Efficient Delivery of DNA Vaccines using Human Papillomavirus Pseudovirions

Shiwen Peng[1], **Archana Monie**[1], **Tae Heung Kang**[1], **Chien-Fu Hung**[1], **Richard Roden**[1,2,4], and **T.-C. Wu**[1,2,3,4]

[1] Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[2] Department of Obstetrics and Gynecology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[3] Department of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

[4] Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA

Abstract

We have examined non-replicative human papillomavirus (HPV) pseudovirions as an approach in the delivery of naked DNA vaccines without safety concerns associated with live viral vectors. In the current study, we have generated HPV-16 pseudovirions encapsidating a DNA vaccine encoding the model antigen, ovalbumin (OVA) (HPV16-OVA pseudovirions). Vaccination with HPV16-OVA pseudovirions subcutaneously elicited significantly stronger OVA-specific CD8+ T cell immune responses compared to OVA DNA vaccination via gene gun in a dose-dependent manner. We showed that a single amino acid mutation in the L2 minor capsid protein that eliminates the infectivity of HPV16-OVA pseudovirion significantly decreased the antigen-specific CD8+ T cell responses in vaccinated mice. Furthermore, a subset of CD11c+ cells and B220+ cells in draining lymph nodes became labeled upon vaccination with FITC-labeled HPV16-OVA pseudovirions in injected mice. HPV pseudovirions were found to infect bone marrow-derived dendritic cells (BMDCs) in vitro. We also showed that pretreatment of HPV16-GFP pseudovirions with furin leads to enhanced HPV16-OVA pseudovirion infection of BMDCs and OVA antigen presentation. Our data suggest that DNA vaccines delivered using HPV pseudovirions represent an efficient delivery system that can potentially impact the field of DNA vaccine delivery.

Keywords

human papillomavirus; pseudovirions; vaccine

Address correspondence to: Dr. T.-C. Wu, Department of Pathology, School of Medicine, Johns Hopkins University, CRBII Room 309, 1550 Orleans Street, Baltimore, Maryland 21231, USA. Phone: (410) 614-3899; Fax: (443) 287-4295; wutc@jhmi.edu.

Introduction

Vaccination has proven to be the most cost-effective method to combat infectious disease, to improve health and to extend life expectancy. Nevertheless chronic infections still exact a terrible toll globally, and it is now clear that infectious agents cause 15–20% of all cancer cases 1. Many chronic viral infections including human papillomavirus (HPV) and Hepatitis B virus (HBV), Epstein-Barr virus (EBV), Kaposi's sarcoma virus (KSV), herpes simplex virus (HSV), Hepatitis C virus (HCV), etc. likely require a potent virus-specific cytotoxic T cell response for clearance. Current subunit vaccines and killed vaccines are safe and effective in inducing neutralizing antibodies and in preventing many new infections, but they have generally not proven effective in generating T cell responses capable of clearing chronic viral infections (for review see 2). On the other hand, live viral vectors are capable of inducing potent cytotoxic T cell immune responses, but they raise significant concerns related to safety.

Genetic vaccination, in which the antigen is expressed within the host cells, has emerged as a potentially promising approach to generate antigen-specific T cell-mediated immune responses. The current paradigm for genetic vaccination is the employment of naked nucleic acid vaccines. This approach has shown great promise in numerous animal models over the last decade, and these vaccines are very stable, simple, inexpensive to manufacture, and thus far safe. However, naked nucleic acid vaccines have generally demonstrated lesser immunogenicity in patients, as compared to animal models, both in our clinical trials using naked DNA vaccines targeting the human papillomavirus E7 antigen 3, and in trials by numerous other groups targeting other antigens (for review see 4). It has become apparent that the poor immunogenicity of naked nucleic acid vaccines in patients likely reflects inefficient delivery of the antigenic gene to professional antigen-presenting cells, most critically dendritic cells (DCs). Thus, an approach capable of efficiently delivering nucleic acid vaccines into dendritic cells of patients to trigger cytotoxic T cell immunity, without the safety concerns related to live vectors, could potentially revolutionize vaccination against chronic infections.

An emerging mode of delivery for DNA vaccines is the employment of non-replicative viral particles, or pseudovirions that exploit viral capsids to facilitate the delivery of the vaccine. Recent advances enable the packaging of DNA plasmids into the papillomavirus capsid proteins to generate a 'pseudovirion' that can efficiently deliver the DNA plasmid into the infected cells, resulting in the expression of the encoded gene. Furthermore, because pseudovirions do not contain the viral genome, they lack many of the safety concerns associated with live viral vectors. HPV pseudovirions carrying a marker gene have been shown to be capable of efficiently infecting dendritic cells. In addition, recent advances in technology related to the generation of high-titer HPV pseudovirions have enabled us to generate large quantities of pseudovirions for cutaneous vaccination 5. Thus, gene delivery using the papillomavirus pseudovirion system represents a potentially promising non-viral gene delivery system to trigger potent immune responses against viral infections and cancer.

In the current study, we have used the HPV pseudovirion system to explore the possibility of in vivo delivery of DNA vaccines in vivo. We have generated HPV-16 pseudovirions

encapsidating DNA encoding ovalbumin (OVA) (HPV16-OVA pseudovirions). We chose OVA because it is a commonly used model antigen with well-characterized available immunological assays. Our data suggest that DNA vaccines delivered using HPV pseudovirions represent an efficient delivery system that can potentially impact the field of DNA vaccine delivery.

Results

Vaccination with HPV-16 pseudovirions containing OVA DNA elicits strong OVA-specific CD8+ T cell immune responses in a dose-dependent manner

In order to determine if OVA-specific CD8+ T cell immune responses may be generated by vaccination with HPV-16 pseudovirions containing OVA DNA (HPV16-OVA pseudovirions), C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA pseudovirions or HPV16-pcDNA3 pseudovirions at a dose of 5μg L1 protein/mouse via subcutaneous injection. All mice were boosted 7 days later with the same regimen. One week after last vaccination, splenocytes were prepared and stimulated with OVA peptide and then analyzed for OVA-specific CD8$^+$ T cells by intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 1A and B, mice vaccinated with HPV16-OVA pseudovirions generated significantly higher number of OVA-specific CD8+ T cell immune responses compared to mice vaccinated with the control HPV16-pcDNA3 pseudovirions. We did not detect significant OVA-specific CD4+ T cell immune responses in mice vaccinated with HPV16-OVA pseudovirions or HPV16-pcDNA3 pseudovirions (See Supplementary Figure 1). We also examined the OVA-specific antibody responses in mice vaccinated with HPV16-OVA pseudovirions over time. We found that mice vaccinated with HPV16-OVA pseudovirions did not generate detectable levels of OVA-specific antibody responses (See Supplementary Figure 2). Thus, our data indicate that subcutaneous vaccination with HPV-16-OVA pseudovirions effectively presents OVA via MHC class I to generate significant OVA-specific CD8+ T cell immune responses. We also checked the serum titer of HPV-16 neutralizing antibodies in vaccinated mice. We found that the HPV16 neutralizing antibodies could be detected 7 days after the initial vaccination and was significantly elevated 2 weeks after the initial vaccination (See Supplementary Figure 3).

The induction of HPV-specific neutralizing antibodies by the priming dose of pseudovirions may limit the potency of the subsequent booster dose. One way to eliminate this concern is by boosting with pseudovirion derived from a different HPV type, since HPV neutralizing antibodies are primarily type-restricted. We therefore compared the OVA-specific CD8+ T cell immune responses generated by prime-boost vaccination with the same type of pseudovirions (homologous vaccination) with prime-boost vaccination with different types of pseudovirions (heterologous vaccination). C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA pseudovirions via subcutaneous (footpad) injection. 7 days later, one group was boosted with HPV16-OVA pseudovirions (homologous vaccination), and another group was boosted with HPV18-OVA pseudovirions (heterologous vaccination). One week after last vaccination, splenocytes from vaccinated mice were isolated and analyzed for OVA-specific CD8$^+$ T cells by intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 2A and B, mice vaccinated with HPV-16-OVA pseudovirions

by homologous vaccination generated similar number of OVA-specific CD8+ T cell immune responses compared to mice vaccinated by heterologous vaccination. Thus, our data indicate that homologous vaccination with HPV-16-OVA pseudovirions generates comparable OVA-specific CD8+ T cell immune responses compared to heterologous vaccination with different type of HPV pseudovirions when performed one week apart.

