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TriVax: an improved peptide-based therapeutic vaccination strategy against Human Papillomavirus-induced cancers

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Abstract

Background—Therapeutic vaccines for cancer are an attractive alternative to conventional therapies, since the later result in serious adverse effects and in most cases are not effective against advanced disease. Human Papillomavirus (HPV) is responsible for several malignancies such as cervical carcinoma. Vaccines targeting oncogenic viral proteins like HPV16-E6 and -E7 are ideal candidates to elicit strong immune responses without generating autoimmunity because: *i*) these products are not expressed in normal cells; and *ii*) their expression is required to maintain the malignant phenotype. Our group has developed peptide vaccination strategy called TriVax, which is effective in generating vast numbers of antigen-specific T cells in mice capable of persisting for long time periods.

Materials and methods—We have used two HPV-induced mouse cancer models (TC-1 and C3.43) to evaluate the immunogenicity and therapeutic efficacy of TriVax prepared with the immunodominant CD8 T cell epitope HPV16-E7_{49–57}, mixed with poly-IC adjuvant and costimulatory anti-CD40 antibodies.

Results—TriVax using HPV16-E7_{49–57} induced large and persistent T cell responses that were therapeutically effective against established HPV16-E7 expressing tumors. In most cases, TriVax was successful in attaining complete rejections of 6–11 day established tumors. In addition, TriVax induced long-term immunological memory, which prevented tumor recurrences. The antitumor effects of TriVax were independent of NK and CD4 T cells and surprisingly, did not rely to a great extent on type-I or type-II interferon.

Conclusions—These findings indicate that the TriVax strategy is an appealing immunotherapeutic approach for the treatment of established viral-induced tumors. We believe that these studies may help to launch more effective and less invasive therapeutic vaccines for HPV-mediated malignancies.

Keywords

Peptide vaccines; HPV; cervical cancer; CD8 T cells; PIVAC 11

Introduction

Cervical cancer is the second most prevalent cancer among women. Human Papillomavirus (HPV) causes 99% of cervical cancer (CC) and the HPV16 and HPV18 genotypes account

for 70% these cases [1]. Worldwide, CC is responsible for ~ 250 000 deaths per year and for causing a huge economic burden in related health care costs [2,3]. The current approved prophylactic vaccines for HPV are not useful for treating established malignant disease, nor can protect already infected individuals from developing cancer [4–6]. Most importantly, a significant proportion of women, especially in the third world, have not nor will receive the prophylactic vaccines and therefore, will continue to be at high risk of developing CC [7]. Because, conventional therapies for CC are usually devastating, invasive, toxic and associated with 10% recurrence [8], there is an urgent need for developing alternative treatments such as immunotherapy and more specifically, therapeutic vaccines.

CD8 T lymphocytes are the most effective components of the adaptive immune system capable of recognizing and destroying viral-infected and transformed malignant cells [9–11]. The antigens recognized by CD8 T cells on their target cells are small peptides derived from viral or tumor-associated antigens (TAAs) that associate with cell surface class I products of the major histocompatibility complex (MHC-I). In the case of cells transformed by HPV, peptide sequences derived from the oncogenic E6 and E7 viral proteins have been shown to represent suitable TAAs for CC and are considered as ideal candidates for developing therapeutic vaccines [12–18]. Synthetic peptides representing these TAAs have been tested in numerous ways in human patients and mouse cancer models for their ability to generate anti-tumor T cell responses capable of exhibiting anti-tumor effects [17,19,12,20,21,16]. However, in most instances only modest T cell responses capable of dealing with very early disease stages were obtained indicating that improved peptide-based immunization strategies need to be developed to have a significant impact against established and advanced disease stages.

