CLINICAL AND VACCINE IMMUNOLOGY, May 2008, p. 817–824 Vol. 15, No. 5
1556-6811/08/$08.00+0 doi:10.1128/CVI.00343-07

Single-Dose, Therapeutic Vaccination of Mice with Vesicular Stomatitis Virus Expressing Human Papillomavirus Type 16 E7 Protein▽

John B. Liao,[1,2]† Jean Publicover,[3] John K. Rose,[4] and Daniel DiMaio[5]*

Department of Obstetrics, Gynecology, and Reproductive Sciences,[1] Investigative Medicine Program,[2] Microbiology Graduate Program,[3] Department of Pathology,[4] and Departments of Genetics, Molecular Biophysics and Biochemistry, and Therapeutic Radiology,[5] Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

Received 21 August 2007/Returned for modification 20 November 2007/Accepted 12 February 2008

We are developing recombinant attenuated vesicular stomatitis virus (VSV) as a vaccine vector to generate humoral and cell-mediated immune responses. Here, we explore the use of VSV vaccines for cancer immunotherapy. Immunotherapy targeting high-risk human papillomavirus (HPV) lesions has the potential to benefit HPV-infected individuals and cervical cancer patients by generating cytotoxic T cells that kill tumor cells that express viral antigens. A single dose of VSV expressing the HPV type 16 (HPV16) E7 oncogene was used for therapeutic vaccination of mice bearing TC-1 syngeneic tumors, which express HPV16 E7. HPV16 E7-specific T cells were generated and displayed cytotoxic activity against the tumor cells. By 14 days postvaccination, average tumor volumes were 10-fold less in the vaccinated group than in mice that received the empty-vector VSV, and regression of preexisting tumors occurred in some cases. This antitumor effect was CD8 T-cell dependent. Our results demonstrate antitumor responses to HPV16 E7 and suggest that recombinant-VSV-based vaccination should be explored as a therapeutic strategy for cervical carcinoma and other HPV-associated cancers.

Persistent infection with high-risk types of human papillomavirus (HPV) leads to the development of cervical cancer, the second most common cancer in women worldwide and a significant global health problem, with nearly 500,000 new cases each year and over 270,000 deaths annually (10, 37). The great majority of cervical cancer occurs in the developing world, where limitations in health care infrastructure and resources hamper the implementation of effective screening tests, such as the Pap smear. High-risk HPV infection is also associated with a significant fraction of other anogenital cancers and some head-and-neck squamous cell carcinomas. Prophylactic vaccines that prevent infection by the two most common high-risk types, HPV type 16 (HPV16) and HPV18, hold much promise for reducing cancer incidence. These vaccines are based on virus-like particles, which, when injected with adjuvant, generate neutralizing antibodies to prevent infection (21, 29). Given the massive global vaccination effort needed to combat this disease and the long time between infection and cancer development, it will be decades before there is a significant impact on cancer incidence (13). Moreover, because these vaccines are type specific, even complete vaccination for HPV16 and -18 will provide limited protection against the one-third of cervical cancers caused by other high-risk types. Furthermore, virus-like-particle vaccination appears to be of little benefit to women who harbor active HPV infections (23), and treatment options for patients who already have HPV-associated cancer are unsatisfactory. Therefore, there is still great need for new approaches to treat patients with HPV infections or HPV-induced tumors.

AIDS patients, transplant patients receiving immunosuppressive therapy, and individuals with T-cell deficiencies have increased rates of HPV persistence, HPV-induced anogenital warts, and cervical cancer (14, 20, 36, 38). In addition, infiltrating lymphocytes and HPV-specific T cells are present in spontaneously regressing human papillomas (6, 11, 16, 25). Taken together, these findings strongly suggest that the cellular immune response plays an important role in controlling HPV infection and associated disease. Therefore, several groups have attempted to generate a therapeutic cellular immune response to HPV-infected cells.

HPV E6 and E7 viral oncoproteins have been the main candidate targets for therapeutic-vaccination strategies for cervical cancer, because they are consistently expressed in precancerous cells, as well as cancer cells, and are not present in uninfected cells. The majority of therapeutic approaches have used the E7 protein. Initial studies of viral gene expression in cervical cancer cell lines showed the E7 protein to be present at higher steady-state levels than E6 (1). Furthermore, the HPV16 E7 protein contains peptides that have a high affinity for the most common major histocompatibility complex (MHC) class I allele in humans, HLA-A2, and for MHC class I and class II in the mouse strain C57BL/6 (26). Finally, E7 variants were extremely rare in HPV16-positive invasive cervical carcinomas and were detected only in combination with E6 variants, suggesting that the E7 protein is well conserved in vivo (48). In contrast, E6 variants were extremely common and became even more so as lesions progressed to cervical carci-

* Corresponding author. Mailing address: Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510. Phone: (203) 785-2684. Fax: (203) 785-6765. E-mail: daniel.dimaio@yale.edu.

† Present address: Department of Ob/Gyn, University of Pennsylvania, 421 Curie Blvd., Philadelphia, PA 19104.

▽ Published ahead of print on 12 March 2008.

noma, with fully 94% of invasive cervical cancers possessing variants. These findings suggest that E7 protein would be a good fixed target against which to direct an immunotherapeutic response.

Purified recombinant HPV proteins and HPV DNA vaccines have been successfully used to generate cell-mediated immune responses in experimental animals and humans (5, 12, 43, 45, 47). A number of viral vectors have also been tested in murine models to deliver E7 for vaccination: vaccinia virus (30, 33), adenovirus (22), and alphaviruses (9, 46). All of these studies demonstrated that vaccinated mice were able to reject a subsequent challenge with syngeneic tumor cells expressing HPV16 E7 protein. Two studies also tested therapeutic vaccination of mice with preestablished syngeneic tumors. These studies showed 50% and 67% regression rates (30, 46) following two intraperitoneal or three intramuscular weekly injections, respectively. CD8-mediated cellular immune responses appeared to be required for antitumor responses in all of these studies.

Vesicular stomatitis virus (VSV) is a negative-strand RNA virus of the family *Rhabdoviridae*. It causes a self-limiting disease in livestock, and infection of humans is rare, typically resulting in only mild flu-like symptoms. Recombinant, attenuated forms of VSV have been developed as vectors for high-level expression of foreign genes and tested as experimental vaccines in animals. These vaccines generate robust humoral and cytotoxic-T-lymphocyte (CTL) responses against a variety of cellular and viral antigens (18, 19, 40). As is the case with some other live viral vectors, VSV has the potential to be delivered using a needle-free route of infection (41), which would overcome one of the limitations to vaccine delivery in the developing world.

The utility of VSV for therapeutic vaccination against papillomavirus-induced tumors has been tested in the cottontail rabbit papillomavirus (CRPV) system. These experiments showed that vaccination with VSV expressing various CRPV proteins caused the regression of skin warts in rabbits, with CRPV E7 being the most effective immunogen (3, 4). However, because of the outbred nature of rabbits, it is difficult to perform immune studies with cells from these animals, and the ability of VSV to generate an anti-E7 response has not been documented at the cellular level. The aim of this study was to test whether VSV-mediated delivery of HPV E7 generates therapeutic cell-mediated immune responses in a mouse model of HPV-associated cancer. We show that a single dose of VSV expressing HPV16 E7 is sufficient to generate a therapeutic E7-specific cellular immune response.

