Therapeutic Peptide-Based Vaccination Strategies Against HPV-Induced Cancers

Kelly Barrios Marrugo
University of South Florida, ktbarrios@gmail.com

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Therapeutic Peptide-Based Vaccination Strategies Against HPV-Induced Cancers

by

Kelly Barrios-Marrugo

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Molecular Medicine
College of Medicine
University of South Florida

Major Professor: Esteban Celis, M.D., Ph.D.
Amer Beg, Ph.D.
Peter Medveczky, M.D.
Kenneth Ugen, Ph.D.
Xue-Zhong Yu, M.D.

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Dedication

I dedicate this dissertation to my mom Arinda Marrugo, who’s constant love and support sustained me through the toughest times, mom you are the strongest woman I know and I am lucky to be your daughter.

I would like to thank Hernando Barrios my Brother, and my two nieces Valentina and Isabella you have been an endless source of advice and inspiration. Special thanks to my friends Valentina Schneeberger, Milene Lara and Kenrick Semple you have been a great support system for me over the years, thanks for the laughter, counsel, getting me out of the house and the lab, allowing me to vent and rooting for me all along, I needed that. Lastly, to my furry friend Maggie for your unconditional love and over the top cuteness, you make me smile every time I see you.
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Abstract

There is an urgent need for the development of an effective therapeutic vaccine against cancer caused by human papilloma virus (HPV). I focused on HPV-induced malignancies because of their high worldwide prevalence (e.g., cervical carcinoma and head & neck cancer). A successful therapeutic vaccine could prevent the 250,000 deaths/year worldwide and the 2.25 billion dollars that are expended in related care in the US.

We used an HPV-induced mouse cancer model to test vaccines composed of a CD8 T cell peptide epitope administered with potent adjuvants designed to generate vast numbers of high avidity cytotoxic T lymphocytes specific for the HPV16-E7 antigen. One vaccination strategy (TriVax) consists of intravenous administration of synthetic peptide HPV16-E749-57 administered together with a Poly-IC (a TLR3 agonist) and anti-CD40 monoclonal antibody (αCD40 mAb) while the second more simple strategy (BiVax) comprises solely of peptide plus Poly-IC. We used an E7 peptide as antigen in the vaccination strategies because expression of the viral E6 and E7 proteins is required to maintain oncogenic phenotype and because normal cells do not express E6/E7, therefore a therapeutic vaccine targeting these proteins has several advantages: a) a strong immune response can be induced since immune tolerance to these
foreign antigens does not exist and b) the strong immune response should not inflict damage to normal cells.

TriVax and BiVax generate a high number of antigen specific CD8 T cells capable clear subcutaneous tumors and prevent recurrences; both vaccines are efficient through the i.v. and i.m. route. TriVax (prime-boost) clears tumor in 100% of mice while BiVax clears tumor in 50% of mice, this differential effect is due to the number of antigen specific CD8 T cells and increasing the number of booster shot makes BiVax as immunogenic and efficient in clearing tumors. In the absence of type-I IFN signaling (in IFNαβR KO mice), TriVax is less effective in generating sufficient numbers of CD8 T cells that could be necessary for total disease eradication. We observed a significant anti-tumor effect of TriVax in the absence of interferon gamma (IFNγ), however the cytokine may play some role in the overall effectiveness of TriVax to completely reject the tumors. Immune responses produced by BiVax are highly dependent on the simultaneous administration of peptide and Poly-IC, on the peptide composition, vaccine formulation and route of administration. The magnitude of the response is dependent on the expression of the Poly-IC receptors TLR3 and melanoma differentiation-associated protein 5 (MDA5). Interestingly, the magnitude and duration of the CD8 T cell responses generated by peptide and Poly-IC mixtures does not rely on the presence of CD4 T cells, scavenger receptor-A (SR-A) or type-I IFN signals and was minimally affected by the absence of CD40 signaling. The present findings could facilitate the development of simple and effective subunit vaccines for diseases where CD8 T cells may hold a therapeutic benefit.
Chapter 1: Introduction