Our laboratory has recently described an improved peptide vaccination strategy capable of generating in mice vast numbers of CD8 T cells capable of persisting for long time periods [22,23]. This vaccine called TriVax consists of a synthetic peptide corresponding to the minimal T cell epitope, poly-IC adjuvant and costimulatory monoclonal anti-CD40 antibodies (α CD40 mAb), which are mixed together and administered intravenously. After two sequential TriVax immunizations (prime/boost) with the well-known Ova_{257–264} T cell epitope up to 80% of all the CD8 T cells in blood were antigen specific and more than half of these cells persisted for at least 60 days [23]. The goal of the present study was to evaluate the efficacy of TriVax in an HPV cancer mouse model. The results demonstrate that TriVax using peptide HPV16-E7_{49–57} induced large and persistent T cell responses that were effective against two different tumors expressing HPV16-E7. Interestingly, the antitumor effects of TriVax in this tumor model appeared to be independent of NK and CD4 T cells and did not rely to a great extent on either type-I or -II interferons. We believe that the results from these studies may help to develop more effective therapeutic vaccines for CC.

Material and methods

Mice

Six- to nine-week-old female C57BL/6 (B6), mice were obtained from the National Cancer Institute/Charles River program (Wilmington, MA). Interferon-gamma (IFN γ) knockout (KO) mice in the B6 background were purchased from Jackson Laboratories (Bar Harbor, ME). IFN $\alpha\beta$ receptor-KO (IFN $\alpha\beta$ R KO) mice also in the B6 background were obtained from Dr. Philippa Marrack (National Jewish Medical and Research Center, Denver, CO). All animal care and experiments were conducted according to our institutional animal care and use committee guidelines.

Cell lines

TC-1 tumor cells, obtained from Dr. T-C Wu (Johns Hopkins University, Baltimore, MD), were derived from primary lung epithelial cells of B6 mice and express HPV16-E6 and E7 proteins [24]. The C3.43 tumor cell line obtained from Dr. W. M. Kast (University of Southern California, Los Angeles, CA) is an aggressive derivative of the C3 line (B6 background), which was transformed using a pRSVneo-derived plasmid containing the complete HPV16 genome [19]. The EL4 cell line was purchased from the American Type Culture Collection (Manassas, VA). All cells were maintained in tissue culture following the recommendations of the providers.

Peptides, MHC-I tetramer and antibodies

The synthetic peptide RAHYNIVTF from HPV16-E7 (E7_{49–57}), defined as an immunodominant H₂Db-restricted CD8 T cell epitope [19,25], and control peptide NAYVFKGL from chicken ovalbumin (Ova_{176–183}) were purchased as >95% pure from A&A Labs (San Diego, CA). Rat anti-mouse CD40 (FGK45.5) anti-NK1.1 (PK136), anti-CD4 (GK1.5) and anti-CD8 (2.43) monoclonal antibodies were prepared from hybridoma culture supernatants. The E7_{49–57}/H-2Db tetramers labeled with Alexa 647 were provided by the National Institute of Allergy and Infectious Disease Tetramer Facility at the Emory University (Atlanta, GA from NIH). Fluorochrome labeled antibodies specific for mouse CD8a (53–6.7) and MHC-II (M5/114.15.2) were from eBioscience, Inc (San Diego, CA).

Immunizations

Mice were usually vaccinated via the *i.v.* route (unless otherwise noted). TriVax consisted of a mixture of 30 µg of the E7_{49–57} peptide, 100 µg αCD40 mAb and 50 µg of poly IC (Poly-ICLC, Oncovir, Inc.). BiVax contained only the peptide and poly-IC at the same amounts. In all cases, mice are given two sequential vaccinations 13 days apart (prime and boost). In some cases mice received peptide alone or peptide with αCD40 mAb.

