T-cells, suggest the possibility of using therapeutic HPV vaccine as another promising option to fight against cervical cancer and a good candidate for combination therapy [5-10].

In China, high-risk type 16 (HPV16) was found to be positive in more than 60% of cervical intraepithelial neoplasia 2 or 3 (CIN2/3) and squamous cell carcinoma (SCC) samples [11, 12]. The genome integration and the interaction of non-structural proteins E6-p53, E7-pRb are thought to play important roles in the HPV-associated cervical carcinogenesis by disrupting the regulation of the cell cycle [13, 14]. Meanwhile, E7 protein is highly conserved genetically in pre-cancer/cancer patients, which makes it the optimum tumor specific antigen for a therapeutic vaccine design [15]. The relatively slow progression from CIN stages to advanced cancer also provides adequate time for the therapeutic intervention [14]. Multiple approaches have so far been taken to develop therapeutic HPV vaccines. Some of them targeted HPV16 E7 and/or E6 which have shown therapeutic benefits in CIN2/3 or cervical cancer [16-19].

In this study, we evaluated a previously-reported therapeutic vaccine candidate (VR_{111}), based on HPV16E7-HBcAg-Hsp65 fusion protein, formulated it with aluminum hydroxide adjuvant. The antigen HPV16 E7 nonstructural

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[DOI: 10.52547/vacres.8.1.60]

protein and Mycobacterium bovis Bacille Calmette-Guérin (BCG) heat shock protein (Hsp65) were genetically fused to the N and C termini of the core antigen from hepatitis B virus (HBcAg), respectively. The self-assembly HBcAg is a flexible carrier to form virus-like particles (VLP) and Hsp65 was used to enhance antigen presentation as well as antigen specific cytotoxic T-lymphocyte responses [20-23]. In the previous study, VR_{111} was shown to induce significant dose-dependent cellular (E7-specific) and humoral immune responses [24]. Thus, we hypothesized that VR_{111} therapeutic vaccine could have a potent anti-tumor efficacy against the HPV16-induced cancer. The anti-tumor activity of VR_{111} was evaluated in tumor-harboring mice and the results showed VR_{111} could significantly inhibit the tumor growth alone or combined with cisplatin (an effective anticancer agent) [25, 26]. Moreover, the $CD8^+$ T-cells played a major role in VR_{111}-induced immune responses against TC-1 tumor cells and the adoptive lymphocyte transfer therapy also showed anti-tumor effects.

MATERIALS AND METHODS

Ethical Statement

Ethical approval for treatment of the mice was granted by the Institutional Animal Care and Use Committee (IACUC) of National Center for Safety Evaluation of Drugs (NCSED) and National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). The animal use was in line with the 3R (Reduction, Replacement, Refinement) principle and followed the Guide for the Care and Use of Laboratory Animals.

Mice and Cell Lines

Six to eight weeks old female C57BL/6 mice were purchased from Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China. Animals were cared for under the standard protocols of the institutional committee. All animals were maintained under specific-pathogen-free conditions. TC-1 cells were provided by Shanghai Zerun Anke Biopharmaceutical company and were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin (100U/ml each) and L-glutamine (2mM) at 37˚C with 5% CO_2. This cell line was derived from mouse lung epithelial cells and co-transformed by HPV16 E6/E7 and activated c-Ha-Ras [27].