1.1 Overview

According to Dr. Weinberg’s group the 8 hallmark of cancer include “sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction” [1]. In some cases viral infections can be directly linked to cancer, examples of these are Epstein-Barr virus (Hodgkin’s, Burkitt’s lymphoma, and nasopharyngeal carcinoma), hepatitis B virus (liver cancer), Hepatitis C virus (liver cancer), human T cell lymphotropic virus type 1 (adult T-cell leukemia/lymphoma), human herpesvirus 8 (Kaposi sarcoma / lymphoma), Merkel cell polyomavirus (Merkel cell carcinoma) and human papillomavirus (head and neck carcinoma, anal, vulvar, vaginal, penile and cervical cancer) [2-9]. Amongst all these, cervical cancer (CC) stands out in importance because worldwide is the 2nd most prevalent cancer among women [10]. HPV causes 99% of cervical cancer worldwide and HPV-16 and -18 subtypes account for 80% those cases. There are 2 prophylactic vaccines that are used to prevent HPV infection but are not intended to treat neither established tumors, nor protect infected people from developing CC [11-13], and they do not protect against
infection with all oncogenic HPV genotypes. Furthermore, not all women have or will receive the vaccine and therefore are not protected from the development of CC [14]. Finally, conventional therapy for CC is ablative and associated with 10% recurrence; thus, there is an urgent need for the development of an effective therapeutic vaccine against cancer caused by HPV. A successful therapeutic vaccine could prevent the 250 000 deaths that occur every year in the US from this disease alone and the 2.25 billion dollars that are expended in related care in the US [15, 16].

Immune-based therapies for cancer such as therapeutic vaccines may be an attractive alternative over radiation and chemotherapy for several reasons: Radiation and chemotherapy result is serious adverse effects and in many cases are not effective against large tumors or disseminated (metastatic) disease. On the other hand, a potential problem with immunotherapy is finding a good antigen. Since expression of the viral E6 and E7 proteins is required to maintain oncogenic phenotype and because normal cells do not express E6/E7, a therapeutic vaccine targeting these proteins has several advantages: (i) the tumor cell cannot lose their expression as an immune evasion mechanism; (ii) there is no immune tolerance generated against them and an effective immune response in not likely to generated autoimmunity that could compromise healthy cells. These benefits contrast with other vaccines that target tumor-associated antigens (TAA) that are also present in normal cells (e.g., melanoma, using tyrosinase as an antigen). Despite of this, immunotherapy for virus-induced cancer has not been successful in the clinic [17-22].
Our goal is to develop therapeutic peptide based vaccines against HPV induced cancers. The results of the work shown here indicate that the TriVax and BiVax strategies are appealing immunotherapeutic approaches 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.

In the following paragraphs I will discuss relevant aspects of HPV including their classification, structure, cycle, oncogenic proteins and the diseases caused by the virus. I will also address the nature of the immune response against HPV infection and neoplasia caused by the virus, the FDA approved prophylactic vaccines and current approaches to the development of experimental therapeutic vaccines against HPV induced cancers, making an emphasis in our approach: peptide based vaccines.

1.2 HPV

Papillomaviruses (PVs) are highly species specific and can cause cancer in animals (non human primates, cattle, hamsters, sheep and dogs, among others [23-29]) and in humans [30]. The species infecting humans, HPV, has over 100 different genotypes worldwide, from these, types 6 and 11 are the most common cause of genital warts while genotypes 16 and 18 account for over 80% of the CC induced by HPV [31-35].

Structurally, HPV is a double stranded DNA, non enveloped virus with icosahedral symmetry, the viral DNA is associated with proteins similar to
histones, wrapped in 72 capsomeres composed of two structural proteins, L1 and L2 [36, 37]. HPV’s genome is around 7900 bp long and is grouped into the Alpha, Beta, Gamma, Mu and Nu papillomavirus genera. The Alpha genus infects mucosa and skin and can be further classified in high risk (HR) and low risk (LR) types [38].

1.2.1 High risk vs. low risk HPV types

The classification of high and Low risk types is based on the ability of a particular HPV genotype to induce malignant transformation in humans. In fact, persistency of HR types is the single most important risk factor for the development of cancer [39-46]. HR genotypes include type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, 23, 53 and 66, being types 16 and 18 the most common [47].

The main differences between HR and LR genotypes are on the genes that encode the E6 and E7 proteins, which in turn impacts how efficient they are in malignant transformation. E6 and E7 genes from HR genotypes are very efficient in immortalizing primary genital keratinocytes and interacting with negative regulators of the cell cycle[42, 48, 49], thus allowing the persistency of episomal genomes in undifferentiated cells and productive replication of genomes or amplification in differentiated cells $^{41,47,48}$. E7 from HR binds to retinoblastoma (Rb) tumor suppressor family of proteins with higher affinity (10 fold) than LR, it also binds to Histone deacetylases 1 2 and 3 that are important
for activating cellular genes that contribute to stable viral genome [50]. The importance of these proteins is highlighted on the virus's life cycle described below.