Immunological assays

For tetramer staining, either peripheral blood samples (~3–5 drops) taken from the submandibular vein, or splenocytes were stained with a mixture of antibodies to MHC-II, CD8a (eBioscience; San Diego, CA), and tetramer for 40 min in ice. After washing with three times, the fluorescence was evaluated using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Ashland, OR). Results are presented as % tetramer positive cells of the CD8+/MHC-II negative population. To determine if CD8 T were able to recognize tumor cell lines (TC-1, C3.43) expressing the naturally processed peptide, IFNγ, enzyme-linked immunosorbent spot (EliSpot) assays were performed as described [26]. Briefly, CD8 T cells from spleens of vaccinated mice were purified by positive selection using antibody-coated magnetic (Miltenyi Biotec, Auburn, CA). Responder (CD8-purified) cells were incubated at 3×10⁵, 1×10⁵, and 3×10⁴ per well, together with 1×10⁵ stimulator cells (EL4, plus/minus peptide, TC-1 and C3.43 cells pretreated or not for 24 h with 100 ng/ml IFNγ). Cultures were incubated at 37 °C for 20 h, and spots (IFNγ producing cells) were developed as described by the EliSpot kit manufacturer (Mabtech, Inc., Mariemont, OH). Spot counting was done with an AID EliSpot Reader System (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Evaluation of therapeutic anti-tumor effects

Mice received 3×10⁵/mouse tumor cells (TC-1 or C3.43) *s.c.* in a shaved rear flank 6 or 11 days (as noted) before their first immunization. In some instances, survivor mice were re-challenged with the same number of tumor cells (in opposite flanks). To determine the contribution of different subsets of lymphocytes the anti tumor effect of the vaccine, NK,

CD4 and CD8 cell antibody-depleted mice and KO mice were compared with B6 wild type (WT) mice. For cell depletions each mouse received 300 μ g anti-NK1.1, 300 μ g anti-CD4 or 500 μ g anti-CD8 twice on days -2 and 0 before immunization. Depletions were confirmed by analysis of blood samples using flow cytometry (data not presented). Tumor growth was monitored every 2–4 days in individual tagged mice by measuring 2 opposing diameters with a set of calipers. Mice were euthanized when the tumors area reached 400 mm². Results are presented as the mean tumor size (area in mm²) $\pm$ SD for every treatment group at various time points until the termination of the experiment.

Statistical analyses

Statistical significance to assess numbers of antigen specific CD8 T cells (EliSpot), cytokine levels (ELISA) absolute number of lung tumor nodules unpaired Student's *t* tests. Tumor sizes between 2 populations throughout time were analyzed for significance using 2-way ANOVA tests. All analysis and graphics were done using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA).

Results

Evaluation of TriVax immunization using a peptide epitope from HPV16-E7

Residues 49–57 of the HPV16-E7 protein (RAHYNIVTF) correspond an immunodominant CD8 T cell epitope restricted by the H-2Db MHC-I molecule [19,25]. We first determined the ability of synthetic peptide E7_{49–57} representing this sequence to elicit an immune response when administered to mice in combination with poly-IC and α CD40 mAb, a vaccine formulation known as TriVax. In addition, we compared the immunogenicity of vaccines containing peptide alone, peptide plus poly-IC (BiVax) or peptide plus α CD40 mAb. Antigen-specific immune responses were measured using tetramer analysis six days after the prime and booster immunizations and at various time points thereafter. As shown in Figure 1a TriVax and BiVax induced very high number of antigen specific (tetramer positive) CD8 T cells after the booster immunization, which persisted at high levels for several weeks (Fig. 1b). On the other hand, administration of the E7_{49–57} peptide alone or in combination with α CD40 mAb failed to generate a significant immune response. More importantly, spleen CD8 T cells from TriVax and BiVax immunized mice were shown to be very effective in recognizing E7_{49–57} peptide-pulsed target cells (EL4) and TC-1 tumor cells, which express the HPV16-E7 protein and naturally process the antigen (Fig. 1c).