In order to determine the dose response of OVA-specific CD8+ T cell immune responses induced by vaccination with HPV16-OVA pseudovirions, C57BL/6 mice (5 per group) were vaccinated with increasing doses of HPV16-OVA pseudovirions (0.1, 0.5, 1, 2.5, 5 μg) via subcutaneous injection. All mice were boosted 7 days later with the same regimen. One week after last vaccination, splenocytes from vaccinated mice were isolated and analyzed for OVA-specific CD8$^+$ T cells by intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 3A and B, mice vaccinated with the highest dose of HPV-16-OVA pseudovirions generated the highest number of OVA-specific CD8+ T cell immune responses. Thus, our data indicate that the level of OVA-specific CD8+ T cell immune responses increased with increasing dose of HPV16-OVA pseudovirion vaccination.

The infectivity mediated by the L2 minor capsid protein on the HPV16-OVA pseudovirion is essential for the generation of antigen-specific CD8+ T cell responses in vaccinated mice

L2 minor capsid protein has been shown to be crucial for the infection of cells by papillomavirus pseudovirions [6,7]. In order to determine if infection mediated by L2 plays an essential role in the generation of antigen-specific CD8+ T cell immune responses in mice vaccinated with HPV16 pseudovirions, we have generated a HPV16-OVA pseudovirion which has a single amino acid mutation (amino acid 28 from Cysteine to Serine) in the L2 protein of the pseudovirion (HPV16L1mtL2-OVA pseudovirion), which abolishes the infectivity of pseudovirions [7]. 293-Kb cells were infected with HPV16L1L2-OVA or the mutant HPV16L1mtL2-OVA pseudovirus, incubated with OVA-specific CD8+T cells and then analyzed by intracellular IFN-γ staining. As shown in Figure 4A, 293-Kb cells infected with L2 mutated HPV16-OVA pseudovirus demonstrated significant reduction in their ability to activate OVA-specific CD8+ T cells compared to cells infected with wild-type HPV16-OVA pseudovirus. This data suggests that an intact L2 is essential for infection of 293-Kb cells by pseudovirion to lead to MHC class I presentation of OVA antigen.

In order to determine if the intact L2 in the pseudovirions is essential for the generation of antigen-specific CD8+ T cell immune responses in vaccinated mice, C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA pseudovirions or the mutant HPV16L1mtL2-OVA pseudovirions via footpad injection. All mice were boosted 7 days later with the same regimen. 1 week after last vaccination, splenocytes were prepared and stimulated with OVA peptide and analyzed for OVA-specific CD8$^+$ T cells by intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 4B and C, mice vaccinated with the mutant HPV16L1mtL2-OVA pseudovirions generated significantly decreased number of OVA-specific CD8+ T cell immune responses compared to mice vaccinated with the wild type HPV-16L1L2-OVA pseudovirions. Taken together, our data indicate that the infectivity

of the HPV pseudovirions mediated by the intact L2 is essential for their ability to generate antigen-specific CD8+ T cell immune responses in vaccinated mice.

Vaccination with HPV-16 pseudovirions containing OVA DNA leads to strong protective antitumor effects against OVA-expressing tumors in vaccinated mice

In order to assess the cytotoxic activity of OVA-specific CD8+ T cell immune responses generated by vaccination with HPV16-OVA pseudovirions, C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA or HPV16-pcDNA3 via footpad injection. The mice were boosted twice with the same regimen at day 7 and day 14. One week after last vaccination, the mice were injected with B16-OVA cells subcutaneously. Tumor growth was monitored twice a week. As shown in Figure 5A, mice vaccinated with HPV16-OVA pseudovirions demonstrated significantly higher percentage of tumor-free mice compared to mice vaccinated with HPV16-pcDNA3 pseudovirions. For antibody depletion of specific immune cell subsets, the mice were treated with antibodies against mouse CD4, CD8 and NK1.1 at the same time of last vaccination via intraperitoneal injection. Depletion of CD8+ T cells in mice vaccinated with HPV16-OVA pseudovirions significantly lowered the percentage of tumor-free mice compared to vaccinated mice with CD4 or NK1.1 depletion or no depletion (Figure 5B). Thus, our data indicate that vaccination with HPV-16 pseudovirions containing OVA DNA leads to strong protective antitumor effects against B16-OVA tumors in vaccinated mice and that CD8+ T cells play a major role in the antitumor effects.

Vaccination with HPV16-OVA pseudovirions elicits significantly stronger OVA-specific CD8+ T cell immune responses compared to intradermal vaccination with naked OVA DNA

Intradermal vaccination with naked DNA via needles or gene gun routes of administration are used to generate potent antigen-specific immune responses by naked DNA vaccines in preclinical and clinical studies 8 (For review see 9). In order to compare the OVA-specific immune responses generated by HPV16-OVA pseudovirion vaccination with intradermal vaccination with naked OVA DNA, C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA pseudovirions via subcutaneous injection or with pcDNA3-OVA DNA via gene gun. All mice were boosted 7 days later with the same dose and regimen. One week after last vaccination, splenocytes from vaccinated mice were isolated and analyzed for OVA-specific CD8$^+$ T cells by intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 6A and B, mice vaccinated with HPV16-OVA pseudovirions generated significantly higher number of OVA-specific CD8+ T cell immune responses compared to mice vaccinated with naked OVA DNA vaccination. Thus, our data indicate that vaccination with HPV16-OVA pseudovirions generates a significantly higher number of OVA-specific CD8+ T cell immune responses than vaccination with naked OVA DNA.

HPV pseudovirions can efficiently infect bone marrow derived dendritic cells in vitro and can be taken up by CD11c+ and B220+ cells in the draining lymph nodes of vaccinated mice

In order to determine if HPV pseudovirions can infect bone marrow derived dendritic cells (BMDC), we cultured BMDCs in the presence of GM-CSF for 4 days and added HPV16 pseudovirions containing DNA encoding GFP or OVA to the culture. After 72 hours,

BMDCs were harvested and GFP expression was examined by flow cytometry analysis. As shown in Figure 7A, a significant percentage of CD11c+ bone marrow-derived dendritic cells infected with pseudovirions containing GFP DNA, but not OVA DNA, demonstrated GFP expression.

In order to determine whether mice vaccinated with HPV16 pseudovirions containing GFP can lead to the expression of GFP in the draining lymph nodes, C57BL/6 mice (5 per group) were vaccinated with HPV16 pseudovirions carrying GFP or OVA DNA via footpad injection. After 72 hours, draining lymph nodes were harvested, total RNA was isolated and RT-PCR was performed to detect GFP mRNA expression. As shown in Figure 7B, mice vaccinated with HPV16 pseudovirions carrying GFP DNA, but not pseudovirions carrying OVA DNA demonstrated detectable expression of GFP in draining lymph nodes.

In order to further determine the type of cells that can carry HPV16-OVA pseudovirions into draining lymph nodes, we conjugated HPV16-OVA pseudovirions with FITC and injected the labeled pseudovirions into C57BL/6 mice via subcutaneous injection. The draining lymph nodes of the injected mice were harvested after 48 hours and the presence of FITC-labeled pseudovirions within the cells in the draining lymph nodes was analyzed by flow cytometry. As shown in Figure 7C and D, the B220+ cells and CD11c+ cells in draining lymph nodes demonstrated significant percentage (2.27% CD11c+ cells and 0.24% B220+ cells) of the FITC+ cells indicating uptake of the HPV16-OVA pseudovirions. Thus, our data indicate that dendritic cells in draining lymph nodes can significantly uptake FITC-labeled HPV16-OVA pseudovirions and a subset of B220+ cells in draining lymph nodes can uptake FITC-labeled HPV16-OVA pseudovirions to a lesser extent.

Taken together, our data suggest that HPV pseudovirions can efficiently infect bone marrow derived dendritic cells in vitro. Furthermore, administration of HPV pseudovirions *in vivo* can lead to the uptake of pseudovirions by CD11c+ cells and B220+ cells in draining lymph nodes, resulting in the expression of the encoded protein.