MATERIALS AND METHODS

Plasmids and viruses. The DNA sequence encoding HPV16 E7 was amplified from the complete HPV16 genome using the forward primer 5′-CCG CTC GAG AAC ATG CAT GGA GAT ACA CC-3′ and the reverse primer 5′-CGG GCT AGC TTA TGG TTT CTG AGA ACA G-3′. The DNA sequence encoding HPV16 E7 deletion 21-24 inactivated at the retinoblastoma (Rb) binding motif was amplified with the same primers from an HPV16 E7 mutant clone obtained as a gift from Denise Galloway (University of Washington). These primers place XhoI and NheI restriction sites (underlined) upstream and downstream, respectively, of the coding sequence. The PCR product was purified, sequentially digested with XhoI and NheI, and ligated into a pVSV1-XN plasmid digested with the same enzymes to construct pVSV1-16E7 and pVSV1-16E7del, expressing the wild-type or deleted version of HPV16 E7, respectively (40). The insert sequences were verified by the Yale Keck DNA Sequencing Facility.

VSV preparation and titering. VSV recovery was performed as previously described (31, 44). In brief, baby hamster kidney cells (BHK-21; ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The cells were grown on 10-cm tissue culture dishes to approximately 80% confluence and then infected with a vaccinia virus expressing T7 RNA polymerase at a multiplicity of infection of 10. One hour later, 10 μg of either pVSV1-16E7 or pVSV1-16E7del was cotransfected with 3 μg of pBS-N, 5 μg of pBS-P, 1 μg of pBS-L, and 4 μg of pBS-G using a transfection reagent composed of dimethyldioctadecyl ammonium bromide and phosphatidylethanolamine. Supernatants from transfected cells were collected after 48 h at 37°C. The supernatants were filtered through a 0.2-μm filter onto fresh BHK-21 cells (2.5×10^6) to remove the vaccinia virus and placed back at 37°C to allow the VSV to grow. The supernatants were collected 24 h later upon the appearance of cytopathic effects. The recovered virus supernatant was collected and filtered through a 0.1-μm filter and plaque purified in BHK-21 cells, and stocks were grown in BHK-21 cells from individual plaques. The stocks were stored at −80°C. The titers of the virus stocks were determined as previously described (31, 44).

Metabolic labeling and SDS-PAGE. Approximately 5×10^5 BHK-21 cells were seeded in 35-mm-diameter tissue culture dishes and infected with VSV recombinants at a multiplicity of infection of 10 to 20. After 5 h, the culture medium was replaced with methionine-free DMEM including 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (NEN Life Sciences, Boston, MA). The cells were incubated for 1 hour. The medium was removed, and the cells were washed with phosphate-buffered saline (PBS) and lysed with detergent solution (1% Nonidet P-40, 0.4% deoxycholate, 50 mM Tris-HCl, pH 8, 62.5 mM EDTA) on ice for 5 min and collected into 1.5-ml Eppendorf tubes. The nuclei were removed by centrifugation at $16{,}000 \times g$ for 2 min at room temperature in an Eppendorf centrifuge. Protein extracts (10 μl of each sample) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% acrylamide), and the proteins were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescence. BHK-21 cells on coverslips were infected with VSV1-16E7 or VSV1-16E7del at a multiplicity of infection of 10. After 8 hours, the cells were fixed with 3% formaldehyde and incubated with mouse monoclonal anti-HPV16 E7 (8C9; Zymed, South San Francisco, CA) primary antibody, followed by Alexafluor 594 goat anti-mouse immunoglobulin G secondary antibody, and visualized by fluorescence microscopy.

Mice and cell lines. Seven- to 10-week-old female C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). C57BL/6 mice deficient in CD4 and CD8 cells were obtained from Jackson Laboratories (Bar Harbor, ME). The mice were housed under specific-pathogen-free conditions, and all protocols were approved by the Yale Institutional Animal Care and Use Committee. Tumor studies were performed using TC-1 cells obtained from the ATCC (Manassas, VA). These are syngeneic mouse tumor cells expressing HPV16 E7 protein and the *ras* oncogene (33).

Tumor therapy studies. TC-1 cells (5×10^4) were injected subcutaneously into the right flanks of mice at one site per mouse. Tumor growth was measured weekly on three axes using a digital caliper. One week after tumor cell injection, the mice were vaccinated intramuscularly in the upper right thigh with 5×10^6 PFU of recombinant VSV (rVSV) in 100 μl of PBS. In one experiment (see Fig. 8), an inoculum of 2.5×10^4 TC-1 cells per mouse was used.

ELISPOT assay for IFN-γ secretion by splenocytes. One week after vaccination, the mice were sacrificed, and splenocytes were harvested by disrupting spleens between the frosted ends of two microscope slides. The red blood cells were removed using red blood cell lysis buffer (Roche, Basel, Switzerland). The splenocytes (10^6 per well) were plated in 96-well plates coated with antibody recognizing mouse gamma interferon (IFN-γ) (R&D Systems, Minneapolis, MN) that had been preincubated at room temperature with 200 μl of sterile DMEM per well. The plates were then incubated for 24 h with and without 10^{-7} M $E7_{49\text{-}57}$ peptide, which corresponds to the known immunodominant MHC class I epitope for *H2-D^b* mice (12, 28) (the peptide was synthesized by the W. M. Keck Biotechnology Resource Laboratory, Yale University), using an enzyme-linked immunospot (ELISPOT) kit for detection of mouse IFN-γ (EL485; R&D Systems, Minneapolis, MN). The plates were developed according to the manufacturer's protocol, counted on an automated ELISPOT plate reader, and analyzed using Immunospot software version 3.1 (Cellular Technology Ltd., Cleveland, OH).

Intracellular-cytokine staining of CD8$^+$ cells. Splenocytes (10^6) were plated in 96-well flat-bottom tissue culture plates and incubated for 6 hours at 37°C in 100 μl sterile DMEM with 50 μl of a 1:250 dilution of brefeldin A (to a final concentration of 10 μg/ml), to prevent the secretion of cytokines, and with and without 10^{-7} M $E7_{49\text{-}57}$ peptide. After incubation, the cells were transferred to

96-well U-bottom plates and spun at 1,650 rpm for 2.5 min. The supernatants were discarded, the plates were briefly vortexed, and the cells were stained for surface markers at a 1:200 dilution of fluorescein isothiocyanate-conjugated anti-mouse CD8+a, clone 53-6.7 (eBioscience), for 30 min. The cells were washed with 200 μl of fluorescence-activated cell sorter (FACS) buffer (1% FBS in PBS) and permeabilized by incubation on ice for 15 min in the dark after resuspension in 50 μl of Cytofix/Cytoperm (BD Pharmingen). The cells were washed twice in Permwash (BD Pharmingen) and then stained for intracellular IFN-γ with allophycocyanin-conjugated anti-mouse IFN-γ, clone XMG1.2 (eBioscience), diluted 1:200 in Permwash for 1 hour on ice in the dark. The cells were washed three times with Permwash and once with FACS buffer and then analyzed on a FACS Calibur flow cytometer.