Tumor Implantation and Treatment

TC-1 cells in log phase were implanted with 2×10^5 cells/mouse subcutaneously (s.c.) in the right flank of each mouse on Day 0. Diameters of each tumor were measured every 3 or 4 days using digital caliper. Tumor volume was calculated using formula V=1/2*a*b*b, whereby “V” is volume, “a” is longer diameter and “b” is shorter diameter. In all experiments, mice were sacrificed when became moribund. Two days after tumor implantation, appropriate groups (n=10) were intramuscularly (i.m.) injected with VR_{111}, negative (normal saline) control or constituent controls twice with a 2-week interval. All antigens were expressed in *Eschericia coli* (SE1 strain) expression system and manufactured at Shanghai Zerun Biotechnology Co., Ltd [24]. Briefly, the codon optimized VR_{111} fusion protein (823a.a.) gene sequence was constructed in pStaby1.2 vector and the expression was induced by IPTG (isopropyl-β-d-thiogalactoside). The recombinant bacteria were cultured in a 30 L fermentor. Recombinant protein was purified by cation and anion exchange chromatography and washed by TritonX-100. The final endotoxin content was controlled to below 20 EU/mg. The purified antigen bulk (720 μg/ml) was then gently mixed with aluminum hydroxide diluent (2700 μg/ml) in a 2:1 volume ratio at 2 to 8℃ for 15~20 h to prepare the final bulk. Each dose of VR_{111} bulk was 0.5ml that contained 240 μg of fusion protein and 450 μg of aluminum hydroxide adjuvant in 50mM phosphate buffer (pH = 7.5). The constituent protein controls were also formulated with aluminum hydroxide adjuvant with the same concentrations. In the cisplatin combination treatment, 5 mg/kg cisplatin (Qilu Pharmaceutical, China) was intraperitoneally (i.p.) injected alone or combined with 10 μg i.m. immunized VR_{111} vaccine. To visibly verify the anti-tumor growth, tumor tissues were dissected 17 days after tumor implantation followed by two times of injections.

Cytotoxic T lymphocytes (CTL) Assay

C57BL/6 mice harboring TC-1 tumor were treated as before and sacrificed two weeks after the second immunization. Splenocytes were collected and lymphocytes were isolated by using mouse lymphocytes separation medium (Hao Yang Bio, Tianjin, China). The lymphocytes were stimulated with 100 μg/ml $E7_{49-57}$ peptide (GL Biochem, Cat. 51582) in RPMI 1640 medium with 10% FBS for 3 days. Target TC-1 cells were labeled with ^{51}Cr (PerkinElmerTM) by mixing 5×10^4 cells/well with 3.7 MBq $Na_2{}^{51}CrO_4$ in a 24-well plate and incubate at 37℃ overnight. The effector T cells were diluted and added to incubate together with labeled target TC-1 cells at ratios (effector:target) of 30:1, 10:1, 3:1 for 6-8 hours at 37°C. The radioactivity was measured in 100 μl of cell culture supernatant. The percentage of lysis was calculated as previously described [28].

T-Cell Proliferation Assay

The lymphocytes were isolated as before and stimulated as triplicates with 100 μg/ml $E7_{49-57}$ peptide, 10 μg/ml Con A (Sigma) or blank negative control for 3 days at 37℃. After that, 0.2 μCi tritiated thymidine (^{3}H-TDR, PerkinElmer^{TM}) was added and incubated for another 24 hours. The T cell proliferation was determined by measuring methy-^{3}H thymidine uptake in a liquid scintillation counter (PerkinElmer^{TM}) as previously described [29]. The proliferation index was calculated using formula, proliferation index = (experimental signal - background) / (negative control signal – background).

$CD4^+/CD8^+$ T -Cell Depletion

Mice were intraperitoneally injected with 150 μg of either anti-CD4 (H129.19, BD Biosciences) or anti-CD8a (53-6.7, BD Biosciences) or both once a day in the first 3 days and then every other day till the end. Ten days after antibody injection, the $CD4^+$ and $CD8^+$ T lymphocytes were verified in one mouse from each group using flow cytometry assay. T lymphocytes were isolated and stained with PE-anti-CD4 and FITC-anti-CD8 (BD Biosciences). The percentages of each cell subset were measured by the Flow Cytometer (FC500MCL, Beckman). One day post depletion, mice were implanted with TC-1 cells and immunized with VR_{111} as previously described. Tumor volumes and animal survival were recorded.

Adoptive Cell Transfer Assay

Donor mice were intramuscularly immunized twice at a 2-week interval. One week after the second immunization, the lymphocytes were isolated as before and 4×10^6 cells were injected to the recipient mice through tail intravenous.

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Recipient mice were implanted TC-1 tumor cells 1 day before. The tumor volumes were followed about 40 days and calculated as previously.

Statistical Analysis

All statistical parameters and significance between groups were calculated using GraphPad Prism 6.0 software (San Diego, CA). Statistical significance was determined by ordinary two-way ANOVA or student *t*-test ($p<0.05$ was considered statistically significant).