Treatment of HPV16 pseudovirions with furin leads to enhanced pseudovirion infection and improved antigen presentation in infected cells

Several previous studies have implicated furin in the process of papillomavirus infection 7,10–12. It was recently found that infectious entry of papillomaviruses is dependent upon the cleavage of the L2 protein by furin (for review see 13). Thus, in order to determine if HPV16 pseudovirion infection can be enhanced by pretreatment with furin, DC-1 cells were infected with HPV16-GFP pseudovirions with or without pretreatment with furin. The infection of DC-1 cells by HPV16-GFP pseudovirions was analyzed by characterization of GFP expression in DC-1 cells using flow cytometry. As shown in Figure 8A, DC-1 cells infected with HPV16-GFP pseudovirions in the presence of furin demonstrated significantly higher percentage of GFP+ cells compared to DC-1 cells infected with HPV16-GFP pseudovirions without furin. Thus, our data indicate that treatment of HPV16 pseudovirions with furin leads to enhanced pseudovirion infection.

In order to determine if the enhanced pseudovirion infection can be translated into improved antigen presentation in the infected cells, DC-1 cells were infected with HPV16-OVA

pseudovirions with or without the treatment with furin. The infected cells were collected 72 hours after infection, and co-cultured with OVA-specific CD8+ OT-1 T cells (E:T ratio at 1:1) overnight. Activation of OT-1 T cells was analyzed by IFN-γ intracellular staining followed by flow cytometry analysis. As shown in Figure 8B, cells infected with HPV16-OVA pseudovirions in the presence of furin demonstrated significantly higher percentage of activated IFNγ-secreting CD8+ T cells compared to cells infected HPV16-OVA pseudovirions without furin. This indicates that treatment of HPV16 pseudovirions with furin leads to enhanced antigen presentation in the infected cells. Thus, our data suggest that treatment of HPV16 pseudovirions with furin leads to enhanced pseudovirion infection of DC-1 cells, resulting in improved antigen presentation in infected cells.

In order to determine if furin pretreatment leads to enhanced antigen presentation, producing a stronger immune response, C57BL/6 mice were vaccinated with HPV16-OVA pseudovirions with or without furin treatment. All mice were boosted 7 days later with the same dose and regimen. One week after last vaccination, splenocytes were prepared and stimulated with OVA peptide and analyzed for OVA-specific CD8$^+$ T cells by intracellular cytokine staining followed by flow cytometry analysis. As shown in Figure 8C, the difference in the OVA-specific CD8+ T cell immune responses generated in mice vaccinated with HPV16-OVA pseudovirions treated with furin compared to mice vaccinated with HPV16-OVA pseudovirions without furin treatment was not statistically significant (p=0.1057). Taken together, although treatment of HPV16 pseudovirions with furin led to enhanced pseudovirion infection and improved antigen presentation in DC-1 cells, it did not significantly increase the OVA-specific CD8+ T cell immune responses in vaccinated mice.

Discussion

In the current study, we found that vaccination with HPV16-OVA pseudovirions elicits strong OVA-specific CD8+ T cell immune responses in a dose-dependent manner. Our data also indicate that pseudovirions are also capable of infecting a subset of bone marrow derived dendritic cells. In addition, vaccination with HPV16-OVA pseudovirions was found to elicit significantly stronger OVA-specific CD8+ T cell immune responses compared to OVA DNA vaccination administered intradermally via gene gun. Our data suggest that DNA vaccines delivered by pseudovirions may represent an opportunity to improve DNA vaccination that may be suitable for different antigenic systems.

The current technology using pseudovirions to deliver DNA vaccines is quite different from previously employed delivery systems using virus-like particles (VLPs) to deliver DNA. The delivery of DNA using VLPs would require either the binding of DNA or the in vitro assembly of DNA within the VLPs 14–18. These approaches in general may not require the minor capsid protein L2 or infection by papillomavirus particles for gene delivery. In comparison, the delivery of DNA using current pseudovirion technology will require the packaging of DNA by the papillomavirus capsid protein within the cell and requires the inclusion of L2 protein for efficient infection of target cells (See Figure 4). It will be of interest to explore the efficiency of gene delivery using the current delivery approach compared to the other papillomavirus particle-related delivery systems.

In our study, we observed that in some of the experiments (such as Figures 1, 3 and 6) the absolute number of OVA-specific CD8+ T cells was quite variable among the different sets of experiments while the mice received the same dose and regimen of the pseudovirion vaccination. Since the different sets of experiments were performed at different times and with different conditions, some degree of variation may occur in these experiments. Meanwhile, the standard error within each set of experiments was low (For example, Figures 1B and 3B). Thus, the data from the different groups within each set of experiments is consistent and comparable. Thus, we believe that our results are reproducible and support our conclusions.

Our data demonstrated that infection is essential for the ability of HPV pseudovirions to generate potent antigen-specific CD8+ T cell immune responses in vaccinated mice (Figure 4). The minor capsid protein, L2 has been shown to play an essential role in papillomavirus infection. Previously, it has been shown that a single amino acid from Cys to Ser can lead to significant reduction of infection without impacting virion assembly 6. This reduced infectivity caused by the mutated L2 presumably causes a significant reduction in the number of infected cells as well as generation of the encoded antigen, resulting in poor immunogenicity of the DNA vaccine delivery by pseudovirions.

The current study uses HPV type 16 for the development of pseudovirions. However, many individuals may have been previously infected or vaccinated with HPV type 16, which may preclude repeated vaccination with HPV-16 pseudovirions. Fortunately, there are several other types of human papillomaviruses and papillomaviruses from different species such as bovine papillomavirus (BPV) that can potentially be used for the preparation of pseudovirions for the delivery of DNA vaccines. Thus, there is a wide spectrum of different types of papillomaviruses that might be used to enhance the delivery of DNA vaccines even among those patients who have received the current HPV vaccines, assuming that the tropism of these other types is not distinct from HPV16.

Our data showed that homologous vaccination with HPV16-OVA pseudovirions elicits comparable OVA-specific CD8+ T cell immune responses compared to heterologous vaccination with HPV16-OVA and HPV18-OVA pseudovirions (Figure 2). We observed that vaccination with HPV-16 pseudovirions was capable of generating detectable levels of HPV-16 L1 VLP-specific neutralizing antibodies one week after the initial vaccination (at the time of booster) (See Supplementary Figure 3). The fact that homologous and heterologous vaccination generated comparable levels of OVA-specific CD8+ T cell immune responses suggests that the presence of a low level of L1 VLP-specific neutralizing antibodies does not compromise the boost of the antigen-specific CD8+ T cell immune responses generated by booster with homologous HPV pseudovirions. One possible explanation is that the low level of antibodies may not be sufficient to neutralize the booster of the same HPV pseudovirion. Another potential explanation is that the binding of neutralizing antibodies to the HPV pseudovirions does not compromise the delivery of the DNA vaccine by HPV pseudovirions. It will be important to determine the mechanism for the observed phenomenon in future studies. Nevertheless, homologous vaccinations with HPV pseudovirions are more practical for future clinical translation since it would require the manufacture of only one reagent.

We also showed that pretreatment of HPV16-GFP pseudovirions with furin leads to enhanced pseudovirion infection and improved antigen presentation of the infected cells (Figure 8). However, we showed that treatment of HPV16-GFP pseudovirions with furin only slightly increases the OVA-specific CD8+ T cell immune responses in vaccinated mice. One potential explanation is that the extracellular furin levels are diluted by the culture medium, whereas in vivo the endogenous furin may be able efficiently render the HPV pseudovirions equally infective compared to pseudovirions treated with furin before vaccination. A better understanding of the role of various factors involved in pseudovirion infection such as furin may provide an insight into the mechanism of HPV pseudovirion infection, creating opportunities to improve vaccine potency using HPV pseudovirions.

Materials and Methods

Mice

C57BL/6 mice (5- to 8-week-old) were purchased from the National Cancer Institute (Frederick, MD). OT-1 transgenic mice on C57BL/6 background were purchased from Taconic. All animals were maintained under specific-pathogen free conditions, and all procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Peptides, antibodies and regents

The H-2K^b-restricted Ovalbumin (OVA) peptide, SIINFEKL was synthesized by Macromolecular Resources (Denver, CO) at a purity of 80%. FITC-conjugated rat anti-mouse IFN-γ, PE-conjugated anti-mouse CD8, PE-Cy5 conjugated anti-mouse B220 and APC-conjugated anti-mouse CD11c antibodies were purchased from BD Pharmingen (BD Pharmingen, San Diego, CA). A horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin G(IgG) antibody was purchased from Zymed (San Francisco, CA). OVA protein was purchased from Sigma.