In vivo CTL assay. The CTL assay was performed as previously described (7). Target cells were prepared from the spleen of a naïve mouse. Erythrocytes were removed from the splenocyte cell suspension by osmotic lysis. The cells were washed and split into two populations, one labeled with a high concentration (5 μM) of 5,6-carboxyfluorescein diacetate succinimidyl ester ($CFSE^{hi}$) (Molecular Probes, Eugene, OR) and one labeled with a low concentration (0.5 μM) ($CFSE^{lo}$). The high-concentration cells were pulsed with 10^{-6} M $E7_{49-57}$ peptide for 45 min at 37°C, and the low-concentration cells were not. These populations were mixed together in equal numbers, and 5×10^7 total cells in PBS were injected intravenously into tumor-bearing mice vaccinated 1 week previously with VSV1-16E7 or control VSV. The mice were sacrificed after 20 h, and their spleens were harvested. Splenocytes were subjected to flow cytometry to determine the CFSE fluorescence intensities (7). To calculate the percent target-specific lysis, the following formula was used: [1 − ($CFSE^{lo}$ control/$CFSE^{hi}$ control)/($CFSE^{lo}$ experimental/$CFSE^{hi}$ experimental) × 100]. The results for one mouse for each vaccine treatment are shown (see Fig. 5).

In vitro CTL assay. An in vitro CTL assay using ex vivo expanded splenocytes was performed as previously described (32, 42). Splenocytes were harvested from tumor-bearing mice 1 week after vaccination with empty rVSV or VSV1-16E7 and grown for 4 days in T-25 vented culture flasks in DMEM at 37°C, 5% CO_2, and 95% humidity in the presence of 10^{-7} M $E7_{49-57}$ peptide and 5% leukocyte-conditioned medium (Stem Cell Technologies, Inc., Vancouver, British Columbia, Canada) as a source of interleukin 2 (IL-2). TC-1 tumor cells for use as targets were washed twice in PBS and resuspended in DMEM–5% FCS without phenol red. Calcein AM (Invitrogen/Molecular Probes) was added to a final concentration of 12.5 μM and incubated at 37°C for 1 hour protected from light. Cultured effector splenocytes were harvested from culture, washed, spun down, and resuspended in 3 ml of DMEM with 5% FBS without phenol red.

Target cells were plated into round-bottom 96-well plates at a concentration of 2×10^5 cells per ml. Expanded effector splenocytes were added to produce effector/target ratios of 0.8:1, 2:1, 6:1, and 20:1 in quadruplicate. Culture medium was added to minimum-release wells, and lysis buffer (50 mM sodium borate, 0.1% Triton, pH 9.0) was added to the maximum-release wells. The plates were spun at 600 rpm for 5 min and incubated at 37°C for 4 h protected from light. After incubation, the plates were spun again at 2,000 rpm for 5 min, and the supernatants were transferred to a black fluorimeter 96-well plate. The plates were read using Soft Max Pro software with excitation at 485 nm and emission at 538 nm. The percent target-specific lysis was calculated by using the average number of fluorescence units of experimental samples minus the minimum-release well divided by the maximum-release sample average minus the minimum-release average times 100. The results for effector cells prepared from a single mouse for each vaccine treatment are shown (see Fig. 6).

RESULTS

Expression of HPV16 E7 from recombinant VSV. To construct a VSV recombinant expressing the 98-amino-acid wild-type HPV16 E7 (E7) protein and an HPV16 E7 mutant with a deletion of amino acids 21 to 24, which eliminates the Rb binding motif, we inserted DNA sequences encoding these proteins into the plasmid vector pVSV1-XN at the first position in the viral genome upstream of the VSV N gene. This position gives maximum mRNA expression, because gene transcription is attenuated at downstream positions (24). rVSV was recovered by transfecting BHK cells with the vaccine genome and helper plasmids expressing VSV proteins (44). Expression of the E7 protein following infection of BHK cells with rVSV stocks was verified by metabolic labeling with [^{35}S]methionine and [^{35}S]cysteine, SDS-PAGE, and visualization by PhosphorImager. A band at the known molecular mass of HPV16 E7 of 19 kDa (15) was detected in cells infected with VSV1-16E7 and VSV1-16E7del, but not in mock-infected cells or cells infected with VSV lacking E7 (rVSV) (Fig. 1). Expression of E7 protein and the E7 deletion mutant in infected cells was also demonstrated by indirect-immunofluorescence microscopy with an antibody that recognized both the wild-type and mutant E7 proteins (39) (Fig. 2). As expected, this antibody did not stain mock-infected cells or cells infected with the empty vector, rVSV.

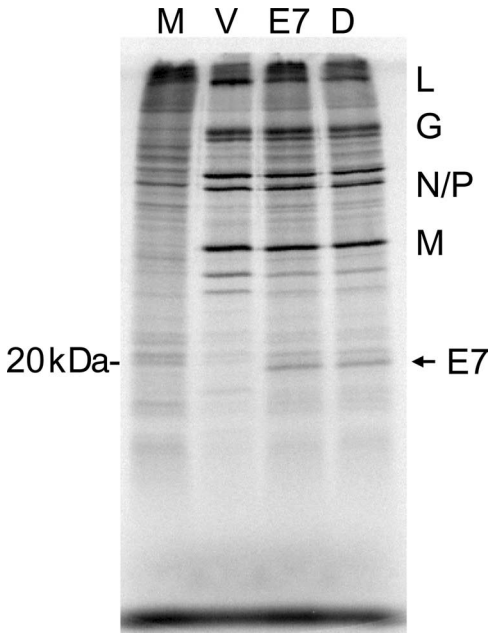

FIG. 1. Expression of HPV16 E7 from VSV vectors. Protein extracts of mock-infected (M) BHK cells or cells infected with empty-vector rVSV (V) or VSV recombinants expressing HPV16 E7 (E7) and the HPV16 E7 deletion mutant (D) labeled with [^{35}S]methionine and cysteine were subjected to gel electrophoresis. VSV proteins L, G, N/P, and M are indicated. The E7 label denotes wild-type and mutant HPV16 E7 proteins.

Generation of E7-specific immune responses with vaccination and tumor priming. Naïve C57BL/6 mice and mice that had been inoculated subcutaneously 1 week previously with syngeneic murine TC-1 tumor cells, which express the HPV16 E7 protein, were inoculated intramuscularly with a single dose of 5×10^6 PFU of rVSV or VSV1-16E7 or left untreated. One week after vaccination, splenocytes were harvested and tested for the presence of E7-specific immune cells by ELISPOT assay. The splenocytes were incubated in the presence or absence of 10^{-7} M peptide (E7 amino acids 49 to 57) that corresponded to the immunodominant HPV16 E7 MHC class I epitope for *H2-D*^{b} C57BL/6 mice (12). In mice injected with TC-1 cells 1 week prior to vaccination, VSV E7 vaccination induced the appearance of approximately 130 E7-specific, IFN-γ-secreting splenocytes per 10^6 total splenocytes in a representative experiment, whereas mice vaccinated with the empty-vector virus contained no E7-specific immune cells (Fig. 3, left). In mice not preinjected with TC-1 cells, E7 vaccination induced the appearance of sixfold-

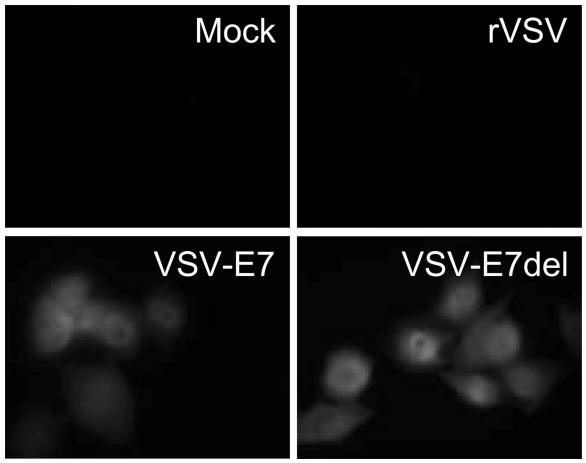

FIG. 2. Indirect-immunofluorescence microscopy for E7 expression. E7 immunofluorescence was performed on mock-infected BHK cells or cells that were infected for 4.5 h with empty rVSV or VSV recombinants expressing HPV16 E7 and HPV16 E7del.