RESULTS

Dose-Dependent Anti-Tumor Efficacy of VR_{111}

In our previous report, VR_{111} induced dose dependent E7 epitope specific IFN-γ T-cell responses in mice [24]. Thus, we assumed that VR_{111} was likely to have potent anti-tumor efficacy *in vivo*. We selected the 3 doses and detected the therapeutic efficacy in TC-1 tumor mice model. As showing in Fig. 1A, implanted TC-1 tumor mice were treated twice with three different doses of VR_{111}. The tumor growth of each mouse was monitored up to 46 days. The tumor volumes increased very rapidly when left untreated. However, two immunizations with VR_{111} could significantly inhibit tumor growth in a dose dependent manner (Fig. 1B, D). Meanwhile, the treatment with the higher doses of the vaccine resulted in higher prolonged survival rates compared to the low dose or untreated mice. Importantly, both the 10 μg and 30 μg of the vaccine induced tumor regression in 70% of the treated mice (Fig. 1C, D). These results indicated that VR_{111} vaccine candidate had significant anti-tumor activity in tumor-harboring C57BL/6 mice.

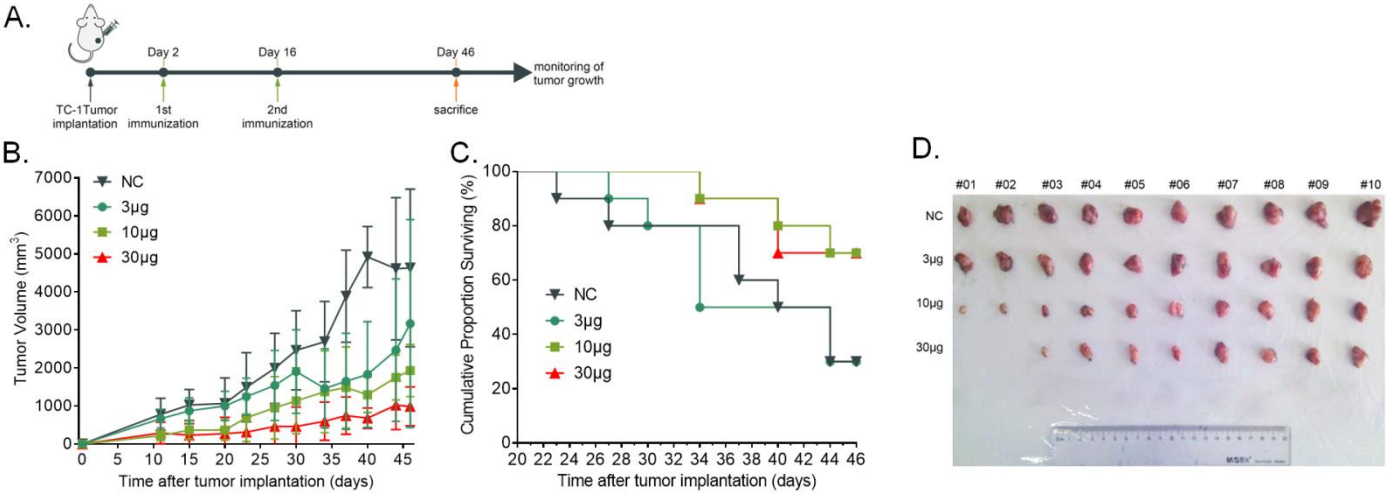

Fig. 1. Dose-dependent therapeutic efficacy of VR_{111} vaccine candidate against TC-1 tumor cells in mice. **(A)** Treatment schedule. C57BL/6 mice were implanted with 2×10^5 cells/mouse on day 0. VR_{111} was intramuscularly injected twice at days 2 and 16. Tumor volume was measured every 3 or 4 days. **(B)** Tumor volumes of mice (n=10) injected with different doses of VR_{111}. The data represent means with standard deviation (SD). **(C)** The Kaplan-Meier plot depicts overall survival. **(D)** Tumor tissues dissected at day 17 after tumor implantation. NC, negative control.