Cells

293TT cells were kindly provided by J. Schiller (NCI, NIH) 19. These cells were generated by transfecting 293T cells with an additional copy of the SV40 large T antigen. Murine melanoma cell line, B16 expressing OVA was described before 20. Both cell lines were grown in complete Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Gemini Bio-Products). The immortalized DC line was provided by Dr. K. Rock (University of Massachusetts, Worcester, MA) 21. With continued passage, we have generated subclones of the DC line, DC-1 that is easily transfected using Lipofectamine 2000 (Invitrogen) 22. EG.7 cell line, derived from murine EL4lymphoma cell transfected with OVA-expressing vector was purchased from ATCC. Both DC-1 and EG.7 cells were cultured in complete RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. OVA peptide, SIINFEKL-specific CD8 T cell line was generated by stimulating splenocytes from OT-1 transgenic mice with irradiated EG.7 cells at the presence of IL-2 (20 IU/ml, Pepro-Tech).

Plasmid construction

The plasmids encoding HPV16 and 18 L1 and L2 (pShell16, pShell18, p16L1 and p16L2) were kindly provided by Dr. John Schiller (NCI). The point mutation HPV16L1mtL2-OVA construct is described in our previous study 7. The generation of ovalbumin-expressing plasmid (pcDNA3-OVA) and GFP-expressing plasmid (pcDNA3-GFP) has been described previously 23,24.

HPV pseudovirion production

HPV16 and HPV18 pseudovirions were made as described previously 19. Briefly, 293TT cells were co-transfected with HPV L1 and L2 expression plasmids and the targeted antigen-expressing plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 hours, the cells were harvested and washed with Dulbecco's PBS (Invitrogen) supplemented with 9.5 mM $MgCl_2$ and antibiotic-antimycotic mixture (DPBS-Mg) (Invitrogen). The cells were suspended in DPBS-Mg supplemented with 0.5% Briji58, 0.2% Benzonase (Novagen), 0.2% Plasmid Safe (Epicentre) at $>100 \times 10^6$ cells/ml and incubated at 37°C for 24 hours for capsid maturation. After maturation, the cell lysate was chilled on ice for 10 minutes. The salt concentration of the cell lysate was adjusted to 850 mM and incubated on ice for 10 minutes. The lysate was then clarified by centrifugation, and the supernatant was then layered onto an Optiprep gradient. The gradient was spun for 4.5 hours at 16°C at 40,000 rpm in a SW40 rotor (Beckman). Furin-precleaved pseudovirion (FPC) was produced as described previously 12. Briefly, 20 U/ml of furin was added to the pseudovirion extract prior to the maturation process. After maturation, the FPC virions were purified as described above.

The purity of HPV pseudovirions was evaluated by running the fractions on 4–15% gradient SDS-PAGE gel. The encapsulated DNA plasmid was quantified by extracting encapsidated DNA from Optiprep factions followed by quantitative real time PCR compared to serial dilutions of naked DNA.

Characterization of the amount of DNA contained in pseudovirions

The extraction of plasmid DNA from pseudovirions for the quantitative Real-time PCR was performed using methods from John Schiller's Group (Laboratory of Cellular Oncology, NCI). Briefly, 100 μl of Optiprep fraction material adding 10 μl of 0.5M EDTA and 2.5 μl of proteinase K (Qiagen) was incubated at 56° C for 30 minutes followed adding 5μl of 10% SDS and another incubation 30 min. After incubation, the solution was massed up 200ul and 200μl of equilibrated phenol-chloroform-isoamylalcohol (Roche) and 200μl of chloroform-isoamylalcohol (Sigma) was used serially for the preparation of extracted lysate. 2.6 volumes of 95% ethanol were added to about 200μl of extracted lysate and precipitate DNA 4°C overnight. After spin down for 60 min at $15,000 \times g$ room temperature, supernatant was removed carefully. Pellet was washed with 800μl of 70% ethanol and dissolved in 50μl of dH_2O. For the quantification of plasmid DNA, the quantitative real-time PCR reactions were performed in triplicates using Bio-Rad iCycler. OVA or No insert plasmid DNA from pseudovirus and naked OVA or No insert were used as a template for amplification using primers for OVA or No insert (OVA: 5′-AATGGACCAGTTCTAATGT-3′, 5′-GTCAGCCCTAAATTCTTC-3′ or No insert: 5′-TAATACGACTCACTATAGGG-3′, 5′-

TAGAAGGCACAGTCGAGG-3′) and amplified products were quantified by fluorescence intensity of SYBR Green I (Molecular Probes). Standard curve method was used to calculate the quantity of pseudovirus plasmid DNA relative to the naked plasmid DNA. Five serial dilutions of naked plasmid (OVA or No insert) were made for the calibration curve and trend lines were drawn using Ct values versus log of dilutions for each plasmid. The quantity of pseudovirus plasmid DNA was calculated using line equations derived from calibration curves. The concentration of pcDNA3 plasmid DNA and pcDNA3-OVA DNA in the pseudovirions was determined to be approx. 6.2 ng of DNA per 1 μg of L1 protein.

HPV pseudovirion labeling and *in vivo* uptake

HPV16-OVA pseudovirions were labeled with FITC using the FluoReporter FITC protein labeling kit (F6434) (Invitrogen). After extensive washing, FITC labeled or unlabeled pseudovirions were injected into the hind footpads of mouse. 48 hours later, Inguinal and popliteal lymph nodes were collected, minced and digested with 0.05 mg/ml Collagenase I, 0.05 mg/ml collagenase IV, 0.025 mg/ml Hyaluronidase IV (Sigma) and 0.25 mg/ml DNase I (Roche) at 37°C for 1 hour. After washing, the cells were stained with anti-mouse B220 and CD11c antibody, labeled with FITC and analyzed with flow cytometry.

Generation of bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated from bone marrow progenitor cells as described previously 25. Briefly, bone marrow cells were flushed from the femurs and tibiae of 5- to 8-week-old C57BL/6 mice. Cells were washed twice with RPMI-1640 after lysis of red blood cells and resuspended at a density of 1×10^6/ml in RPMI-1640 medium supplemented with 2 m*M* glutamine, 1 m*M* sodium pyruvate, 100 m*M* nonessential amino acids, 55 μ*M* β-mercaptoethanol, 100 IU/ml penicillin, 100 g/ml streptomycin, 5% fetal bovine serum, and 20 ng/ml recombinant murine GM-CSF (PeproTech, Rock Hill, NJ). The cells were then cultured in a 24-well plate (1 ml/well) at 37°C in 5% humidified CO_2. The wells were replenished with fresh medium supplemented with 20 ng/ml recombinant murine GM-CSF on days 2 and 4. The cells were harvested as indicated.

In vitro infection with HPV pseudovirions

DC-1 cells were seeded into 24-well plate at the density of 1×10^5/well, and infected with 5 μg (HPV L1 protein amount) of HPV16-GFP or HPV16-OVA pseudovirions. For furin cleavage experiment, 5 units/ml of furin (Alexis Biochemical, San Diego) were added to the cell culture medium. BMDCs were also infected with 5 μg (HPV L1 protein amount) of HPV16-GFP or HPV16-OVA pseudovirions. 72 hours later, the cells were analyzed for GFP expression by flow cytometry or used in T cell activation assay.

In vitro T cell activation assay

OT-1 T cells were co-incubated with HPV16-GFP or HPV16-OVA pseudovirions infected DC-1 cells (E:T ratio 2:1) at the presence of GolgiPlug (BD Pharmingen) at 37°C for 20 hours. T cell activation was analyzed by detecting intracellular IFN-γ production with flow cytometry analysis.

Vaccination with HPV pseudovirions

C57BL/6 mice were vaccinated with indicated HPV pseudovirions (adjusted to 5μg L1 protein amount) at both hind footpads. 7 days later, the mice were boosted with indicated HPV pseudovirions with the same regimen. For antibody detection experiment, sera were collected before and after vaccination at indicated time point. For antigen-specific T cell detection, mouse splenocytes were harvested 1 week after last vaccination.