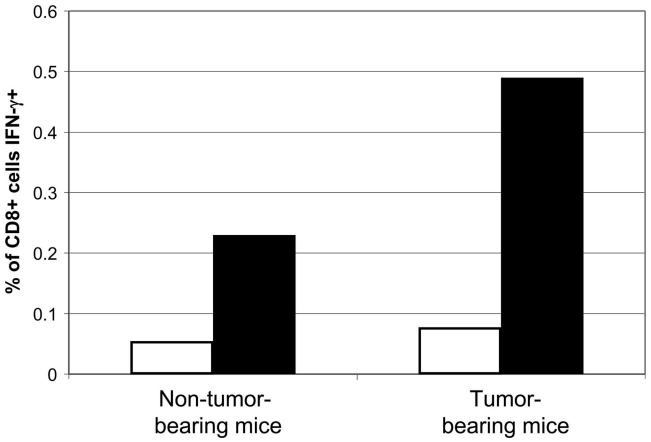

FIG. 4. Intracellular-cytokine staining. Splenocytes from rVSV- or VSV1-HPV16 E7-vaccinated mice were stained for intracellular IFN-γ and cell surface CD8 after a 6-hour stimulation with HPV16 E7 peptide and analyzed by flow cytometry. The graph shows quantitation of IFN-γ-containing $CD8^+$ cells from vector-vaccinated (clear bars) and E7-vaccinated (filled bars) mice.

fewer E7-specific cells than in the tumor-bearing animals. Similar results were obtained in multiple independent experiments.

Intracellular-cytokine staining was also performed with splenocytes harvested 1 week after intramuscular vaccination with empty-vector rVSV or VSV1-16E7. Splenocytes were incubated for 6 hours with the E7 peptide, stained for cell surface CD8, permeabilized, and stained for intracellular IFN-γ. $CD8^+$ IFN-γ^+ cells responding to the E7 peptide were counted by flow cytometry. Tumor-bearing mice vaccinated with E7-expressing viruses showed an approximately sixfold increase in the number of $CD8^+$ IFN-γ^+ cells compared to tumor-bearing mice vaccinated with the empty vector (Fig. 4). As was the case for the ELISPOT assay, there were substantially more E7-specific $CD8^+$ IFN-γ^+ cells when vaccination was performed in tumor-bearing mice than in naïve mice. Similar results were obtained in two independent experiments.

These results demonstrated that a single intramuscular injection with a VSV HPV16 E7 vaccine vector generated a readily detectable anti-E7 immune response, as assessed by ELISPOT assay for IFN-γ secretion and by intracellular IFN-γ staining. This response was more robust in mice previously inoculated with tumor cells than in mice without tumors, a phenomenon we refer to as tumor priming.

E7-specific CTL activity after VSV-E7 vaccination. The experiments described above demonstrated an increase in E7-specific $CD8^+$ IFN-γ^+ cells following E7 vaccination. To determine whether this responding population displayed cytolytic activity (8), we assessed CTL activity in vaccinated animals by using a CFSE-based in vivo assay (7). To generate target cells, two populations of splenocytes from an unvaccinated, tumor-free mouse were labeled with CFSE, one at a high concentration of CFSE and one at a low concentration of CFSE. The high-concentration cells were then loaded with the E7-specific peptide $E7_{49-57}$. High- and low-concentration cells were mixed in equal numbers and inoculated into tumor-bearing mice that had been vaccinated 7 days earlier with the empty-vector rVSV or VSV1-16E7. Twenty hours later, splenocytes were harvested and analyzed by flow cytometry for CFSE fluorescence (Fig. 5). These experiments revealed a reduced proportion of $CSFE^{hi}$ (i.e., E7 peptide-loaded) target cells in mice vaccinated with VSV1-16E7 compared to mice vaccinated with the control

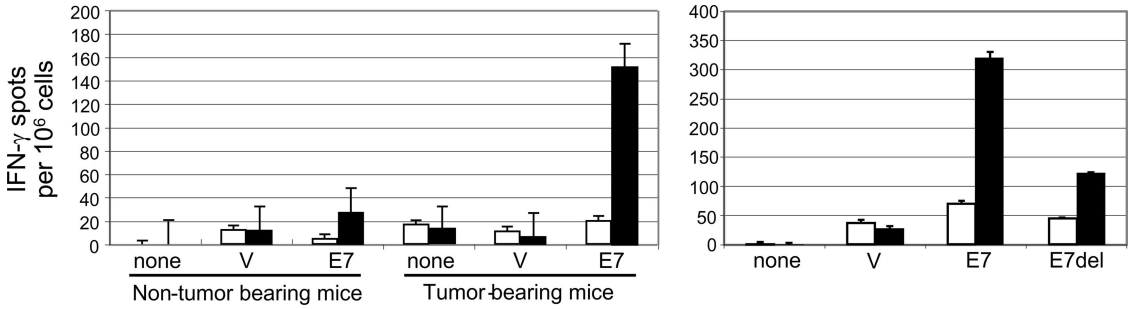

FIG. 3. ELISPOT assay. (Left) An ELISPOT assay for mouse IFN-γ was performed on splenocytes harvested 7 days after vaccination of non-tumor-bearing mice or mice inoculated 1 week earlier with TC-1 cells. None, mock vaccination; V, empty-vector rVSV vaccination; E7, VSV1-16E7 vaccination. Splenocytes were stimulated with peptide corresponding to the known MHC class I HPV16 E7 epitope for 24 hours (filled bars) or left unstimulated prior to assay (clear bars). (Right) Similar ELISPOT assay with cells from tumor-bearing mice after mock vaccination (none) or vaccination with empty-vector rVSV (V), VSV1-16E7 (E7), or VSV1-E7del (E7del). The error bars represent standard deviations.