### 1.2.2 HPV life cycle

The life cycle of HPV is directly related to the cellular differentiation program of the host cell, establishing infection in the basal layer of the cervix where the stem cells (SC) reside and assembling the virions in the upper, terminally differentiated layers of the cervical epithelia. During the virus's life cycle the order of expression of its proteins is highly regulated and follows the pattern showed below:

First, HPV virions infect cells in the basal epithelial layer that become exposed through micro wounds or abrasions like those produced by sexual intercourse. Then, viral capsids bind to the basement membrane possible through integrin and heparin sulfate and/or laminin 5, the virions enter keratinocytes and the virus genome is replicated in the nucleus to about 100 episomal copies per cell [51, 52]. Later, E1 binds to the HPV origin of replication prior to the initiation of DNA synthesis, E2 recruits E1 to the origin of replication and regulates the transcription of E6 and E7 from the early promoter and at some point the E2 gets disrupted leading to the deregulation of the E6 and E7 proteins. Deregulation of E6 and E7 leads to immortalization and cell transformation [53-55]. E6 activities lead to dysregulation of organization, differentiation, and
chromosomal integrity of HPV infected epithelial cells, most importantly, HR E6 is able to bind the tumor suppressor protein, p53 and induces its rapid turnover by forming complexes with the E6AP ligase [56, 57]. Up-regulation of p53 leads to expression of Rb tumor suppressor family of proteins, HR E7 binds to and modulates Rb along with cell cycle regulatory proteins, leading to accelerated cellular and viral replication [58]. E7 also targets Rb for ubiquitin mediated degradation and induces abnormal centrosome number, genomic instability, chromosome misalignment and lagging chromosomes during mitosis [59]. At the same time that E6 and E7 are being up regulated, the E5 protein is being expressed and exerts an additive effect on the transforming properties of E6 and E7 [60]. Finally, the virion particles assemble in the outer layers of the epithelia cells that are terminally differentiated causing no inflammation, thus evading the immune system.

1.2.3 HPV related disease

HR HPVs genotypes cause virtually all of the CC worldwide and although most people infected never develop cancer, the high prevalence of HPV infection makes this disease one of the most common malignancy [41, 61, 62]. In fact CC is preceded only by breast cancer as the most common cause of death from cancer among women [63]. The cost of screening, follow up and treatment of CC approximates $6 billion dollars per year in the US alone [64].

Risk factors for HPV induced cancers include smoking, long term oral contraceptive pill use, human immunodeficiency virus (HIV) co-infection, high
parity, HSV and chlamydia trachomatis infection, Phimosis, immune suppression as well as nutritional and dietary factors [65]. The interval from first infection to neoplasia has been estimated to be between 7-10 years.

In addition to causing CC, HPV can cause penile, anal, and head and neck cancers. Head and neck cancer affects over 650 000 patients worldwide each year and 350 000 patients die from this disease, 30-65% of head and neck cancer are caused by HPV [66, 67]. Penile cancer is 10 times less common than CC and untreated patients usually die within 2 years of the diagnosis [68]. Finally, anal cancer with an incidence around 1.5% of all tumors of the digestive system is relatively uncommon with an estimated 690 related deaths annually [69-71].

1.3 Natural immune response to HPV infection and CC
1.3.1 Innate immune response to HPV infection and CC

Cellular mediators of the innate immune responses to genital infection by HPV include Langerhans cells, macrophages and Natural Killer (NK) cells [72]. Macrophages negatively regulate the transcription of the E6 and E7 genes, and have antitumor effect against HPV transformed cells; their presence can be an indicator of the lesion regression [73]. In addition, a small number of NK cells have been observed in Condyloma acuminata, in mild to moderate dysplasias and in severe dysplasias, their activity is reduced in patients presenting stage I or II invasive CC [74].
Cytokines are another important mediators of innate immune response to genital infection; indeed, malignant transformation involves loss of responsiveness to the inhibitory effects of some cytokines. Successful immune response against HPV infection is mediated by: the effects of Interferon gamma (IFNγ), Tumor growth factor β (TGFβ) and IFNα on infected cells [75-78]. IFNγ blocks the expression of the HPV 18 mRNA of CC cells, TGFβ inhibits proliferation of cells infected by HPV and IFNα has been shown to inhibit proliferation of HPV16 immortalized human keratinocytes at concentrations 10 to 100 fold lower than those needed to inhibit growth of normal human keratinocytes [79-81].