DNA vaccination

Gene gun particle-mediated DNA vaccination was performed as described previously [26]. Gold particles coated with pcDNA3-OVA, or pcDNA3 were delivered to the shaved abdominal regions of mice by using a helium-driven gene gun (Bio-Rad Laboratories Inc., Hercules, Calif.) with a discharge pressure of 400 lb/in2. Mice were immunized with 2 μg of the DNA vaccine and boosted with the same regimen1 week later. Splenocytes were harvested 1 week after the last vaccination.

Antibody Neutralization Assays

The HPV pseudovirion in vitro neutralization assay was performed as described earlier [27], and the secreted alkaline phosphatase activity in the cell-free supernatant was determined using *p*-nitrophenyl phosphate (Sigma Aldrich, St Louis, MO)dissolved in diethanolamine, with absorbance measured at 405nm. Neutralizing antibody titers were defined as the reciprocal of the highest dilution that caused a greater than 50% reduction in A_{405}, as described previously [27]. Preimmunesera were used as a negative control and mouse monoclonal antibody RG-1 or rabbit antiserum to L1 VLP as positive controls [28].

Detection of Ovalbumin-specific antibody by ELISA

To detect OVA-specific antibody in vaccinated mouse sera, an ELISA assay was performed. Briefly, maximum absorption 96-well ELISA plate was coated with OVA protein (Sigma) at 1 μg/ml, and incubated at 4°C overnight. After blocking with PBS containing 1% BSA for 1 h at 37 °C, the wells were then washed with PBS containing 0.05% Tween-20. The plate was incubated with serially diluted sera for 2 h at 37 °C. Serum from mouse vaccinated with OVA protein via intramuscular injection plus electroporation (Kang TH, et al. Manuscript in preparation) was used as the positive control. After washing with PBS containing 0.05% Tween-20, the plate was further incubated with 1:2,000 dilution of a HRP-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for 1 h. The plate was washed, developed with 1-Step Turbo TMB-ELISA(Pierce, Rockford, Ill.), and stopped with 1 M H_2SO_4. The ELISAplate was read with a standard ELISA reader at 450nm.

Intracellular cytokine staining and flow cytometry analysis

Before intracellular cytokine staining, pooled splenocytes from each vaccination group were incubated for 20 hours with 1 μg/ml of OVA SIINFEKL peptide at the presence of GolgiPlug (BD Pharmingen, San Diego, CA). The stimulated splenocytes were then washed once with FACS can buffer and stained with PE-conjugated monoclonal rat antimouse CD8a (clone 53.6.7). Cells were subjected to intracellular cytokine staining using the Cytofix/

Cytoperm kit according to the manufacturer's instruction (BD Pharmingen, San Diego, CA). Intracellular IFN-γ was stained with FITC-conjugated rat anti-mouse IFN-γ (clone XMG1.2). Flow cytometry analysis was performed using FACSCalibur with CELLQuest software (BD biosciences, Mountain View, CA).

RT-PCR analysis of in vivo GFP expression

To detect GFP expression in the draining lymph nodes after pseudovirion infection, total RNA was extracted from draining lymph nodes 48 hours after subcutaneous HPV16-GFP or HPV16-OVA pseudovirions infection. RT-PCR was performed as described previously [29]. Briefly, the RNA was extracted from the cells by TRIZOL (Invitrogen, Carlsbad, Calif). RT-PCR was performed using the Superscript One-Step RT-PCR Kit (Invitrogen). One microgram of total RNA was used. Sequences of primers for GFP and GAPDH were as follows: GFP-F (5′-ATGGTGAGCAAGGGCGAGGAG-3′), GFP-R (5′-CTTGTACAGCTCGTCCATGCC -3′), GAPDH-F (5′-CCGGATCCTGGGAAGCTTGTCATCAACGG -3′), and GAPDH-R (5′-GGCTCGAGGCAGTGATGGCATGGACTG -3′). The reaction condition for GFP was 1 cycle (94°C, 30 sec), 35 cycle (94°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec), and 1 cycle (72°C, 10 min). The reaction condition for GAPDH was similar except that amplification was repeated for 20 cycles. The products were analysed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. GAPDH expression was used as positive control and no RT was used as a negative control.

In vivo tumor protection and antibody depletion

C57BL/6 mice (five per group) were vaccinated with the indicated HPV pseudovirions (adjusted with 5μg L1 protein amount) at both hind footpads. 7 days later, the mice were boosted with indicated HPV pseudovirions with the same regimen. 1 week after last vaccination, mice were injected with 1×10^5 B16-OVA tumor cells subcutaneously at the flank site in 100 μLPBS. *In vivo* antibody depletions have been described previously [30]. Briefly, monoclonal antibody (MAb) GK1.5 was used for CD4 depletion, MAb 2.43 was used for CD8 depletion and MAb PK136 was used for NK1.1 depletion. Depletion started 1 week before tumor cell challenge. Growth of tumors was monitored twice a week by inspection and palpation.

Statistical analysis

Data expressed as means ± standard deviations (SD)are representative of at least two different experiments. Comparisons between individual data points were made by 2-tailed Student's *t* test. A *P* value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. zur Hausen H. Infections Causing Human Cancer. JAMA. 2008; 299:837–838.

2. Roden R, Wu TC. Preventative and therapeutic vaccines for cervical cancer. Expert Rev Vaccines. 2003; 2:495–516. [PubMed: 14711335]

3. Trimble CL, Peng S, Kos F, Gravitt P, Viscidi R, Sugar E, et al. A phase I trial of a human papillomavirus DNA vaccine for HPV16+ cervical intraepithelial neoplasia 2/3. Clin Cancer Res. 2009; 15:361–367. [PubMed: 19118066]

4. Donnelly JJ, Wahren B, Liu MA. DNA vaccines: progress and challenges. J Immunol. 2005; 175:633–639. [PubMed: 16002657]

5. Buck CB, Pastrana DV, Lowy DR, Schiller JT. Generation of HPV pseudovirions using transfection and their use in neutralization assays. Methods Mol Med. 2005; 119:445–462. [PubMed: 16350417]

6. Campos SK, Ozbun MA. Two highly conserved cysteine residues in HPV16 L2 form an intramolecular disulfide bond and are critical for infectivity in human keratinocytes. PLoS ONE. 2009; 4:e4463. [PubMed: 19214230]

7. Gambhira R, Jagu S, Karanam B, Day PM, Roden R. Role of L2 cysteines in papillomavirus infection and neutralization. Virol J. 2009; 6:176. [PubMed: 19860897]

8. Trimble C, Lin CT, Hung CF, Pai S, Juang J, He L, et al. Comparison of the CD8+ T cell responses and antitumor effects generated by DNA vaccine administered through gene gun, biojector, and syringe. Vaccine. 2003; 21:4036–4042. [PubMed: 12922140]

9. Gurunathan S, Klinman DM, Seder RA. DNA vaccines: immunology, application, and optimization*. Annu Rev Immunol. 2000; 18:927–974. [PubMed: 10837079]

10. Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proc Natl Acad Sci U S A. 2009; 106:20458–20463. [PubMed: 19920181]

11. Day PM, Gambhira R, Roden RB, Lowy DR, Schiller JT. Mechanisms of human papillomavirus type 16 neutralization by l2 cross-neutralizing and l1 type-specific antibodies. J Virol. 2008; 82:4638–4646. [PubMed: 18305047]

12. Day PM, Lowy DR, Schiller JT. Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. J Virol. 2008; 82:12565–12568. [PubMed: 18829767]

13. Day PM, Schiller JT. The role of furin in papillomavirus infection. Future Microbiol. 2009; 4:1255–1262. [PubMed: 19995186]

14. Malboeuf CM, Simon DA, Lee YE, Lankes HA, Dewhurst S, Frelinger JG, et al. Human papillomavirus-like particles mediate functional delivery of plasmid DNA to antigen presenting cells in vivo. Vaccine. 2007; 25:3270–3276. [PubMed: 17293010]

15. El Mehdaoui S, Touze A, Laurent S, Sizaret PY, Rasschaert D, Coursaget P. Gene transfer using recombinant rabbit hemorrhagic disease virus capsids with genetically modified DNA encapsidation capacity by addition of packaging sequences from the L1 or L2 protein of human papillomavirus type 16. J Virol. 2000; 74:10332–10340. [PubMed: 11044077]