Fusion with HBcAg and HSP65 Enhances Therapeutic Efficacy of VR_{111}

VR_{111} vaccine candidate contains a fusion protein HPV16E7-HBcAg-Hsp65 expressed in *E.coli*. HPV16 E7 and HSP65 proteins were genetically linked to the N and C termi of HBcAg, respectively. Theoretically, the self-assembly of HBcAg could form small particles to enhance the antigenicity and HSP65 could also enhance the antigen presentation. In previous study, the addition of both HBcAg and HSP65 enhanced the E7 specific cellular and humor immune responses [24]. To verify the benefit of fusion protein design in an anti-tumor response context, we compared the efficacy of all the constituents of VR_{111} in TC-1 model as before, either genetically fused or simply mixed. As shown in Fig. 2B, VR_{111} showed the best therapeutic efficacy than any other constituent controls. Interestingly, E7 genetically fused with HSP65 also showed significant tumor growth inhibition (Fig. 2A, B). When we detected the antigen induced CTL lysis to TC-1 target cells or the proliferation capability in vitro, the E7 specific T-cell responses of each group was significantly associated with the tumor growth slowdown (Fig. 2C, D). These data suggest that when genetically fused with HBcAg and HSP65, HPV16 E7 could induce significant improvement in T-cell responses and anti-tumor therapeutic efficacy.

Combination of VR_{111} and Cisplatin Significantly Enhances Treatment Efficacy

Cisplatin is one of the most used regimens in locally advanced cervical cancer and has shown benefits in some combination therapies [4]. We thus hypothesized that combination of VR_{111} and cisplatin could improve the efficacy of anti-tumor immunotherapy. To verify this hypothesis, we evaluated the treatment effects of cisplatin and VR_{111}, either alone or in combination, on tumor growth and survival of TC-1 tumor-harboring mice. While either VR_{111} or cisplatin treatment alone resulted in slight inhibition of tumor growth, the combination treatment showed significant anti-tumor activity as well as a better survival rate (Fig. 3). These results indicate that combination of VR_{111} with cisplatin treatment could be considered as a therapeutic strategy.

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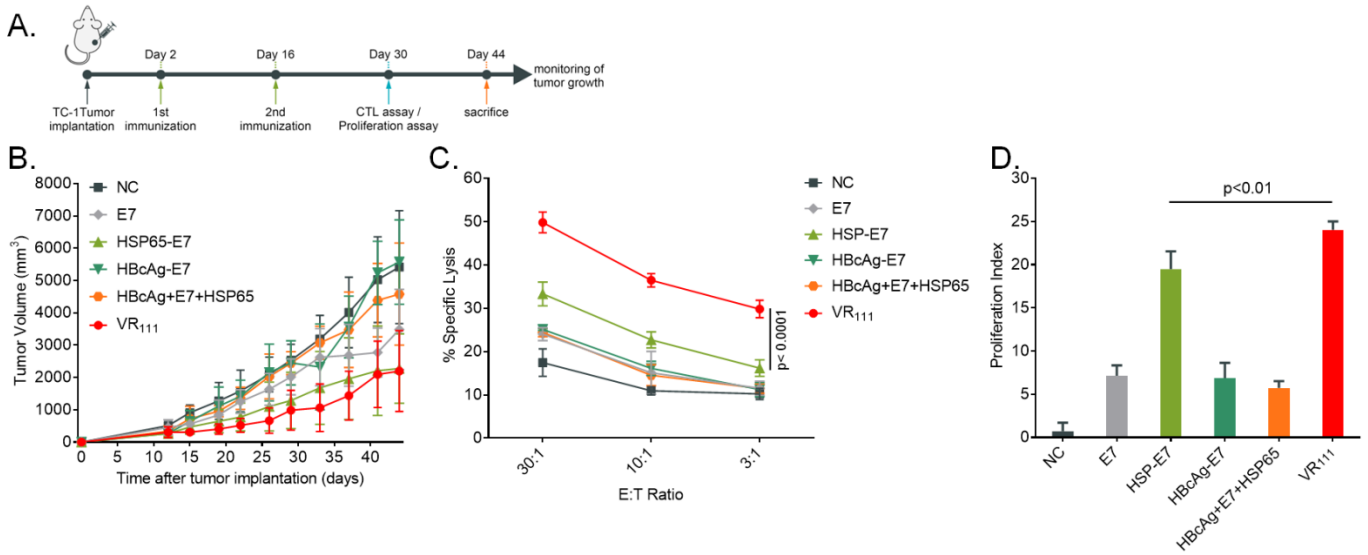