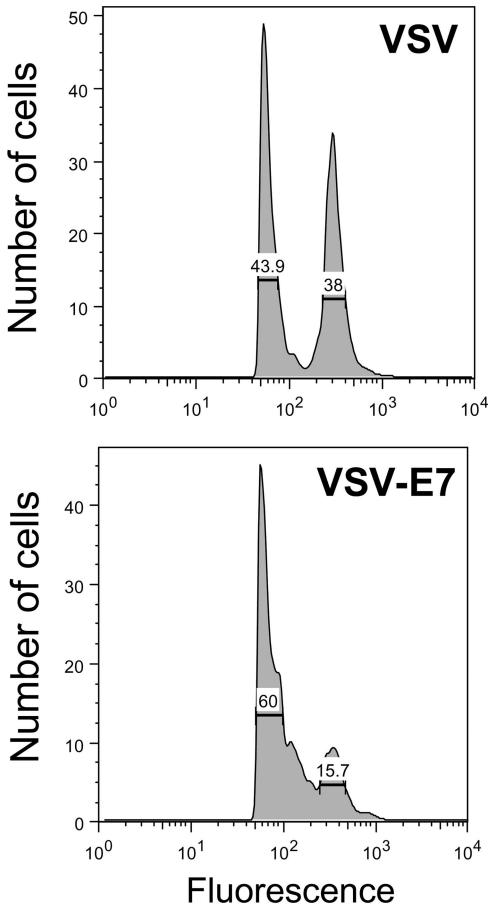

FIG. 5. In vivo CTL assay. Tumor-bearing mice vaccinated with empty-vector rVSV (top) or VSV1-HPV16 E7 (bottom) were injected intravenously with equal numbers of normal splenocytes labeled with CSFE at a high concentration and at a low concentration. Cells at a high CSFE concentration were also pulsed with E7 peptide. Twenty hours later, splenocytes were harvested from the mice and analyzed by flow cytometry for CFSE fluorescence. The numbers indicate the percentage of cells in each peak.

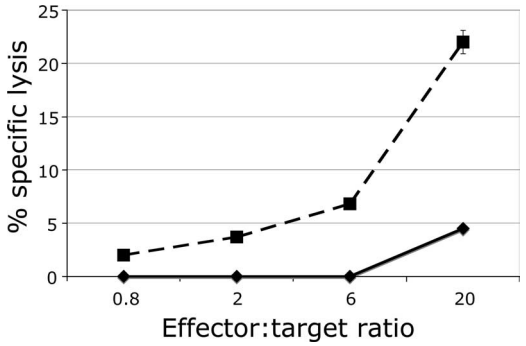

FIG. 6. In vitro CTL assay. Splenocytes from tumor-bearing mice vaccinated with VSV1-HPV16 E7 (dashed line) and empty-vector rVSV (solid line) were grown in culture in the presence of IL-2 and HPV16 E7 peptide to expand any antigen-specific clones. After 4 days, the splenocytes were mixed at the indicated effector/target ratios with calcein-labeled TC-1 target cells. Release of fluorescent dye as evidence of cytolysis was measured in the supernatant. The error bars represent standard errors of samples done in quadruplicate. Target-specific lysis was defined as the percentage of target cells that were recognized and lysed above the background minimum-release level, expressed as a percentage of the maximum possible release.

rVSV, corresponding to a 77% target-specific lysis of the antigen-bearing targets in response to E7 vaccination.

We also performed an in vitro CTL assay against calcein-labeled TC-1 tumor cells. Splenocytes from tumor-bearing mice were harvested 1 week after vaccination with empty VSV vector and VSV1-16E7 and grown for 5 days in culture in the presence of $E7_{49-57}$ and IL-2-conditioned medium. E7-expressing TC-1 tumor cells were incubated with calcein to generate labeled target cells and then incubated with the cultured splenoncytes at various effector/target ratios. Splenocytes from E7-vaccinated mice induced increased dose-dependent release of calcein, indicative of lysis of tumor cells, compared to splenocytes from mice vaccinated with empty VSV vector (Fig. 6). Thus, vaccination with VSV1-16E7 generated cytolytic activity against cells expressing E7, as assessed by in vivo and in vitro assays.

Antitumor effect of therapeutic vaccination. VSV E7 vaccines were studied for their abilities to control TC-1 tumor growth in C57BL/6 mice. The mice were injected subcutaneously with 5×10^4 TC-1 tumor cells. Seven days later, 90 to 100% of the unvaccinated mice developed palpable tumors that grew progressively (data not shown). One week after the inoculation of tumor cells, mice with established tumors were injected intramuscularly with a single dose of 5×10^6 PFU of control rVSV, VSV1-16E7, or VSV1-16E7del. Tumors grew rapidly in mice inoculated with rVSV, whereas tumor growth was significantly retarded in mice that received VSV expressing the E7 protein (Fig. 7, top). Tumor volumes were 10-fold less in the E7-vaccinated group 2 weeks after vaccination. At 14 and 21 days after vaccination, the average tumor volumes of the E7-vaccinated animals were significantly less ($P < 0.01$) than in those vaccinated with the empty vector. Similar results were obtained in three independent experiments with six to eight mice per group.

We constructed VSV1-16E7del in order to test the immunogenicity of an E7 protein unable to bind to the tumor suppressor Rb. This deletion was located outside of the region of the only MHC class I immunodominant epitope in the HPV16 E7. ELISPOT assays carried out 7 days after therapeutic vaccination of tumor-bearing mice showed the generation of E7-specific IFN-γ-secreting splenocytes in mice vaccinated with the E7 deletion, albeit at a lower level than in mice vaccinated with the full-length E7 protein (Fig. 3, right). Vaccination with the E7 deletion VSV had an antitumor effect comparable to vaccination with VSV expressing full-length E7 protein (Fig. 7, top).

When fewer (2.5×10^4) tumor cells were injected, the tumor take rate was 80% (data not shown). Mice with palpable tumors were vaccinated with a single dose of rVSV or VSV1-16E7del. Vaccination with VSV1-16E7del caused the tumors to regress in >80% of the mice (Fig. 8).

Requirement for CD8 T cells for antitumor response. We also determined the requirement for CD8 and CD4 T cells in the antitumor response to vaccination with VSV expressing HPV16 E7. Mice genetically deficient for CD8 or CD4 cells were inoculated with 5×10^4 tumor cells. One week later, these mice were vaccinated with empty-vector rVSV or VSV1-