16. Zhang H, Fayad R, Wang X, Quinn D, Qiao L. Human immunodeficiency virus type 1 gag-specific mucosal immunity after oral immunization with papillomavirus pseudoviruses encoding gag. J Virol. 2004; 78:10249–10257. [PubMed: 15367590]

17. Bousarghin L, Combita-Rojas AL, Touze A, El Mehdaoui S, Sizaret PY, Bravo MM, et al. Detection of neutralizing antibodies against human papillomaviruses (HPV) by inhibition of gene transfer mediated by HPV pseudovirions. J Clin Microbiol. 2002; 40:926–932. [PubMed: 11880418]

18. Combita AL, Touze A, Bousarghin L, Sizaret PY, Munoz N, Coursaget P. Gene transfer using human papillomavirus pseudovirions varies according to virus genotype and requires cell surface heparan sulfate. FEMS Microbiol Lett. 2001; 204:183–188. [PubMed: 11682199]

19. Buck CB, Pastrana DV, Lowy DR, Schiller JT. Efficient intracellular assembly of papillomaviral vectors. J Virol. 2004; 78:751–757. [PubMed: 14694107]

20. Chuang CM, Monie A, Wu A, Pai SI, Hung CF. Combination of viral oncolysis and tumor-specific immunity to control established tumors. Clin Cancer Res. 2009; 15:4581–4588. [PubMed: 19584165]

21. Shen Z, Reznikoff G, Dranoff G, Rock KL. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J Immunol. 1997; 158:2723–2730. [PubMed: 9058806]

22. Kim TW, Hung CF, Boyd DA, He L, Lin CT, Kaiserman D, et al. Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6. Cancer Res. 2004; 64:400–405. [PubMed: 14729651]

23. Kim TW, Hung CF, Ling M, Juang J, He L, Hardwick JM, et al. Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. J Clin Invest. 2003; 112:109–117. [PubMed: 12840065]

24. Hung C-F, Cheng W-F, Hsu K-F, Chai C-Y, He L, Ling M, et al. Cancer immunotherapy using a DNA vaccine encoding the translocation domain of a bacterial toxin linked to a tumor antigen. Cancer Research. 2001; 61:3698–3703. [PubMed: 11325841]

25. Peng S, Kim TW, Lee JH, Yang M, He L, Hung CF, et al. Vaccination with dendritic cells transfected with BAK and BAX siRNA enhances antigen-specific immune responses by prolonging dendritic cell life. Hum Gene Ther. 2005; 16:584–593. [PubMed: 15916483]

26. Peng S, Hung C-F, Trimble C, He L, Yeatermeyer J, Boyd D, et al. Development of a DNA vaccine targeting HPV-16 oncogenic protein E6. J Virol. 2004; 78:8468–8476. [PubMed: 15280455]

27. Pastrana DV, Buck CB, Pang YY, Thompson CD, Castle PE, FitzGerald PC, et al. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology. 2004; 321:205–216. [PubMed: 15051381]

28. Jagu S, Karanam B, Gambhira R, Chivukula SV, Chaganti RJ, Lowy DR, et al. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. J Natl Cancer Inst. 2009; 101:782–792. [PubMed: 19470949]

29. Kim TW, Hung CF, Zheng M, Boyd DA, He L, Pai SI, et al. A DNA vaccine co-expressing antigen and an anti-apoptotic molecule further enhances the antigen-specific CD8+ T-cell immune response. J Biomed Sci. 2004; 11:493–499. [PubMed: 15153784]

30. Lin K-Y, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August T, Pardoll DM, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Research. 1996; 56:21–26. [PubMed: 8548765]
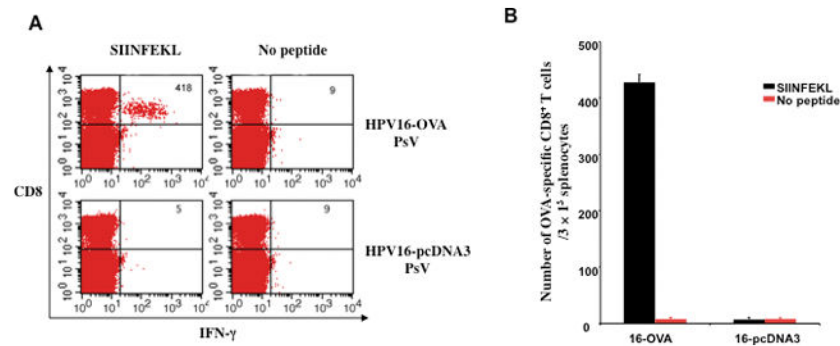
Figure 1. Characterization of OVA-specific CD8+ T cell immune responses generated by HPV-16 pseudovirion vaccination

Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by vaccination with HPV16-OVA or HPV16-pcDNA3 pseudovirions. 5–8 weeks old C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA or HPV16-pcDNA3 pseudovirions (5µg L1 protein/mouse) via footpad injection. All mice were boosted 7 days later with the same regimen. 1 week after last vaccination, splenocytes were prepared and stimulated with OVA peptide, SIINFEKL (1µg/ml) in the presence of GolgiPlug overnight at 37°C. The OVA-specific CD8$^+$ T cells were then analyzed by intracellular cytokine staining followed by flow cytometry analysis. **A)** Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by vaccination with HPV-16-OVA pseudovirions. **B)** Graphical representation of the number of OVA-specific CD8+ T cells/3×10^5 splenocytes.
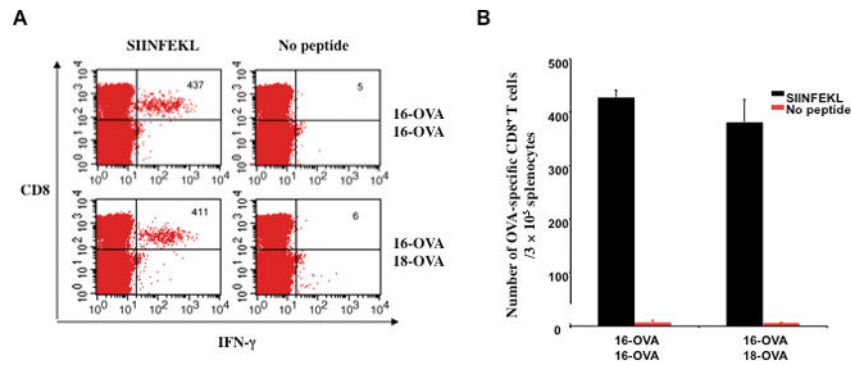
Figure 2. Comparison of OVA-specific CD8^{+} T cell responses induced by homologous or heterologous pseudovirion boost

Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by homologous or heterologous vaccination with HPV-OVA pseudovirions. 5–8 weeks old C57BL/6 mice (5 per group) were vaccinated with indicated HPV16-OVA pseudovirions (5μg L1 protein/mouse) via either intramuscular, or subcutaneous (footpad) injection. 7 days later, one group was boosted with HPV16-OVA pseudovirions, and another group was boosted with HPV18-OVA pseudovirions. 1 week after last vaccination, splenocytes were prepared and stimulated with OVA peptide, SIINFEKL (1μg/ml) in the presence of GolgiPlug overnight at 37°C. The OVA-specific CD8^{+} T cells were then analyzed by staining surface CD8 and intracellular IFN-γ. **A)** Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by homologous or heterologous vaccination with pseudovirions. **B)** Graphical representation of the number of OVA-specific CD8+ T cells/3×10^5 splenocytes.
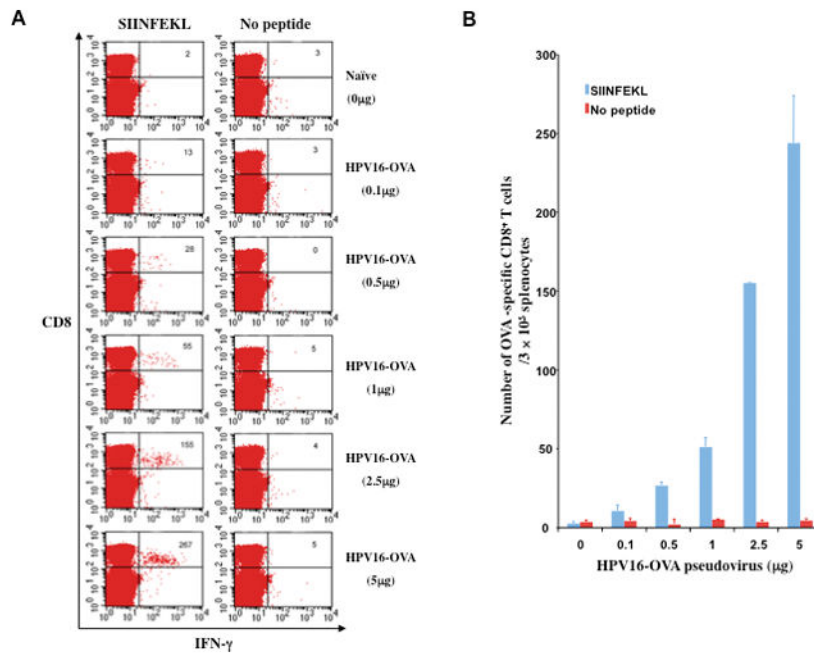
Figure 3. Dose responses of OVA-specific CD8^{+} T cell responses induced by HPV16-OVA pseudovirion vaccination