Fig. 2. Fusion of HBcAg and HSP65 enhances the therapeutic efficacy of VR_{111} vaccine candidate. **(A)** Treatment schedule. TC-1 tumor mice were treated with 10μg of VR_{111} or its constituents 2 days after tumor implantation. **(B)** Tumor volumes (means ± SD) of mice (n=10) treated with VR_{111} or its constituent controls. **(C)** Specific lysis percentages of CTL induced by different VR_{111} treatments under 3 different ratios of effector to target cells. Effector cells, antigen specific T cells. Target cells, TC-1 cells. **(D)** Proliferation index of E7 epitope specific T cells in vitro. The error bar indicates the mean with SD (n=4).

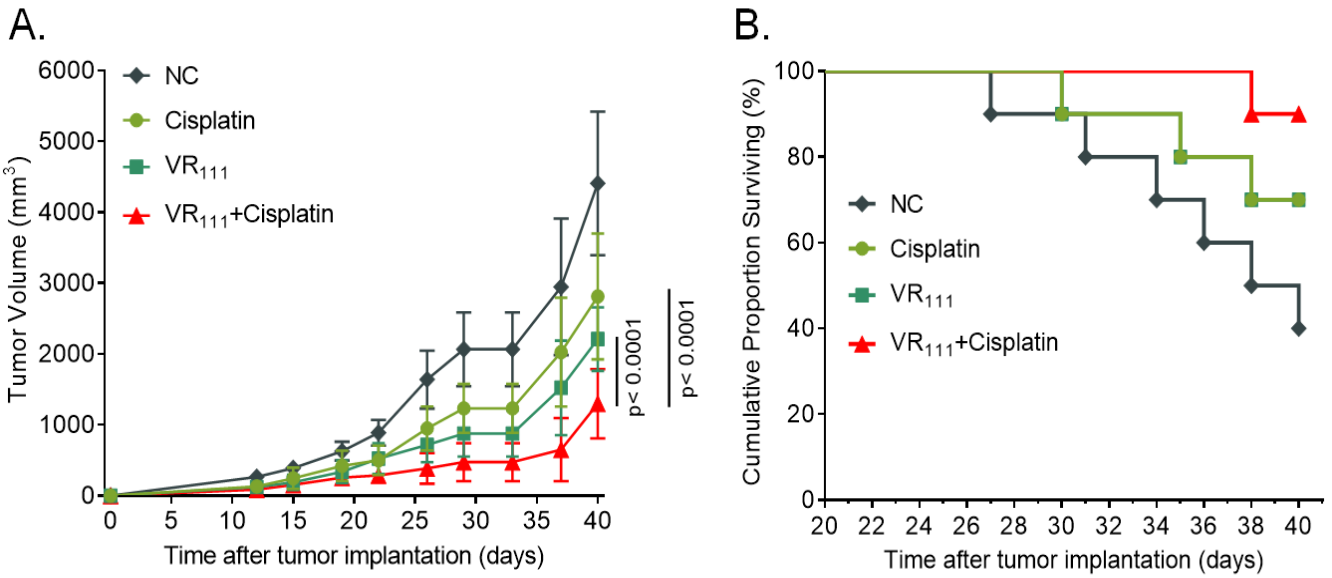

Fig. 3. Combination therapy of cisplatin and VR_{111} vaccine candidate enhances effect of anti-tumor treatment. **(A)** The TC-1 tumor-harboring C57BL/6 mice (n=10) were treated with VR_{111} (10 μg) alone or combined with cisplatin chemotherapy (5 mg/kg). Tumor volumes were recorded every 3-4 days. The error bar indicates the mean with SD. **(B)** The Kaplan-Meier plot depicts overall survival of the mice in each treatment group.