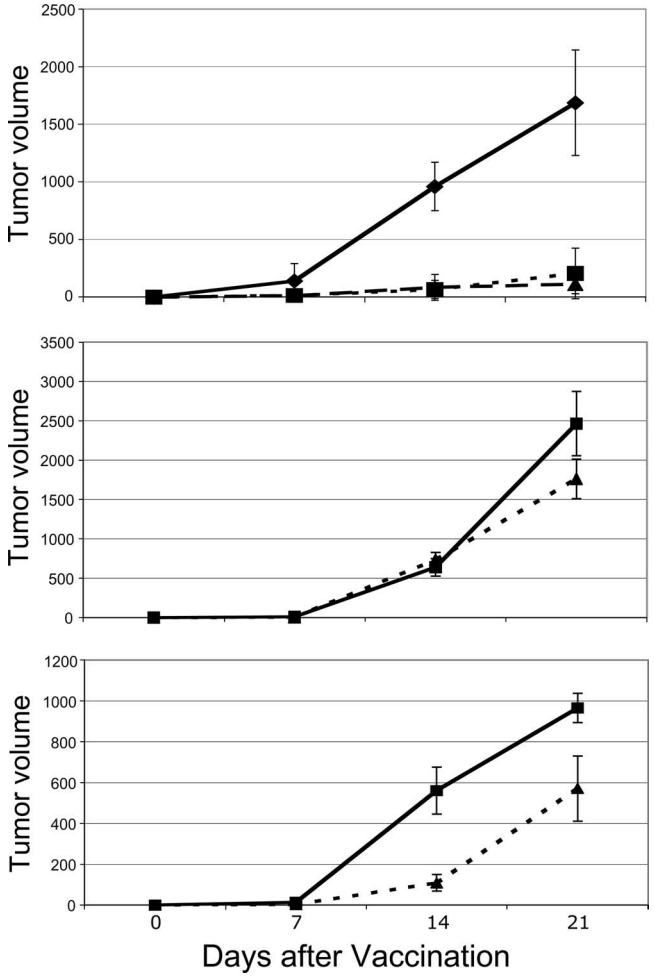

FIG. 7. Antitumor effects of intramuscular vaccination with VSV expressing HPV16 E7 in wild-type mice and in CD4 and CD8 knockout mice. (Top) TC-1 tumor-bearing mice were vaccinated with a single intramuscular injection of VSV1-16E7 (dashed line), VSV1-16E7del (dotted line), or an empty-vector rVSV (solid line). The average tumor volume (mm^3) and standard error for 3 weeks after vaccination are shown. Six to eight mice were used per group. CD8 (middle) and CD4 (bottom) knockout mice injected with TC-1 tumor cells were vaccinated 1 week later with a single intramuscular injection of VSV1-16E7 (dotted line) or an empty-vector rVSV (solid line). The average tumor volume (mm^3) and standard error for 3 weeks after vaccination are shown. Five mice were used per group.

16E7. TC-1 tumor cells grew progressively in mice deficient in CD8 T cells, whether or not they were vaccinated (Fig. 7, middle). At 21 days after vaccination, mean tumor volumes were not statistically different in the treatment group compared to mice receiving the empty vector ($P = 0.21$), indicating that CD8 T cells are required for detectable antitumor responses directed against the HPV16 E7 protein. In contrast, E7 vaccination of mice deficient in CD4 T cells caused an initial delay in tumor formation compared to unvaccinated animals ($P < 0.05$ at 14 days) (Fig. 7, bottom). Tumor growth eventually resumed in E7-vaccinated CD4-deficient mice, suggesting that CD4 cells normally provide a helper function to allow CD8 cells to maintain tumor control.

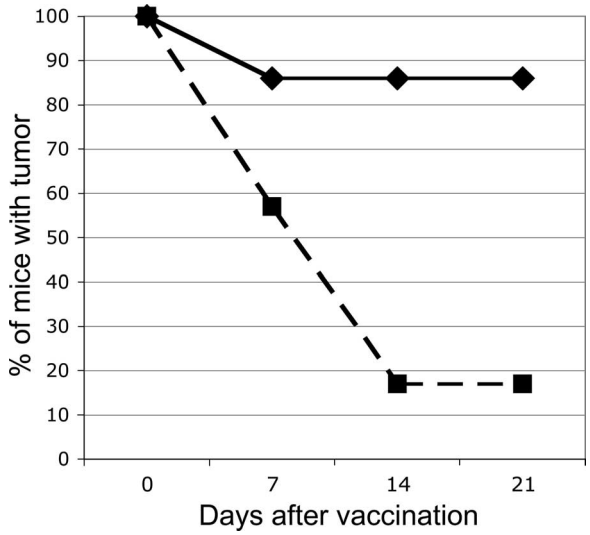

FIG. 8. Therapeutic vaccination with a smaller starting tumor. C57BL/6 mice were injected with 2.5×10^4 TC-1 cells. Mice with palpable tumors were vaccinated 1 week later with a single intramuscular injection of VSV1-16E7del (dashed line) or an empty-vector rVSV (solid line). The mice were examined weekly for tumor growth by palpation for 3 weeks after vaccination. Six to eight mice were used per group.

DISCUSSION

Many tumors express antigens that may form the basis of successful immunotherapy. Although tumor rejection antigens are often poorly defined for sporadically arising tumors, cancers induced by high-risk HPV invariably express the products of two viral oncogenes, E6 and E7, which thus represent well-defined targets for immune attack. Furthermore, continued expression of these proteins is required for the ongoing inactivation of cellular tumor suppression pathways and for the survival and proliferation of the cancer cells, suggesting that cells cannot escape immune killing simply by extinguishing expression of the viral antigens (10, 17). These considerations indicate that HPV-induced tumors may represent good model systems to develop and test therapeutic-vaccination strategies.

Several different viral vector systems can generate robust antitumor immunity in the TC-1 cell–C57BL/6 mouse model of cervical carcinoma. Unfortunately, when these vaccines were tested in humans, they did not demonstrate similar clinical efficacy against cervical cancer or precancerous lesions (2, 27, 34). Thus, it is important to develop and test alternative vaccination strategies. We are developing rVSV vaccine vectors that generate robust humoral immune responses to a variety of antigens in mice, rabbits, and primates. However, there has been relatively little analysis of the abilities of VSV vaccines to generate cellular immunity, particularly in tumor therapy models. We previously reported that VSV vectors expressing CRPV antigens can induce the regression of skin papillomas in rabbits (3, 4). To determine whether this approach could be extended to an antigen of a high-risk HPV in a setting where it is possible to apply cellular measures of immunity, we used the TC-1 cell–C57BL/6 model and showed that a single intramuscular injection of VSV expressing the HPV16 E7 protein generated a readily detectable cellular immune response and

controlled the growth of established E7-expressing tumors in mice. At lower doses of tumor cells, VSV E7 vaccination caused regression of established tumors. Although there was effective tumor control at early times after vaccination, it is possible that tumors might recur during longer follow-up.

While the majority of studies using viral vectors for HPV16 E7 vaccination using mouse models focused on prophylactic vaccination to generate CTLs that could reject a subsequently injected tumor challenge, we conducted therapeutic-vaccination trials, treating mice that already had established tumors. VSV-mediated expression of the HPV16 E7 protein with or without the Rb binding site significantly reduced tumor growth rates in this setting and generated HPV16 E7-specific CTLs, as assessed by a number of immune measures. Therapeutic strategies, such as the use of VSV, which is capable of generating an antitumor response with a single vaccination, should be explored as a way to treat infected patients.

The effectiveness of VSV HPV16 E7 in therapeutically vaccinated mice appears to compare favorably to other published results using the TC-1–C57BL/6 model system. Previous reports of successful therapeutic vaccination using viral vectors to deliver HPV16 E7 have altered the native E7 protein to increase antigen presentation (30, 33) and/or required multiple vaccine doses (30, 46) to demonstrate therapeutic effects. In contrast, our study used a single dose of a vector expressing an unmodified E7 protein. Tumor regression rates were superior to those in all but one earlier study, which used this tumor model with a single dose of a vaccinia virus vaccine expressing an E7 protein fused to a lysosome-associated membrane protein to improve antigen presentation (33). An unaltered E7 protein did not elicit tumor regression in that study. A side-by-side comparison of various vaccination strategies will be required to thoroughly evaluate their relative efficacies in this mouse model.