5–8 weeks old C57BL/6 mice (5 per group) were vaccinated with different doses of HPV16-OVA pseudovirions (0.1–5µg L1 protein/mouse) via subcutaneous (footpad) injection. 7 days later, the mice were boosted with the same amount of HPV16-OVA pseudovirions via footpad injection. 1 week after last vaccination, splenocytes were prepared and stimulated with OVA peptide, SIINFEKL (1µg/ml) in the presence of GolgiPlug overnight at 37°C. The OVA-specific CD8^{+} T cells were then analyzed by intracellular cytokine staining followed by flow cytometry analysis. **A)** Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by vaccination with different doses of HPV16-OVA pseudovirions. **B)** Graphical representation of the number of OVA-specific CD8+ T cells/3×10^5 splenocytes.
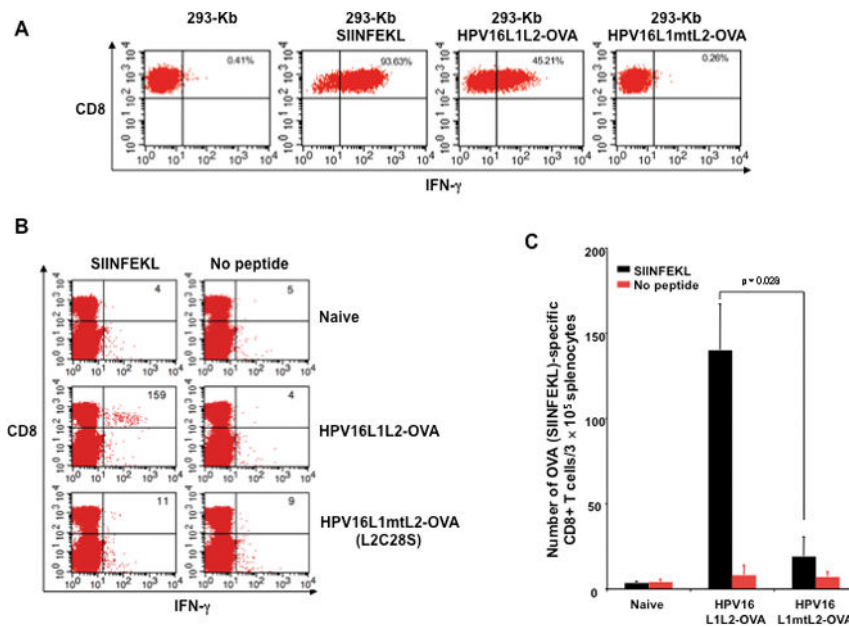
Figure 4. Characterization of OVA-specific CD8+ T cell immune responses generated by HPV-16 L1 mutant L2-OVA pseudovirion vaccination

A) Representative flow cytometry data demonstrating the activation of OVA-specific CD8+ T cells generated by HPV16 L2 mutated or wild-type HPV16-OVA pseudovirus infected 293-Kb cells. 293-Kb cells were infected with HPV16L1L2-OVA or HPV16L1mtL2-OVA pseudovirus (4 μg of L1 protein) for 72 hours. These cells were co-incubated with OT-I T cells at the E:T ratio of 2:1 at the presence of GolgiPlug overnight. OT-I T cell activation was then analyzed with intracellular IFN-γ staining. **B & C.** 5–8 weeks old C57BL/6 mice (5 per group) were vaccinated with HPV16L1L2-OVA or HPV16L1mtL2-OVA pseudoviruses (5 μg of L1 protein/mouse) via footpad injection. All mice were boosted 7 days later with the same regimen. 1 week after last vaccination, splenocytes were prepared and stimulated with OVA peptide, SIINFEKL (1μg/ml) in the presence of GolgiPlug overnight at 37°C. The OVA-specific CD8$^+$ T cells were then analyzed by staining surface CD8 and intracellular IFN-γ by intracellular cytokine staining followed by flow cytometry analysis. **B)** Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by vaccination with the different pseudovirions. **C)** Graphical representation of the number of OVA-specific CD8+ T cells/3×10^5 splenocytes.
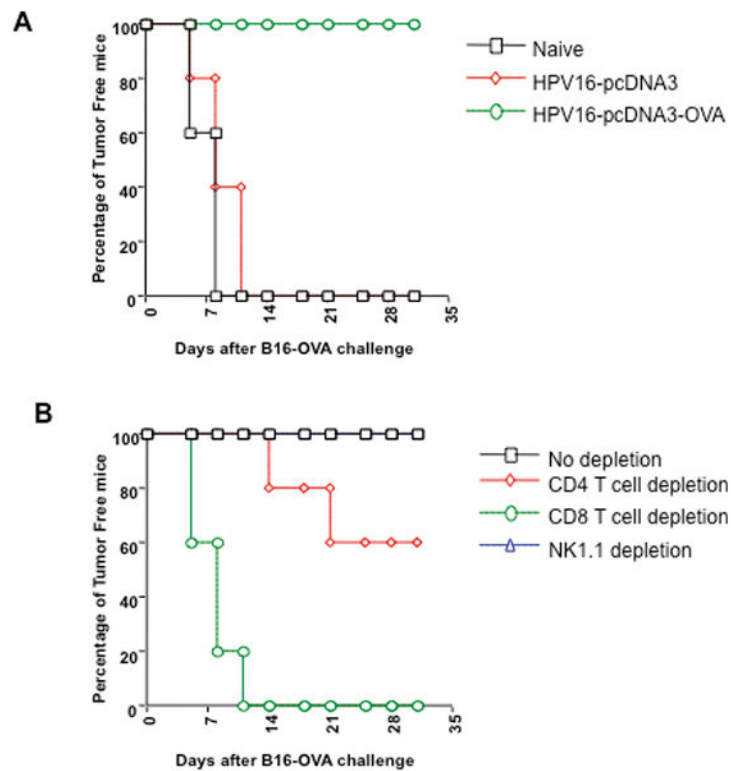
Figure 5. In vivo tumor protection experiments
5–8 weeks old C57BL/6 mice were vaccinated with HPV16-OVA (5 μg of L1 protein/
mouse) or HPV16-pcDNA3 via footpad injection. The mice were boosted twice with the
same regimen at day 7 and day 14. One week after last vaccination, the mice were injected
with 1×10^5 B16-OVA cells subcutaneously. **A)** Kaplan Meier survival analysis of the
groups of mice vaccinated with HPV16-pcDNA3 or HPV16-pcDNA3-OVA. **B)** Kaplan
Meier survival analysis of the groups of mice vaccinated with HPV16-pcDNA3 or HPV16-
pcDNA3-OVA and depleted of CD4, CD8 or NK cells. For the antibody depletion
experiment, mice were treated with antibodies against mouse CD4, CD8 or NK1.1 at the
same time of last vaccination via intraperitoneal injection. One week after last vaccination,
the mice were injected with 1×10^5 B16-OVA cells subcutaneously. Tumor growth was
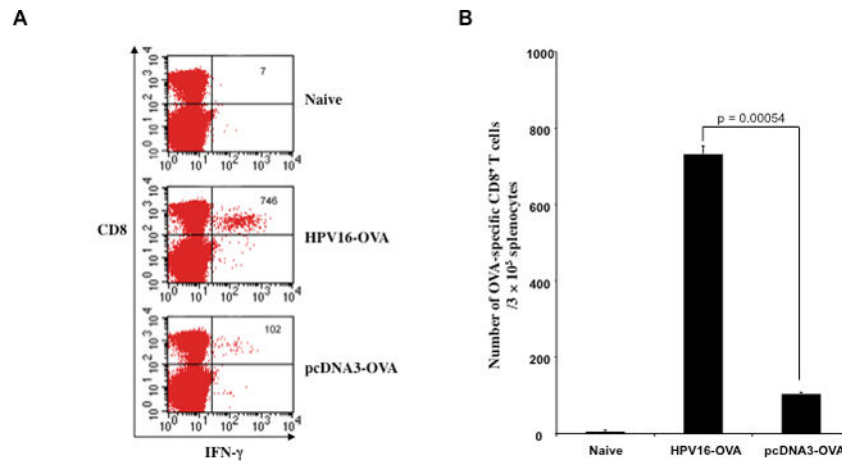monitored twice a week. Representative data from one of three independent experiments are
shown.