1.3.2 Adaptive immune response to HPV infection and CC

Both arms of the adaptive immune system, humoral and cellular, are involved in natural response to HPV infection. The humoral host's immune response to HPV infection is directed to conformational epitopes of the L1 capsid protein, the antibodies produced are mainly of the IgG isotype, the response generated is usually slow, weak and varies considerably among women [82]. Due to the intraepithelial nature of HPV infections, effective immune response against established infection and cancer is dependent on cellular immune response, in fact patients with persistent cell mediated immune defects are at increased risk of persistence of infection and development of HPV related cancer [83-86]. Notably, the most important mediator of cellular immune response to
HPV infection are CD4 T cells, predominance of CD4 cells favors regression, predominance of CD8 lymphocytes favors persistence and progression of the viral infection and cellular transformation. Indeed, The CD8-positive T cell infiltrate far exceeded the CD4-positive cells in the invasive, but not in the preinvasive lesions however they infiltrate the lesion diffusely which is likely the cause of their inefficiency in controlling the disease [87]. It is also possible that the number of the CD8 T cells generated during natural malignant progression is insufficient to control disease or that because progression to malignant lesion is usually slow, the CD8 T cells recruited are exhausted or are in other ways functionally impaired. Immunotherapy can generate a high number of antigen specific cells in a short period of time that could overcome these obstacles.

1.3.3 Immune evasion mechanisms

The life cycle of HPV itself acts as an immune evasion mechanism: (i) the expressions of viral proteins are kept to a minimum and are regulated spatially and temporally, (ii) there is no viremia, (iii) no lysis of infected cells, (iv) and HPV replication and assembly occur in cells already destined for death by anoikis therefore there is no induction of inflammation, which means the immune system does not receive danger signals, a stimuli that usually activates it.

T cells need to see the antigen in the context of a HLA molecule and in the presence of co-stimulatory signals to be activated; the main host cells for HPV infection: keratinocytes, do not express HLA class II molecules and lack expression of co-stimulatory molecules; therefore, these cells are more
susceptible to a state of specific or anergic tolerance [87-89]. In addition, E7 from HR HPV can decrease the expression of HLA I molecules and Transporter associated with antigen processing (TAP) proteins, thus interfering with antigen presentation to CD8 T cells, resulting in down regulation of cellular immune responses, allowing HPV to persist in infected epithelial cells [90]. Likewise, the E5 protein is capable to interfere with antigen presentation by the enhancing the expression of gangliosides on cervical epithelial cells, which also inhibits cytotoxic CD8 T cells (CTL) function locally [91, 92].

Dendritic cells (DCs) are professional Antigen Presenting Cells (APCs) that set in motion a wide range of effector immune responses against pathogens. DCs are not activated by the uptake of HPV capsids constituting another evasion mechanism [93].

Finally, HPV can evade the immune system by manipulating cytokine production. The IFN response to HPV infection, a key antiviral defense mechanism, is actively suppressed by the E6 and E7 proteins of the HR HPVs inhibiting the IFN receptor signaling pathways and the activation of the IFN response genes [94-96]. TNFα synthesis is also diminished in HPV infections, associated with a decrease in the expression of the CD80 co stimulatory molecule resulting in a decrease in the presentation capacity of Langerhans cells [97].

Peptide based vaccines can overcome these obstacles because the immune system is expose to HPV antigens required for malignant transformation but without the transforming capabilities and these antigens are given in
combination with immune adjuvants priming the immune system for a potent response directed specifically to antigens in the tumor cells.

1.4 Prevention of genital neoplasia induced by HPV: Prophylactic vaccines

There are two prophylactic vaccines approved by FDA for the prevention of HPV infection and the associated development of cervical intraepithelial neoplasia (CIN) 2/3 and vulvar intraepithelial neoplasia (VIN): Gardasil and Cervarix. Both vaccines are composed of virus like particles (VLP) these are very immunogenic self-assembling structures form of several subunits of the L1 protein mixed with an adjuvant and administered i.m. over a six months period [98].

Gardasil (MERK) is a quadrivalent vaccine that uses the adjuvant amorphous aluminum hydroxyphosphate sulfate and it is directed against HPV types 16, 18, 6 and 11 although offers some cross protection against HPV types 31, 33, 45, 52 and 58 [12, 99-101]. Seroconversion in 99-100% of vaccinated individuals is achieved 7 months after last immunization and the antibody titers remain stable for at least 5 years [102-104]. Gardasil has a 98% efficacy in preventing CIN 2/3, adenocarcinoma in situ and CC, 100% for VIN 2/3 [101].