The Anti-Tumor Potency of VR_{111} Was Mediated by $CD8^+$ T-Cells

To further investigate the mechanism of immunotherapy induced by VR_{111} vaccination, we depleted $CD4^+$ and/or $CD8^+$ T cells by i.p. injection of anti-CD4 and anti-CD8a antibodies. Next, the implanted TC-1 tumor C57BL/6 mice were injected twice with VR_{111} as before (Fig. 4A). The depletion efficiency was verified using flow cytometry. Nearly, all $CD4^+$ and/or $CD8^+$ T cells were depleted in vivo (Fig. 4B). In tumor treatments, only the mice with $CD8^+$ T cells showed significant better anti-tumor responses and survival rates (Fig. 4C, 4D). These data demonstrate that the $CD8^+$ T cells play the major role in VR_{111}-induced cellular immune responses against TC-1 tumor.

VR_{111} Mediated Adoptive Lymphocytes Transfer Anti-Tumor Therapy

Since VR_{111} inhibited tumor growth by inducing specific T-cell immune responses, adoptive T cell transfer could also be performed to confirm its anti-tumor effect. Therefore, we isolated the lymphocytes from mice immunized with VR_{111} or E7 alone and then transferred the lymphocytes into TC-1 tumor implanted mice through tail intravenous injection (Fig. 5A). As shown in Fig. 2, VR_{111} vaccine could significantly induce a higher T-cell response than E7 treatment. Likewise, VR_{111} showed better tumor growth inhibition compared to E7 alone ($p < 0.05$) and the negative treatment (saline) control ($p < 0.001$). This indicated that the efficacy of adoptive cell transfer therapy

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was highly associated with the antigen-specific cellular immune responses (Fig. 5B).