Our in vitro studies showed that VSV vaccination of mice with preexisting tumors generated a stronger cellular immune response than vaccination of non-tumor-bearing mice. This suggests that tumor cells prime antitumor responses that can be boosted by vaccination with VSV. This property may facilitate the treatment of cervical-cancer patients with tumors that have advanced beyond surgical resection and cure, perhaps as an adjuvant to conventional chemotherapy and radiotherapy. Tumor priming was first observed in the setting of ex vivo expansion of T cells from melanoma patients stimulated with MART-1, a differentiation antigen found in both normal and neoplastic cells of melanocytic origin. There was enhanced CTL response against MART-1 in lymphocytes from tumor-bearing patients compared to those from healthy patients (35). Our observations of a similar phenomenon in VSV vaccination with HPV16 E7 has not been reported in other HPV therapeutic-vaccination studies and needs further dissection.

Therapeutic vaccination using VSV expressing HPV16 E7 shows promise as a treatment strategy for cervical carcinoma and other HPV-associated cancers, such as some head and neck squamous cell carcinomas. The generation of effective antitumor responses with a single vaccine dose suggests that this method may have the potential to eliminate multiple patient visits for vaccine administration. Furthermore, since these responses were generated without alteration of the E7 antigen itself or coexpression of other immune-stimulatory proteins, as described for other therapeutic-vaccination strategies, VSV vaccination may be further improved by using these means of enhancing antigen presentation. These considerations also suggest that VSV may be a suitable vaccine vector in other immunotherapy situations.

ACKNOWLEDGMENTS

J.B.L. is a Berlex-NICHD Scholar of the Reproductive Scientist Development Program supported by NIH grant 5K12HD00849 and the Berlex Foundation. This work was also supported by grants to J.B.L. from the American Cancer Society (IRG-58-012-48) and to D.D. from the NIH (CA16038) and by a generous gift from Laurel Schwartz.

REFERENCES

1. **Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy.** 1987. Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. EMBO J. **6:**989–992.
2. **Baldwin, P. J., S. H. van der Burg, C. M. Boswell, R. Offringa, J. K. Hickling, J. Dobson, J. S. Roberts, J. A. Latimer, R. P. Moseley, N. Coleman, M. A. Stanley, and J. C. Sterling.** 2003. Vaccinia-expressed human papillomavirus 16 and 18 E6 and E7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin. Cancer Res. **9:**5205–5213.
3. **Brandsma, J. L., M. Shylankevich, L. Buonocore, A. Roberts, S. M. Becker, and J. K. Rose.** 2007. Therapeutic efficacy of vesicular stomatitis virus-based E6 vaccination in rabbits. Vaccine **25:**751–762.
4. **Brandsma, J. L., M. Shylankevich, Y. Su, A. Roberts, J. K. Rose, D. Zelterman, and L. Buonocore.** 2007. Vesicular stomatitis virus-based therapeutic vaccination targeted to the E1, E2, E6, and E7 proteins of cottontail rabbit papillomavirus. J. Virol. **81:**5749–5758.
5. **Brinkman, J. A., X. Xu, and W. M. Kast.** 2007. The efficacy of a DNA vaccine containing inserted and replicated regions of the E7 gene for treatment of HPV-16 induced tumors. Vaccine **25:**3437–3444.
6. **Coleman, N., H. D. Birley, A. M. Renton, N. F. Hanna, B. K. Ryait, M. Byrne, D. Taylor-Robinson, and M. A. Stanley.** 1994. Immunological events in regressing genital warts. Am. J. Clin. Pathol. **102:**768–774.
7. **Coles, R. M., S. N. Mueller, W. R. Heath, F. R. Carbone, and A. G. Brooks.** 2002. Progression of armed CTL from draining lymph node to spleen shortly after localized infection with herpes simplex virus 1. J. Immunol. **168:**834–838.
8. **Curtsinger, J. M., D. C. Lins, and M. F. Mescher.** 2003. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. J. Exp. Med. **197:**1141–1151.
9. **Daemen, T., F. Pries, L. Bungener, M. Kraak, J. Regts, and J. Wilschut.** 2000. Genetic immunization against cervical carcinoma: induction of cytotoxic T lymphocyte activity with a recombinant alphavirus vector expressing human papillomavirus type 16 E6 and E7. Gene Ther. **7:**1859–1866.
10. **DiMaio, D., and J. Liao.** 2006. Human papillomaviruses and cervical cancer. Adv. Virus Res. **66:**125–159.
11. **Evans, E. M., S. Man, A. S. Evans, and L. K. Borysiewicz.** 1997. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res. **57:**2943–2950.
12. **Feltkamp, M. C., H. L. Smits, M. P. Vierboom, R. P. Minnaar, B. M. de Jongh, J. W. Drijfhout, J. ter Schegget, C. J. Melief, and W. M. Kast.** 1993. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur. J. Immunol. **23:**2242–2249.
13. **Frazer, I. H.** 2004. Prevention of cervical cancer through papillomavirus vaccination. Nat. Rev. Immunol. **4:**46–54.
14. **Frisch, M., R. J. Biggar, and J. J. Goedert.** 2000. Human papillomavirus-associated cancers in patients with human immunodeficiency virus infection and acquired immunodeficiency syndrome. J. Natl. Cancer Inst. **92:**1500–1510.
15. **Gage, J. R., C. Meyers, and F. O. Wettstein.** 1990. The E7 proteins of the nononcogenic human papillomavirus type 6B (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. J. Virol. **64:**723–730.
16. **Ghosh, A. K., and M. Moore.** 1992. Tumour-infiltrating lymphocytes in cervical carcinoma. Eur. J. Cancer **28A:**1910–1916.
17. **Goodwin, E. C., and D. DiMaio.** 2000. Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc. Natl. Acad. Sci. USA **97:**12513–12518.
18. **Haglund, K., I. Leiner, K. Kersiek, L. Buonocore, E. Pamer, and J. K. Rose.** 2002. High-level primary $CD8^+$ T-cell response to human immunodeficiency virus type 1 gag and env generated by vaccination with recombinant vesicular stomatitis viruses. J. Virol. **76:**2730–2738.
19. **Haglund, K., I. Leiner, K. Kersiek, L. Buonocore, E. Pamer, and J. K. Rose.**