Cervarix (GlaxoSmithKline) is a bivalent vaccine directed against HPV types 16 and 18 and offers some cross protection against HPV types 31, 33 and 45 [104-106]. In contrast to Gardasil the adjuvant used is AS04: Monophosphoryl lipid A (MPL) absorbed to aluminum hydroxide and the vaccine generates greater immune response and immune memory than Gardasil (titers 4-7 folds higher)
Cervarix efficacy is between 92-100% for the prevention of CIN2/3 with a follow up protection of 8.4 years so far.

Both prophylactic vaccines are very efficient in preventing infection by HPV through the generation of neutralizing antibodies, however these vaccines are not effective against established infection [11], established premalignant and malignant lesions and they do not protect against all oncogenic types. In addition, it is estimated that it would take more than 20 years to see an impact on cancer rates as a result of vaccination due to the long latency of HPV infection and the presence of already infected individuals [110]. Also, adherence to vaccination is suboptimal; in fact only 30% of the vaccinated individuals go through with the 3 doses required of the vaccine. Finally, not all the individuals for whom the vaccine is recommended receive the vaccine (for ages 9 to 26) due most likely, in the case in young individuals, to cultural and parental consent issues. Therefore, there is an urgent need to develop therapeutic vaccines against established cancers caused by HPV.

In the following paragraphs different approaches toward the development of experimental therapeutic vaccines against HPV induced cancer and the results of several clinical trials are highlighted.

1.5 Experimental therapeutic vaccines against HPV induced cancers

Conventional therapies against genital diseases caused by HPV are mostly ablative and/or cytodestructive; these include cryotherapy, scissor excision, laser therapy and electrosurgery and although cryotherapy is often
highly effective in the short term with clearances of 70-80 % the rate of recurrence are quite high, around 25-39% and in some cases, like in multifocal lesions, physical removal of the affected tissue can be unpractical or futile [111-114].

There is one therapeutic vaccine against cancers approved by the FDA: Provenge a DC based cancer vaccine supplemented with a cytokine (GM-CSF= granulocytes and monocytes colony stimulating factor) and tumor derived differentiation antigen (PAP= prostatic acid phosphatase) for the treatment of prostate cancer [115, 116]. There is no FDA approved therapeutic vaccine against HPV induced cancers. The reason immunotherapy is so attractive for the treatment of established cancers is that successful immunotherapy provides specific and long lasting immune response directed exclusively against the targeted antigen. As mention before our approach is the use of peptide vaccines, approaches used by others include DNA, vector, DC cells, protein and peptide based vaccines. The following paragraphs describe the most relevant aspects of each strategy, along with some of their advantages and disadvantages.

1.5.1 DNA based cancer vaccines

DNA vaccines are comprised of a bacterial plasmid, which utilizes a promoter that function in mammalian cells to drive expression of a gene encoding the protein of interest.

Major advantages of DNA vaccines are their simplicity and overall safety, which translates to low production costs, overall stability, and ease of storage (no
refrigeration required) thus its large scale production and storage is cheaper and easier than protein based vaccines [117-119]. Because the DNA vaccines consist simply of a plasmid without other antigens, such as a viral or bacterial vector would have, no issues related to prior exposure to the pathogen arise [119].

One disadvantage of DNA based vaccine is their poor immunogenicity specially in humans, when compared with traditional protein based vaccines, due most likely to their inability to amplify and spread in vivo, there is also a small risk of integration to the genome [110]. Strategies to improve DNA vaccine potency include the use of more efficient promoters and codon optimization, addition of traditional or genetic adjuvants, electroporation, intradermal delivery and various prime-boost strategies [118]. Peng et al from Dr. Wu’s group used the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA) in conjunction with E7 DNA vaccination to generate potent antitumor effects in mice challenged with 1x10^5 TC-1 cells s.c. and vaccinated 10/13 and 16 days later with calreticulin/E7 DNA (CRT/E7). Although DMXAA treatment alone, CRT treatment alone and DMXAA/CRT induced anti tumor effect, mice treated with DMXAA/CRT/E7 had significantly smaller tumors [120]. Diniz et al used a DNA vaccine (pgD-E7E6E5) expressing the E7, E6, and E5 of HPV16 genetically fused to the glycoprotein D of the HSV 1, which was administered to mice by the intradermal (i.d.) route using a gene gun to clear tumor in over 70% tumor free mice by day 20 after 2 boosters. In this study
mice were challenged with $5 \times 10^5$ TC-1 tumor cells and vaccinated 8 hours later followed by 2 boosters one week apart [121]. Wu A et al approach was to vaccinate mice with a DNA vaccine encoding Ii-PADRE linked to E6 (Ii-PADRE-E6) 3 and 7 days after tumor challenge with $2 \times 10^5$ TC-1 cells obtaining the best therapeutic anti-tumor effects and best overall survival when compared to the other DNA construct tested (Ii-E6, E6, Ii-PADRE) [122]. Mohit et al vaccinated tumor-bearing mice (7 days after challenge with $1 \times 10^6$ TC-1 cells s.c.) with IP-10 at the same inoculation site of TC-1 along with E7 protein fused to immunostimulatory molecule N terminal gp96 (E7-NT-gp96) delivery by PEI600-Tat as non-viral gene delivery system. Following this strategy the author obtained significant suppression of tumor growth and improved overall survival [123].