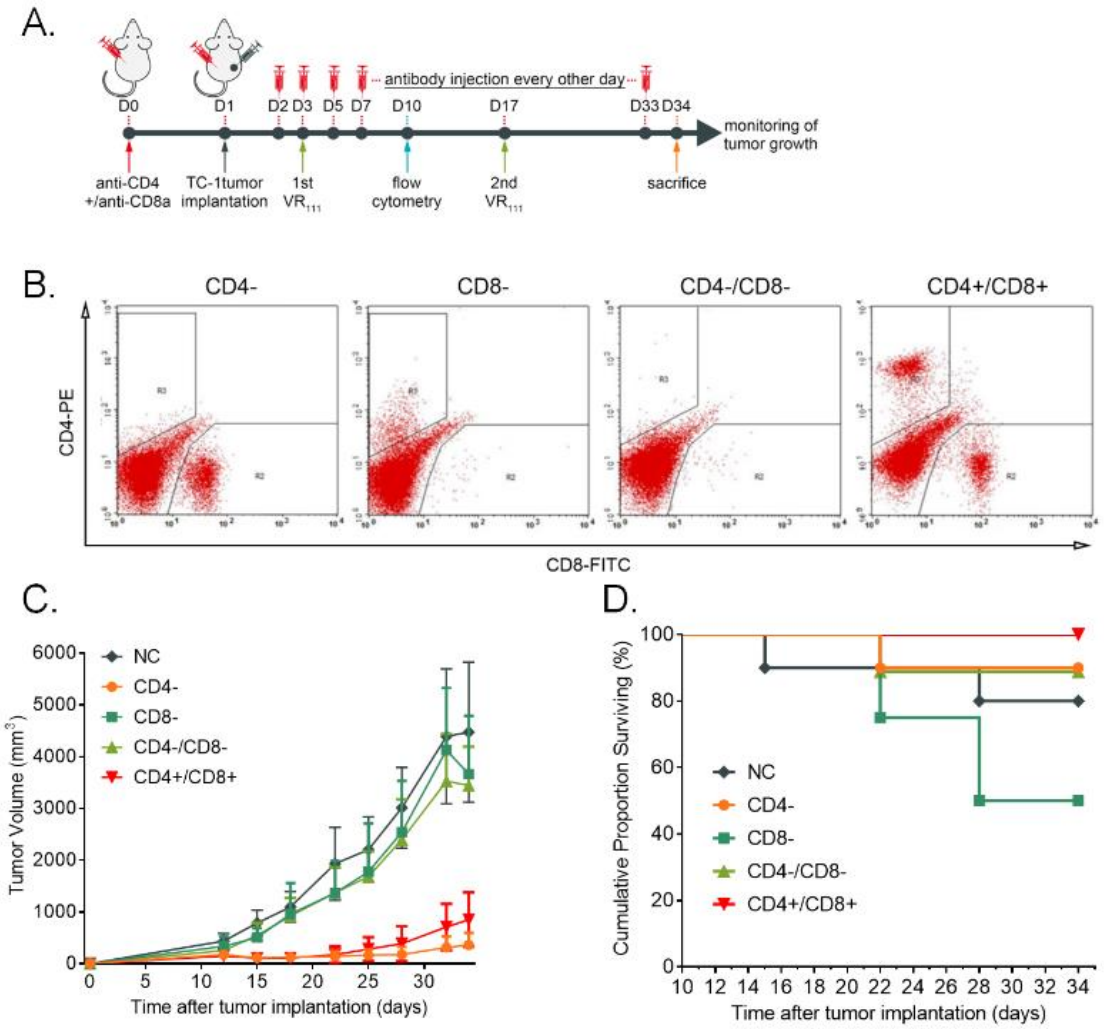

Fig. 4. CD8$^+$ T-cells played a major role in VR_{111} induced immune responses against TC-1 tumor cells. **(A)** Treatment schedule. Mice were intraperitoneally injected with 150 µg of either anti-CD4 antibody or anti-CD8a antibody or both once a day in the first 3 days and then every other day till the end. One day post depletion, mice were implanted with TC-1 cells and immunized with VR_{111} as previously described. **(B)** CD4$^+$ and/or CD8$^+$ T cells subsets depletion verification by flow cytometry. **(C)** Tumor volumes of mice (n=8~10) depleted with either CD4$^+$ or CD8$^+$ T cells or both and then followed with previous described tumor implantation and VR_{111} treatment. The data represent means with SD. **(D)** The Kaplan-Meier plot depicts the overall survival of the mice.

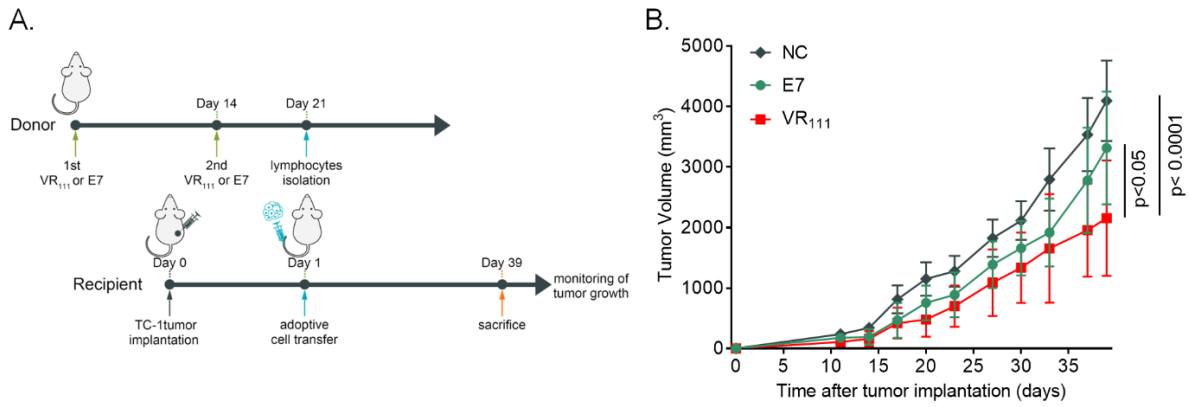

Fig. 5. Adoptive lymphocytes transfer anti-tumor therapy of VR_{111}. (A) Treatment schedule. Lymphocytes from VR_{111} immunized the donor mice (n=4) were isolated and injected into the recipient TC-1 implanted mice (n=10). (B) Tumor volumes of the recipient mice measured were every 3-4 days. The data represent means with SD.