Figure 6. Comparison of OVA-specific CD8$^+$ T cell responses induced by pseudovirion or DNA vaccination

5–8 weeks old C57BL/6 mice (5 per group) were vaccinated with HPV16-OVA pseudovirions (5μg L1 protein/mouse) via subcutaneous (footpad) injection, or vaccinated with 2 μg of pcDNA3-OVA via gene gun delivery. These mice were boosted 7 days later with the same regimen. 1 week after last vaccination, splenocytes were prepared and stimulated with OVA peptide, SIINFEKL (1μg/ml) in the presence of GolgiPlug overnight at 37°C. The OVA-specific CD8$^+$ T cells were then analyzed by intracellular cytokine staining followed by flow cytometry analysis. **A)** Representative flow cytometry data demonstrating the number of OVA-specific CD8+ T cells generated by vaccination with HPV-16-OVA pseudovirions or OVA DNA. **B)** Graphical representation of the number of OVA-specific CD8+ T cells/3×10^5 splenocytes.
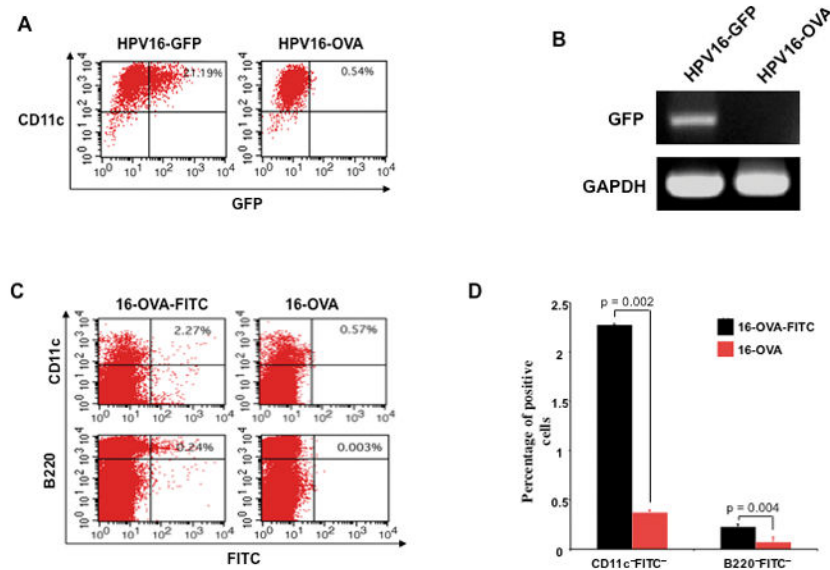
Figure 7. Analysis of cells infected by HPV pseudovirion
A) In vitro infection of BMDCs by HPV pseudovirus. BMDCs were generated from bone marrow progenitor cells and infected with HPV16-GFP or HPV16-OVA pseudovirus at day 4 (4 μg L1 protein). After 72 hours, BMDCs were harvested and GFP expression was examined by flow cytometry. **B)** RT-PCR to demonstrate the expression of GFP mRNA in draining lymph nodes of mice infected with HPV16 pseudovirions containing GFP or OVA. 5–8 weeks old C57BL/6 mice were vaccinated with 10 μg/mouse of HPV16 pseudovirions carrying GFP or OVA DNA subcutaneously. After 72 hours, draining lymph nodes were harvested and total RNA was isolated with TRIzol. RT-PCR was then performed to detect GFP mRNA expression. **C) Representative flow cytometry data depicting the percentage of CD11c+ cells and B220+ cells that uptake the FITC-labeled pseudovirions.** HPV16-OVA pseudovirus was labeled with FITC. 5–8 weeks old C57BL/6 mice were given 10 μg/mouse of HPV16-OVA or HPV16-OVA-FITC pseudovirus subcutaneously. After 72 hours, draining lymph nodes were harvested, and digested with 0.05 mg/ml Collagenase I, 0.05 mg/ml collagenase IV, 0.025 mg/ml Hyaluronidase IV and 0.25 mg/ml DNase I. The cells were then stained with anti-mouse CD11c-APC and PE-Cy5-conjugated anti-mouse B220 followed by flow cytometry analysis. **D) Representative bar graph depicting the percentage of FITC+ CD11c+ cells and FITC+ B220+ cells.**
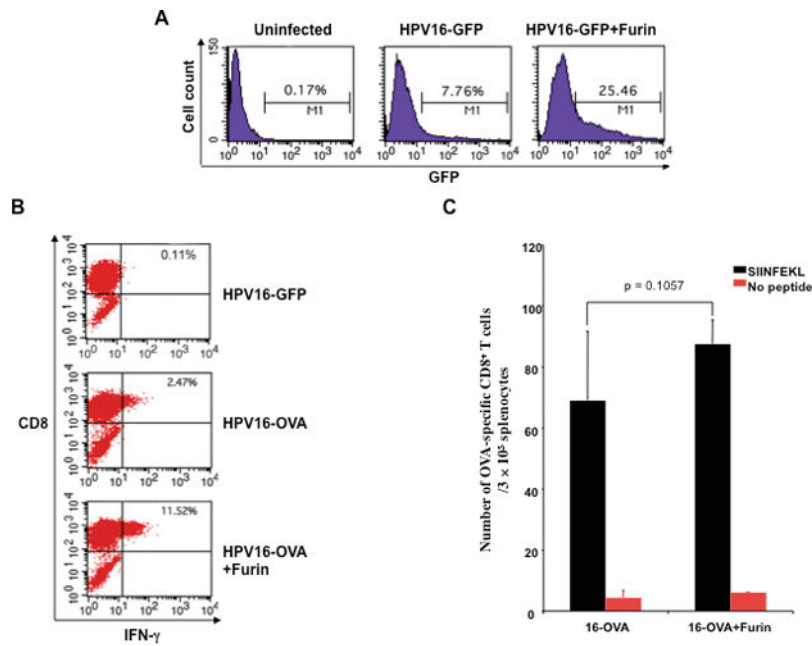
Figure 8. Characterization of the infection and antigen presentation of HPV16-GFP pseudovirions treated with furin

A) Representative flow cytometry data demonstrating the percentage of GFP expressing DC-1 cells. A dendritic cell line, DC-1, was infected with 4 µg (L1 protein) of HPV16-GFP or HPV16-OVA pseudovirions with or without the presence of Furin (5 units). After 72 hours GFP expression by DC-1 cells was analyzed by flow cytometry. **B)** Representative flow cytometry data demonstrating the percentage of activated OVA-specific CD8+ T cells. Infected DC-1 cells were collected 72 hours after infection, and co-cultured with OVA-specific OT-1 T cells (E:T ratio at 1:1) at the presence of GolgiPlug overnight. Activation of OT-1 T cells was analyzed by IFN-γ intracellular staining. **C)** Intracellular cytokine staining followed by flow cytometry analysis to characterize the number of OVA-specific CD8+ T cells in mice vaccinated with HPV16-OVA pseudovirions with or without furin treatment.