### 1.5.2 Vector based cancer vaccines

These strategies exploit the strong immune response generated by viral or bacterial components that function as adjuvant to enhance reactivity against cancer antigens. It is though that these strong immune responses are due to the targeting of DC and subsequent induction of inflammation, in fact several studies have shown that transgenes expressed by a viral vector are more immunogenic than antigen administered with adjuvant[118, 124, 125].

The advantage of viral and bacterial vector vaccines is that they allow HPV epitopes to be delivered more efficiently to target cells and to induce endogenous synthesis of viral and recombinant proteins. Logistically,
recombinant viruses can be produced, administered, and quality controlled easier compared with other immunotherapy strategies. The disadvantage of some vectors is the development of host induced neutralizing antibodies to the vector itself, thus limiting its continued use [110]. Liao et al, developed a vesicular stomatitis virus (VSV) expressing the HPV16 E7 oncogene as a therapeutic vaccine against TC-1 mouse model of cancer. In this study mice were immunized with the viral vector vaccine 7 days after tumor challenge with 5x10^4 tumor cells s.c., 14 days after vaccination mice had tumors 10 fold smaller than mice vaccinated with empty vector [126]. Grasso et al, approach was to use an integrase defective lentiviral vector (IDLV) containing a non-oncogenic HPV16 E7 protein fused to calreticulin (CRT). When mice were challenged with 2x10^5 TC-1 s.c. and vaccinated 14 days later, 4/5 rejected tumor compared to 0/5 of the vector/GFP control group, using the same scheme but vaccinating when tumor were 3-4 mm diameter the authors achieved rejections in 1/8 treated mice [127]. Zhao et al, developed a bivalent recombinant vaccinia virus expressing modified E7E6 fusion proteins of HPV16 and 18 (rVVJ16/18E7E6) based on the vaccinia virus Tiantan strain. After challenging mice with 1 × 10^4 TC-1 cells at day 0 and then vaccinating them i.p. at day 1 and day 11 mice showed delayed tumor growth compared to non treatment or treatment with control vector group [128]. Sewell et al designed a bacterial vector vaccine by transforming an attenuated Listeria strain with an E7 expression cassette, which consisted of the HPV-16 E7 sequence fused to the Listeria protein ActA (Lm-ActA-E7), with this strategy the authors obtained complete regression of HPV-positive tumors in 6 out of 8 mice.
These mice were vaccinated 7 and 14 days after tumor challenge with $2 \times 10^5$ TC-1 cells s.c [129].

1.5.3 DC based cancer vaccines

The approval of Provenge by the FDA is a milestone in the immunotherapy field of DC vaccines, as mentioned before, in this approach, DCs are isolated from a cancer patient, loaded with antigens (peptides or even tumor cell lysates) ex vivo, activated and then reinfused into the patient through intradermal, subcutaneous, intravenous, or intranodal injections [130, 131]. To potentiate further the efficacy of DC vaccines these cells can be modified to upregulate the expression of costimulatory molecules, cytokines, chemokines, and anti-apoptotic molecules, or down regulate the expression of inhibitory molecules [132]. One of the main drawbacks of DC based vaccines is that they require leukapheresis and cell culture processing of peripheral blood mononuclear cells (PBMCs), and thus a limited number of vaccinations can be used. Several preclinical studies has been conducted using DC based vaccines, Kim et al for instance, used a DC pulsed with E7 peptide transfected with and anti-apoptotic siRNA targeting BIM; these treatment yield smaller size tumor as compared to no treatment when mice were immunized 3 days after s.c. inoculation of $5 \times 10^5$ TC-1 tumor cells, although no rejections were achieved [133]; the same group later showed using the same number of tumor cells and the same vaccination schedule, that by transfecting bone marrow DCs pulsed
with the E7<sub>49-57</sub> peptide with IL10 receptor siRNA (and effectively blocking IL10 actions on the BM-DC) they could achieve tumor clearance in 80% of treated mice [134]. Yin et al, used DC loaded with the E7<sub>49-57</sub> peptide fused to the KDEL sequence, an endoplasmic reticulum (ER) retrieval signal, obtaining tumor clearance in 50% of mice challenged with 2x10<sup>5</sup> TC-1 cells s.c. and vaccinated 10 days later [135]. Finally, Kang et al showed that vaccination mice inoculated with 2x10<sup>4</sup> TC-1 cells <i>i.v.</i> with DCs expressing an endosomal/lysosomal signal fused to the E7 peptide (Sig/E7/LAMP-1) yield a significantly lower number of pulmonary tumor nodules when compared to DC expressing the E7 peptide alone or no peptide at all [136]. However, despite the encouraging results of these and other preclinical studies, most clinical trials using DC based vaccines have failed to achieve satisfactory clinical results [132, 137-139].