Anti-tumor effectiveness of TriVax

The therapeutic anti-tumor effects of the 4 modes of peptide vaccination were assessed. Mice bearing 6-day established TC-1 tumors received the peptide vaccines (prime/boost) and tumor sizes and immune responses were measured at various time points. As shown on Figure 2a, tumor growth was effectively controlled in those mice that received TriVax, BiVax and to some extent, peptide E7_{49–57} with α CD40 mAb, as compared to mice vaccinated with E7_{49–57} alone (where tumors grew at an accelerated rate and mice had to be euthanized by day 22). Notably, 100% of the mice that received TriVax and 33% of those receiving BiVax completely rejected their tumors, while in the case of the mice immunized with E7_{49–57} with α CD40 mAb, although the tumors grew at a slow rate, none were rejected. Measurements of the immune responses elicited by these vaccines in the tumor-bearing mice (Fig. 2b) indicated that both TriVax and BiVax induced high numbers of persisting E7_{49–57}-specific CD8 T cells. Interestingly, the differences between TriVax and BiVax were less apparent as compared to those observed in tumor-free mice (Fig. 1b). E7_{49–57} with α CD40 mAb induced a small CD8 T cell response (between 3 and 5% tetramer positive cells) but apparently strong enough to reduce the tumor growth. No appreciable immune response was observed in mice that received E7_{49–57} alone. The therapeutic effect

of TriVax with E7₄₉₋₅₇ was antigen specific since TriVax prepared with an irrelevant peptide (Ova₁₇₆₋₁₈₃) was not effective in reducing the rate of tumor growth (Fig. 2c). The therapeutic effects observed with E7₄₉₋₅₇ BiVax (Fig. 1a) were confirmed in an additional experiment using a larger number of mice (6), where in this case, this therapeutic vaccination strategy resulted 50% complete tumor rejections (Fig. 2d). Moreover, when BiVax was administered three times (prime plus 2 boosts, 7 days apart), rejections were observed in 100% of the mice (data not presented).

The impressive therapeutic anti-tumor effects observed with E7₄₉₋₅₇ TriVax was evaluated against larger and more established TC-1 tumors. The results shown in Figure 3a demonstrate that therapeutic immunization with TriVax 11 days post tumor inoculation resulted in 100% complete rejections and increased survival. The therapeutic effectiveness of E7₄₉₋₅₇ TriVax was also examined using a different tumor cell line called C3.43, which also expresses the HPV16- E7 antigen. As presented in Figure 3b, TriVax using the E7₄₉₋₅₇ peptide resulted in complete rejection of 6-day established C3.43 tumors. Furthermore, at the conclusion of this experiment (day 30) considerable numbers of E7₄₉₋₅₇-specific CD8 T cells remained present on the spleens of the TriVax immunized mice (Fig. 3c). Moreover, the spleen CD8 T cells from the TriVax immunized mice that rejected the C3.43 tumors were effective in recognizing C3.43 and TC-1 tumors and such recognition was increased to some extent by interferon-gamma (IFN γ) pretreatment of the tumor cells (Fig. 3d), which enhances the expression levels of MHC-I on both tumors (data not shown).

TriVax generates immunological memory that prevents tumor recurrences

An effective therapeutic vaccination strategy should generate immune responses capable, not only of eliminating tumor masses, but also should be successful in preventing tumor recurrences by generating long-term immune memory. Thus, we evaluated whether E7₄₉₋₅₇ TriVax treated mice that had successfully rejected TC-1 tumors, would be able to respond to subsequent tumor re-challenges. The experiment shown in Figure 3e shows that ~1 month after rejecting their original TC-1 tumors, TriVax immunized mice successfully resisted second tumor challenges using TC-1 and C3.43 cells, which were given separately to each mouse in opposite posterior flanks. Unvaccinated control mice developed both tumor challenges, which grew at an accelerated rate.