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DISCUSSION

Although the implementation of prophylactic HPV vaccines may gradually decrease the incidence of cervical cancer or high-grade squamous intraepithelial lesion (HSIL) resulted from high-risk HPV (hrHPV) infections in the future, cervical cancer is still one of the leading causes of cancer deaths for women in more than 30 countries [3]. HPV16 remains among the most prevalent hrHPV genotypes of cervical lesions [30]. The progression of cervical cancerogenesis results from hrHPV gene integration, following by sustained expression of nonstructural proteins E6 and E7, which lead to uncontrolled cellular proliferation by mediating the degradation of p53 and pRB [2]. The highly significant expression of E7 in clinically- malignant lesions of the uterine cervix, makes E7 antigen an ideal tumor-associated antigen for HPV therapeutic vaccine development [31]. HPV16 E7-based therapeutic vaccines, either alone or combined with E6 or L2 protein, have been evaluated in clinical trials. A covalently linked HPV16 E7 to Hsp65 (a therapeutic vaccine candidate; HspE7) was previously shown to prime a potent E7-specific $CD8^+$ T cell response and showed moderate therapeutic efficacy in treatment of CIN3[32, 23]. Novel adjuvants including imiquimod, GPI-0100 or CpG have been evaluated in E7-based recombinant protein vaccines to enhance the antigen specific T cell responses[33-35]. Moreover, the self-assembly and high degree of immunogenicity of hepatitis B virus core protein has already been used as an antigen carrier and shown protective efficacy in animal models of malaria, influenza, Lyme disease as well as TC-1 tumor mice model when carrying HPV 16 E7 epitopes[20, 36]. Therefore, we assayed a previously-constructed therapeutic vaccine candidate (VR_{111}), formulated with HPV16E7-HBcAg-Hsp65 fusion protein and aluminum hydroxide adjuvant. In our previous investigation, VR_{111} was able to form macromolecules with a z-average radius of about 46.04 nm and induce significant E7-specific cellular and humoral immune responses in mice [24].

In this study, the anti-tumor activity of VR_{111} vaccine was evaluated in TC-1 tumor-bearing mice. The results indicated potent therapeutic efficacy of VR_{111} vaccine candidate which was associated with E7 specific $CD8^+$ T cell immune response. Since cisplatin alone or in combination with radiation is still considered as the standard effective treatment for patients with advanced or recurrent cervical cancer in many, other combinations with vaccines or monoclonal antibodies are also attractive combination therapies[37]. Our results also indicated that a combination treatment with VR_{111} and cisplatin could enhance the overall antitumor effects. Moreover, an adoptive cell transfer therapy showed significant tumor growth inhibition in our animal model.

Nevertheless, there are some limitations for this study. The anti-tumor effects might be limited to the murine model, based on the transformed TC-1 cell line and not due to a natural hrHPV persistent infection. In the present design of VR_{111} vaccine candidate, E7 was chosen to be the only target tumor-associated antigen. However, since E6 and E7 are the induction factors of the cervical cancer progression, therapeutic vaccines targeting both E6 and E7 might be more efficient [38]. Furthermore, more adjuvants other than aluminum hydroxide could be screened; as Poly (I : C) or CpG oligodeoxynucleotide adjuvants formulated with HPV synthetic peptides are shown to enhance the specific $CD8^+$ T cell responses and tumor growth inhibition [39, 40].

Similar to other therapeutic vaccine-based combination treatments, such as PD-1 immune checkpoint blockade, VR_{111} vaccine candidate, combined with the anticancer drug “cisplatin" demonstrated better anti-tumor response with synergistic effects [41]. Our $CD4^+$ and/or $CD8^+$ T-cells depletion experiment indicated that CD8+ T cells plays a major role in VR111-induced tumor growth inhibition. Previous results from an adoptive lymphocyte transfer therapy experiment has also confirmed the T-cell mediated cancer immunotherapy and suggested potential optimized adoptive cell transfer therapy using in vitro amplified antigen specific T cells [42].

Altogether, VR_{111} as a therapeutic vaccine candidate showed efficient anti-tumor effects and increased survival rates in tumor-harboring C57BL/6 mice by improving treatment dose and cellular immune responses. These results suggest that VR_{111} is a promising candidate for cervical cancer immunotherapy.

ACKNOWLEDGMENT

This work was supported by National Science and Technology of China (no. 2009ZX09103-611), the National Great Science and Technology project (no. 2013ZX09102-059) and the Shanghai Science and Technology Committee (no. 0902H111900). We also appreciated the support of Donghou Li and his crew from the Experimental Laboratory of Pathology, Institute of Radiation Medicine, Academy of Military Medical Sciences, China.

CONFLICTS OF INTEREST

All the authors are current employees of Shanghai Zerun Biotechnology Co., Ltd.

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