### 1.5.4 Protein-based cancer vaccines

Major advantages of protein-based cancer vaccines is their ease of production, stability, safety profile and because they usually also contain both CD4 and CD8 epitopes and are processed by the host there is no concern about HLA restriction [110]. On the other hand purified proteins are inefficient in inducing CD8 T cell response because they are mostly processed through the MHC II pathway, which directs the immune response towards the Th2 phenotype if they are not coupled with the appropriate adjuvants, these vaccines tend to be more costly than peptide-based vaccines [140]. Sharma et al, used a chimeric
form of the 4-1BBL costimulatory molecule engineered with core streptavidin (25 µg) plus modified E7 protein (50 µg) to effectively eradicating established tumors in approximately 70% of mice. In this study mice were inoculated with $1 \times 10^5$ TC-1 cells s.c. and immunized 6 days later [141]. Wick et al on the other hand used Pentarix (100 µg) a recombinant protein-based vaccine directed at the E7 proteins from HPV16, 18, 31, 45 and 52 plus Poly I:C (10 µg) or CpG oligonucleotide (10 µg) to cure mice challenged with $1 \times 10^5$ TC-1 tumor cells [142].

1.5.5 Peptide-based cancer vaccines

As mentioned before, our approach to the development of therapeutic vaccine against HPV induced cancer is the use synthetic peptides containing a CD8 T cells epitope of HPV 16 E7 protein to generate a large number of antigen-specific CD8 T cells in a mouse model of cancer. Peptide vaccines offer the advantage of being safe, stable, easy to manufacture under good manufacturing practice (GMP) and therefore can readily be taken to the clinic. Peptides are not very immunogenic by themselves but their antigenicity can easily be improved by substituting amino acids at specific positions within the peptide sequence.

Because small peptides that target CD8 T cells along can be poor immunogens, some investigators propose the use of long peptides (20 mer) that are somewhat longer than those that bind to MHC I (10-12 mer). These peptides
are thought to be more efficient at generating effector T cells, in the presence of immune adjuvants perhaps because some processing may be required [143]. Another approach is to link chemically multiple immunogenic epitopes to form stable linear complexes [144]. The major advantage of this approach is based on the easy chemical synthesis thus high purity of the manufactured peptides. Because CD4 T cell help is important to generate and sustain the MHC I restricted, CD8 T cell responses some researches use MHC II restricted epitopes derived from the same protein as the CD 8 T cell antigen in their vaccine preparation [144, 145].

Small peptides can be immunogenic if administrated with the appropriated adjuvants [146]. Different groups have tested several adjuvants for their ability to induce high, long lasting, antigen specific immune response against tumor antigens (Table 1 and [147]). There have been some encouraging results in mouse model of cancer, for example the group of Dr. Kast used four E7 peptides combined with the PADRE peptide and CpG plus ISA51 as adjuvants in a vaccine preparation called VacciMax®, using these approach the group achieved eradication of 700mm³ tumors [148]. Dr. Guillen group used a combination of the E7₄⁹-₅⁷ peptide, very small size proteoliposomes (VSSP) and incomplete Freund's adjuvant (IFA) to eradicate subcutaneous TC-1 tumors [149]. Dr. Wu’s group used the E7₄⁹-₅⁷ and PADRE peptides and Poly I-C to generate to treat tumor-bearing mice; the combination of E7 peptide, PADRE and Poly I-C generated the highest immune response and better survival of tumor bearing mice compared to control mice. The group also evaluated the route of
immunization and found that Intra tumor immunization is superior to subcutaneous route, with higher tumor infiltrating lymphocytes (TILS) and E7 specific CD8 T cells and better survival [150]. The results of both in preclinical (Table 1) and clinical studies (Table 2) show that peptide based vaccines are a viable approach to the treatment of established HPV induced cancers; however, as mentioned before, there is no FDA approved immunotherapy for these diseases. The results of my dissertation work shows that TriVax and BiVax are an appealing immunotherapeutic approaches 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.