Effect of route of administration in TriVax

The results presented so far were obtained using immunizations that were administered intravenously (*i.v.*) since the goal was to generate a systemic immune response. However, since vaccines are generally not administered via the *i.v.* route, we examined whether TriVax would be effective against 6-day established TC-1 tumors if it were administered intramuscularly (*i.m.*). As shown in Figure 4a *i.m.* E7₄₉₋₅₇ TriVax induced tumor clearance of 100% of treated mice while tumors in the non-vaccinated mice grew at a fast rate. Notably, the frequency of antigen-specific (tetramer positive) CD8 T cells in blood of these mice, measured 11 days after the booster immunization was rather low (range 2.3 – 10.5 %, Fig. 4b), compared to the values we routinely observed in mice immunized *i.v.* (>50%). In addition, one of the six *i.m.* vaccinated mice in this experiment (the one with the lowest numbers of antigen-specific CD8 T cells), developed a tumor recurrence at the original injection site on day 66 (not shown) suggesting that the tumor had not been totally eradicated.

Mechanism of anti-tumor effects of TriVax

Lastly, to assess the roles of various lymphocyte subsets and the requirements of effector cytokines, the anti-tumor efficacy of E7₄₉₋₅₇ TriVax was examined in CD8, CD4 and NK depleted mice and in IFN γ KO and IFN $\alpha\beta$ R KO mice. The results in Figure 5a show, as

expected, that mice depleted of CD8 T cells were unable to control tumor growth and closely resembled the non-vaccinated mice. On the other hand, 100% of the CD4 and NK cell depleted mice rejected their tumors. Surprisingly, the TriVax immunized IFN γ KO and IFN $\alpha\beta$ KO mice were able to control tumor growth to a great degree and in some instances complete rejections were observed (Fig. 5b). When the intensity of the CD8 T cell responses in immunized IFN γ $\alpha\beta$ and IFN $\alpha\beta$ KO tumor-free mice was evaluated after TriVax immunization (prime/boost), it became evident that absence of IFN γ had little effect, while the role of type-I IFN in generating high CD8 T cell numbers was clearly more important (Fig. 5c).

Discussion

The goal of the present studies was to assess the effectiveness of a new and potent peptide vaccination strategy for its therapeutic effectiveness against tumors expressing the HPV16-E7 oncogene product. This viral product is an ideal TAA for developing T cell based immunotherapy against HPV transformed cells because it is recognized as a foreign antigen. Thus, the lack of immunological tolerance allows the generation of high avidity T cell responses, which contrasts with lower avidity T cells generated against other types of TAAs that are expressed by normal tissues (*e.g.*, melanosomal products, p53, HER2/*neu*). We have reported that TriVax using epitopes from TAA such as Trp1 and Trp2 that are expressed on normal cells although generate large numbers of antigen-specific T cells that diminish tumor growth, are incapable of rejecting established tumors in WT mice [23,27].

Because the HPV16-E7 protein plays a critical role in maintaining the transformed phenotype of the tumor cells [28,29] the possible appearance of antigen-loss mutants, which is often seen in immunotherapy [10] should be diminished. The results presented here using two HPV16 mouse tumor models (TC-1 and C3.43) demonstrate that immunization with a synthetic peptide representing an exact CD8 T cell epitope in combination with poly-IC and α CD40 mAb (TriVax) resulted in extensive antigen-specific T cell responses that were durable and capable of eradicating established tumors. Others have reported that vaccines prepared with the minimal CD8 T cell epitopes, especially when administered systemically, are ineffective because the peptides can be presented by non-professional antigen presenting cells (APCs) resulting in T cell tolerance [30–32]. For this reason, some investigators advocate the use of long synthetic peptides that require antigen processing by professional APCs [20]. The present results and previous work by our group [22,23] demonstrate that vaccines containing minimal CD8 T cell epitopes can be highly immunogenic when provided together with Poly-IC and α CD40 mAb and in some cases with Poly-IC alone (Fig. 1). In fact to our best knowledge, the magnitude of the responses we have observed with several minimal CD8 T cell epitopes using TriVax (HPV16-E7_{49–57}, Trp1_{455–463/9M}, Trp2_{180–188}, Ova_{257–264}, rNEU_{66–74}) is far superior to what has been reported using other peptide vaccines (with either short or long synthetic peptides), when administered with conventional adjuvants (*e.g.*, IFA), pulsed onto DCs or when using recombinant DNA vaccines. Although the magnitude of the T cell responses achieved with TriVax in mice is impressive, we do not know if similar effects can be accomplished in humans following the same strategy. Moreover, it will be important to assess and closely monitor those potential flu-like toxic effects that could accompany the generation of large numbers of activated T cells.