2002. Robust recall and long-term memory T-cell responses induced by prime-boost regimens with heterologous live viral vectors expressing human immunodeficiency virus type 1 Gag and Env proteins. J. Virol. **76:**7506–7517.
20. **Halpert, R., R. G. Fruchter, A. Sedlis, K. Butt, J. G. Boyce, and F. H. Sillman.** 1986. Human papillomavirus and lower genital neoplasia in renal transplant patients. Obstet. Gynecol. **68:**251–258.
21. **Harper, D. M., E. L. Franco, C. Wheeler, D. G. Ferris, D. Jenkins, A. Schuind, T. Zahaf, B. Innis, P. Naud, N. S. De Carvalho, C. M. Roteli-Martins, J. Teixeira, M. M. Blatter, A. P. Korn, W. Quint, and G. Dubin.** 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet **364:**1757–1765.
22. **He, Z., A. P. Wlazlo, D. W. Kowalczyk, J. Cheng, Z. Q. Xiang, W. Giles-Davis, and H. C. Ertl.** 2000. Viral recombinant vaccines to the E6 and E7 antigens of HPV-16. Virology **270:**146–161.
23. **Hildesheim, A., R. Herrero, S. Wacholder, A. C. Rodriguez, D. Solomon, M. C. Bratti, J. T. Schiller, P. Gonzales, G. Dubin, C. Porras, S. E. Jimenez, D. R. Lowy, and the Costa Rican HPV Vaccine Trial Group.** 2007. Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection: a randomized trial. JAMA **298:**743–753.
24. **Iverson, L. E., and J. K. Rose.** 1981. Localized attenuation and discontinuous synthesis during vesicular stomatitis virus transcription. Cell **23:**477–484.
25. **Kadish, A. S., G. Y. Ho, R. D. Burk, Y. Wang, S. L. Romney, R. Ledwidge, and R. H. Angeletti.** 1997. Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia. J. Natl. Cancer Inst. **89:**1285–1293.
26. **Kast, W. M., R. M. Brandt, J. W. Drijfhout, and C. J. Melief.** 1993. Human leukocyte antigen-A2.1 restricted candidate cytotoxic T lymphocyte epitopes of human papillomavirus type 16 E6 and E7 proteins identified by using the processing-defective human cell line T2. J. Immunother. Emphasis Tumor Immunol. **14:**115–120.
27. **Kaufmann, A. M., P. L. Stern, E. M. Rankin, H. Sommer, V. Nuessler, A. Schneider, M. Adams, T. S. Onon, T. Bauknecht, U. Wagner, K. Kroon, J. Hickling, C. M. Boswell, S. N. Stacey, H. C. Kitchener, J. Gillard, J. Wanders, J. S. Roberts, and H. Zwierzina.** 2002. Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 E6 and E7 genes, in women with progressive cervical cancer. Clin. Cancer Res. **8:**3676–3685.
28. **Khammanivong, V., X. S. Liu, W. J. Liu, S. J. Rodda, G. R. Leggatt, R. W. Tindle, I. H. Frazer, and G. J. Fernando.** 2003. Paucity of functional CTL epitopes in the E7 oncoprotein of cervical cancer associated human papillomavirus type 16. Immunol. Cell Biol. **81:**1–7.
29. **Koutsky, L. A., K. A. Ault, C. M. Wheeler, D. R. Brown, E. Barr, F. B. Alvarez, L. M. Chiacchierini, and K. U. Jansen.** 2002. A controlled trial of a human papillomavirus type 16 vaccine. N. Engl. J. Med. **347:**1645–1651.
30. **Lamikanra, A., Z. K. Pan, S. N. Isaacs, T. C. Wu, and Y. Paterson.** 2001. Regression of established human papillomavirus type 16 (HPV-16) immortalized tumors in vivo by vaccinia viruses expressing different forms of HPV-16 E7 correlates with enhanced $CD8^+$ T-cell responses that home to the tumor site. J. Virol. **75:**9654–9664.
31. **Lawson, N. D., E. A. Stillman, M. A. Whitt, and J. K. Rose.** 1995. Recombinant vesicular stomatitis viruses from DNA. Proc. Natl. Acad. Sci. USA **92:**4477–4481.
32. **Lichtenfels, R., W. E. Biddison, H. Schulz, A. B. Bogt, and R. Martin.** 1994. CARE-LASS (calcein-release-assay), an improved fluorescence-based test system to measure cytotoxic T lymphocyte activity. J. Immunol. Methods **172:**227–239.
33. **Lin, K. Y., F. G. Guarnieri, K. F. Staveley-O'Carroll, H. I. Levitsky, J. T. August, D. M. Pardoll, and T. C. Wu.** 1996. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. Cancer Res. **56:**21–26.
34. **Liu, M., B. Acres, J. M. Balloul, N. Bizouarne, S. Paul, P. Slos, and P. Squiban.** 2004. Gene-based vaccines and immunotherapies. Proc. Natl. Acad. Sci. USA **101:**14567–14571.
35. **Marincola, F. M., L. Rivoltini, M. L. Salgaller, M. Player, and S. A. Rosenberg.** 1996. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of in vivo priming by tumor cells. J. Immunother. Emphasis Tumor Immunol. **19:**266–277.
36. **Palefsky, J., and E. Holly.** 2003. Immunosuppression and co-infection with HIV. J. Natl. Cancer Inst. Monogr. **31:**41–46.
37. **Parkin, D. M., F. Bray, J. Ferlay, and P. Pisani.** 2005. Global cancer statistics, 2002. CA Cancer J. Clin. **55:**74–108.
38. **Petry, K. U., D. Scheffel, U. Bode, T. Gabrysiak, H. Kochel, E. Kupsch, M. Glaubitz, S. Niesert, H. Kuhnle, and I. Schedel.** 1994. Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. Int. J. Cancer **57:**836–840.
39. **Psyrri, A., R. A. DeFilippis, A. P. B. Edwards, K. E. Yates, L. Manuelidis, and D. DiMaio.** 2004. Role of the retinoblastoma pathway in senescence triggered by repression of the human papillomavirus E7 protein in cervical carcinoma cells. Cancer Res. **64:**3079–3086.
40. **Publicover, J., E. Ramsburg, and J. K. Rose.** 2004. Characterization of nonpathogenic, live, viral vaccine vectors inducing potent cellular immune responses. J. Virol. **78:**9317–9324.
41. **Reuter, J. D., B. E. Vivas-Gonzalez, D. Gomez, J. H. Wilson, J. L. Brandsma, H. L. Greenstone, J. K. Rose, and A. Roberts.** 2002. Intranasal vaccination with a recombinant vesicular stomatitis virus expressing cottontail rabbit papillomavirus L1 protein provides complete protection against papillomavirus-induced disease. J. Virol. **76:**8900–8909.
42. **Roden, M. M., K. H. Lee, M. C. Panelli, and F. M. Marincola.** 1999. A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology. J. Immunol. Methods **226:**29–41.
43. **Santin, A. D., S. Bellone, M. Palmieri, A. Ravaggi, C. Romani, R. Tassi, J. J. Roman, A. Burnett, S. Pecorelli, and M. J. Cannon.** 2006. HPV16/18 E7-pulsed dendritic cell vaccination in cervical cancer patients with recurrent disease refractory to standard treatment modalities. Gynecol. Oncol. **100:**469–478.
44. **Schnell, M. J., J. E. Johnson, L. Buonocore, and J. K. Rose.** 1997. Construction of a novel virus that targets HIV-1 infected cells and controls HIV-1 infection. Cell **90:**849–857.
45. **Vambutas, A., J. DeVoti, M. Nouri, J. W. Drijfhout, G. B. Lipford, V. R. Bonagura, S. H. van der Burg, and C. J. Melief.** 2005. Therapeutic vaccination with papillomavirus E6 and E7 long peptides results in the control of both established virus-induced lesions and latently infected sites in a preclinical cottontail rabbit papillomavirus model. Vaccine **23:**5271–5280.
46. **Velders, M. P., S. McElhiney, M. C. Cassetti, G. L. Eiben, T. Higgins, G. R. Kovacs, A. G. Elmishad, W. M. Kast, and L. R. Smith.** 2001. Eradication of established tumors by vaccination with Venezuelan equine encephalitis virus replicon particles delivering human papillomavirus 16 E7 RNA. Cancer Res. **61:**7861–7867.
47. **Wu, T. C.** 2007. Therapeutic human papillomavirus DNA vaccination strategies to control cervical cancer. Eur. J. Immunol. **37:**310–314.
48. **Zehbe, I., E. Wilander, H. Delius, and M. Tommasino.** 1998. Human papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res. **58:**829–833.