Although the combination of all three components of TriVax was clearly the most effective therapeutic strategy, possibly because it generated the strongest immune responses (Fig. 1a–b), immunization with peptide and poly-IC (BiVax) or peptide plus α CD40 mAb were also quite effective in eliciting CD8 T cell responses that resulted in significant therapeutic benefit (Figs. 1–2). These results indicate that the α CD40 mAb potentiates the effectiveness

of TriVax, but is not essential and that poly-IC plays the major role of adjuvant in this vaccination strategy. It is possible that with additional immunization boosters, that the efficacy of BiVax could improve to the level of TriVax to attain 100% tumor rejections by increasing T cell numbers. On the other hand, we cannot rule out that the α CD40 mAb may facilitate the generation of CD8 T cells with increased function and that simply inducing high numbers of T cells will be insufficient to achieve maximal therapeutic responses. Notwithstanding, our results indicate that even low numbers of antigen-specific CD8 T cells, which were induced by peptide plus α CD40 mAb, and TriVax administered *i.m.* were sufficient to effectively control the rate of tumor growth. However, complete and durable rejections in all mice were only achieved when high numbers of antigen-specific CD8 T cells were produced.

The rationale for the use of α CD40 mAb in the generation of CD8 T cell responses is to provide the strong costimulatory signal to antigen-presenting dendritic cells (DCs), which is usually supplied by CD40 ligand (CD40L) expressing CD4 T helper cells. The CD40/CD40L costimulatory interaction has been proposed to be critical for the generation of memory CD8 T cells capable of persisting for long time periods [33]. Nevertheless, BiVax showed to be effective in generating high numbers of long-lasting CD8 T cell responses, presumably in the absence of CD40 ligation. Furthermore, depletion of CD4 T cells does not decrease (but slightly increases) the generation of antigen-specific CD8 T cells by BiVax (data not presented), ruling out that this vaccine somehow also stimulates CD4 T cells. The ability of BiVax to trigger strong T cell responses to the E7₄₉₋₅₇ peptide appears to be somewhat unique since other potent CD8 T cell epitopes such as Ova₂₅₇₋₂₆₄ (SIINFEKL) and melanoma Trp_{1455/9M} (TAPDNLGYM) are ineffective and require α CD40 mAb in addition to poly-IC (E. Celis, unpublished). Specific characteristics such as solubility that could influence the formation of micro-aggregates and serum protease resistance affecting the peptide's pharmacokinetics are likely to determine in great part whether a peptide is able to trigger T cell responses when administered in the BiVax format. Poly-IC is a double stranded synthetic RNA that stimulates TLR3 and cytoplasmic RNA helicases (RIG-I and MDA5) resulting in the activation of DCs and the generation of high amounts of type-I IFN [34,35], which is considered important for the induction of effective T cell responses [36]. Our results indicate that in the absence of type-I IFN signaling (in IFN α β R KO mice), TriVax was less effective in generating sufficient numbers of CD8 T cells that could be necessary for total disease eradication (Fig. 5c). Nevertheless, it was interesting to note a substantial anti-tumor effect of TriVax in the absence of type-I IFN signals, which could be due to the participation of other T cell stimulatory cytokines such as IL-12 generated by the combination of poly-IC and α CD40 mAb. It was somewhat unexpected to observe a significant anti-tumor effect of TriVax in the absence of IFN γ (Fig. 5b) since this cytokine is considered to be critical for the anti-tumor effects of CD8 T cells [37-39]. Nevertheless, our results indicate that IFN γ may play some role in the overall effectiveness of TriVax to completely reject the tumors. These results contrast with our recent findings in the B16 melanoma system in which IFN γ was shown to play a negative role in the anti-tumor effects of TriVax using Trp_{1455/9M} and Trp₂₂₈₀₋₂₈₈, where complete rejections of established tumors were observed in IFN γ KO mice and not in WT mice [27]. Thus, it is possible that IFN γ plays an effector role with the TC-1 tumors, limiting cell proliferation or that the increase MHC-I expression induced by IFN γ in these cells enhances T cell recognition.

In summary, the results presented herein in a mouse model of HPV-induced cancer demonstrate the feasibility of a novel and potent peptide vaccination strategy that could be adopted for CC or other HPV-induced malignancies. Both poly-IC and α CD40 mAb for human clinical use are in development and together with known human CD8 T cell epitopes could be administered using the TriVax format with the goal of reducing tumor growth, and perhaps as shown here in mice, eradicate established disease.

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Abbreviations

αCD40 mAb	anti-CD40 monoclonal antibodies
CC	Cervical carcinoma
DC	Dendritic cell
HPV	Human Papillomavirus
MHC-I	Major histocompatibility complex I
IFNγ	Interferon-gamma
IFN$\alpha\beta$R	Interferon alpha/beta receptor
KO	Knockout
TAA	Tumor associated antigen
TLR	Toll-like receptor

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Fig. 1. Synergy for the potentiation of the immunogenicity of E7₄₉₋₅₇ peptide by poly-IC and α CD40 mAb. **a** Frequencies of antigen specific CD8 T cells in blood measured by tetramer analysis 6 days after prime and boost induced by immunization using various vaccine formulations. Numbers below the oval gates represent the % of tetramer positive cells of the CD8 positive population. Dot plots showing the percentage of tetramer positive cells in blood of a mouse from each group (3 mice/group). **b** Average percentage of tetramer positive cells in the blood of mice in each group measured at different time points (same experiment as in panel a). **c** Separate experiment where CD8 T cells were purified from pooled splenocytes 7 days after the boost and antigen-induced responses to various target

cells was evaluated using IFN γ EliSpot assays. Stimulator cells: EL4 cells pulsed or not with E7₄₉₋₅₇ peptide and TC-1 cells (expressing HPV16-E7) were used to evaluate CD8 T cell responses from TriVax (*top row*) and BiVax (*bottom row*) vaccinated mice. In this experiment each well contained 1×10^4 CD8 T cells and 1×10^5 stimulator cells. Numbers represent the total spots present in each well. Representative results of data obtained from two different experiments.

Fig. 2.

Therapeutic effects induced by TriVax and BiVax immunization against established TC-1 tumors. **a** Mice ($n = 3$ per group) were inoculated (*s.c.*) with 3×10^5 TC-1 cells and vaccinated (*i.v.*) 6 days later with E7₄₉₋₅₇ peptide alone, BiVax, peptide plus αCD40 mAb or TriVax. Identical booster immunizations were given on day 13. Tumor growth was measured (two opposing diameters) and recorded twice at week. None of mice from the E7₄₉₋₅₇ and E7₄₉₋₅₇ + αCD40 mAb groups rejected their tumors. One mouse from the BiVax group and all 3 mice from the TriVax group rejected their tumors. **b** The percentage of antigen-specific (tetramer positive) CD8 T cells in blood of the mice from the experiment shown on panel “a” measured at different time points. Only 2 measurements were done in the mice immunized with peptide alone because these mice did not survive beyond day 24. **c** Mice ($n = 4$ per group) were inoculated (*s.c.*) with 3×10^5 TC-1 cells and vaccinated (*i.v.*) 6 days later with TriVax prepared with either E7₄₉₋₅₇ or Ova₁₇₆₋₁₈₃ as indicated. Identical booster immunizations were given on day 13. A Non-vaccinated group (No Vax) was included as control. **d** The therapeutic effects of E7₄₉₋₅₇ BiVax were confirmed using a larger number of mice (6/group). In this experiment, half (3/6) of the mice in the BiVax vaccinated group rejected their tumor, while all mice in the control group did not.

Fig. 3.

Therapeutic effects induced by TriVax against HPV16-E7 expressing tumors. **a** Anti-tumor effects against large TC-1 tumors. Mice (6/group) were inoculated (*s.c.*) with 3×10^5 TC-1 cells and 11 days later were immunized with TriVax, and a booster was given 13 days after prime. All of the mice immunized with TriVax rejected their tumors. **b** Anti-tumor effects against C3.43 tumors. Mice (6/group) were inoculated with 3×10^5 C3.43 cells (*s.c.*) and vaccinated 6 and 13 days later with TriVax. All of the mice in the TriVax immunized mice rejected their tumors. **c** Frequency of antigen-specific CD8 T cells in the spleens of mice from the TriVax immunized group shown in panel “b” was determined 30 days after the prime. Numbers below each oval gate represent % tetramer positive cells of the CD8 population. **d** CD8 T cells were purified from pooled splenocytes of TriVax vaccinated mice

shown in panel “c” and tumor cell recognition was evaluated using IFN γ ELiSpot assays. Stimulator cells were: EL4 cells loaded or not with E7_{49–57} peptide, TC-1 and C3.43 tumor cells previously treated or not with IFN γ (100 U/ml, 24 h, to increase MHC-I expression). Results represent the average number of spots from triplicate wells with SD (*error bars*) of the means. **e** TriVax immunization confers long-term protection against tumors. Mice (6/group) were inoculated (*s.c.*) with 3×10^5 TC-1 cells and 6 and 13 days later received TriVax. By day 25 all mice in the TriVax group rejected their tumors. On day 47 the mice were re-challenged with 3×10^5 TC-1 on their right flanks and 3×10^5 C3.43 on their left flanks. None of the tumors grew. As controls (CTRLs), 3 naïve mice were inoculated with the same number of tumor cells.

Fig. 4. Antitumor efficacy of intramuscularly administered TriVax. **a** Mice were inoculated *s.c.* with 3×10^5 TC-1 cells. Six days later were vaccinated *i.m.* with TriVax (TriVax IM) and an identical booster was given on day 13. Tumor growth was measured as described previously. By day 25, all mice (6/6) vaccinated with TriVax rejected tumor, but by day 66, one of the mice developed a tumor mass at the original site (not shown). All of the mice (3/3) non-vaccinated mice developed large tumors and did not survive. **b** Frequency of antigen-specific CD8 T cells (tetramer positive) in the blood of the mice shown in panel “a” measured 11 days after boost. Numbers below the oval gates represent the % tetramer positive cells of the CD8 T cell population.

Fig. 5.

Mechanism of therapeutic anti-tumor effects of TriVax. **a** Tumor growth in mice depleted of NK cells, CD4 T cells and CD8 T cells vaccinated (*i.v.*) with TriVax. Mice (6/group) were inoculated with 3×10^5 TC-1 cells and 6 and 13 days later received TriVax. One and three days before the TriVax prime mice received depleting antibodies via an *i.p.* injection as described in “Materials and methods”. **b** Tumor growth in IFN γ KO and IFN $\alpha\beta$ R KO vaccinated with TriVax in the same manner as described in panel “a”. Non vaccinated mice and TriVax vaccinated wild type (WT) mice were used as controls in each experiment. All of the TriVax WT, CD4 and NK cell depleted mice rejected their tumors. One out of 6 mice in the IFN γ KO group and 2/4 mice from the IFN $\alpha\beta$ R KO group had complete tumor rejections. **c** Immune responses elicited by TriVax in naïve IFN γ KO and IFN $\alpha\beta$ R KO mice. In a separate experiment, non-tumor bearing wild type (WT), IFN γ KO and IFN $\alpha\beta$ R KO mice were vaccinated with TriVax, (prime/boost, 13 days apart) and the percentage of tetramer positive cells in blood was determined 7 days after boost.