In the following paragraphs ways of improving immunogenicity of peptide vaccines including the major characteristics of some of the most commonly studied adjuvant, stimulation of the CD40-CD40L pathway and blockage of immunosuppressive factors are highlighted.
Table 1. Preclinical studies of peptide-based vaccines against HPV induced cancers: Mouse models.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Experimental design</th>
<th>Major findings (related to therapeutic effects)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7 long peptide 10µg+ Poly I:C (vaccine) 10µg+ DMXAA 20µg/g of body weight</td>
<td>Subcutaneous tumor model= 1x10⁵ TC-1 cells - Vaccination s.c. = D13 and D20 after tumor inoculation - DMMXAA (i.p.) treatment= D20 after tumor inoculation - DC maturation= Vaccination s.c. = D0, D7 Immunooassay= D14 - Cervicovaginal tumor model= Intravaginal challenge TC-1= D0 Vaccination s.c. = D4, D8 DMXAA treatment= D8</td>
<td>- DMXAA augmented immune response, anti tumor effect. DC maturation, and production of TNFα, IFNγ and IP-10 in the tumor effect of the DMXAA was dependent on time of administration - Introduction of novel aggressive cervicovaginal tumor model that recapitulates many aspect of CC tumor progression [151]</td>
</tr>
<tr>
<td>E7εβ-57 20µg+ PADRE 20µg + Poly I:C 20µg</td>
<td>Subcutaneous tumor model= 1x10⁵ TC-1 cells Inoculation of tumor cells= D0 Vaccination s.c. = D3 and weekly until mice died or tumor reached 2 cm Intratumor vaccination = D3 and every 5 days until mice died or tumor reached 2 cm</td>
<td>- The combination of E7 peptide, PADRE and Poly I:C generates the highest immune response and better survival of tumor bearing mice. - Intra tumor immunization is superior to subcutaneous route, with higher TILS and E7 specific CD8 T cells and better survival [150]</td>
</tr>
<tr>
<td>E7εβ-57 10µg + CGN 10µg</td>
<td>Subcutaneous tumor model= 1x10⁵ TC-1 cells Tumor challenge= D0 Vaccination s.c. = D4, D8, D12, D16</td>
<td>- E7 peptide combined with CGN generates better immune responses in naïve mice, better anti tumor effect and improved survival - CGN mechanism of action is through TLR4 - Co-administration of CGN may lead to enhance activation and migration of DCs to lymph nodes. - Other TLR4 ligands (Dextran, MPL A) produce comparable results to CGN [152]</td>
</tr>
<tr>
<td>E7εβ-57 50µg + SA-4-1BBL 25µg</td>
<td>Subcutaneous tumor model= 1x10⁵ TC-1 cells Tumor inoculation= D0 Vaccination s.c. = D10</td>
<td>- SA-4-1BBL has pleiotropic effects on DCs, T effector cells, and T regs - Co-administration of SA-4-1BBL lead to eradication of tumor in 75% of mice with long term immune memory, increase in memory CD8 pool (CD44hi) - Co-administration of SA-4-1BBL restored tumor killing response in mice bearing bigger tumors - Co-administration of SA-4-1BBL was more efficient than MPL, LPS in eradicating tumor [153]</td>
</tr>
<tr>
<td>E7εβ-57 25µg + E7εβ-50 25µg + E7εβ-50 25µg + PADRE 25µg + CpG ODN 1826 50µg+ ISA51 (VacciMax®)</td>
<td>Subcutaneous tumor model= 1x10⁵ TC-1/A2 cells Tumor inoculation in HLA 12mo old mice= D0 Vaccination s.c. = D19</td>
<td>- Eradication of 700mm² tumors within 3 weeks after vaccination - Chemical linkage of antigen to PADRE was no necessary for vaccine success [148]</td>
</tr>
<tr>
<td>E7εβ-57 fused to PADRE 50µg+ CpG ODN 1826 50µg+IFA or ISA51 (VacciMax®)</td>
<td>Subcutaneous tumor model= 5x10⁵ C3 cells Tumor inoculation= D0 Vaccination s.c. = D4, D5, D6, D9, D14</td>
<td>- Eradication of D4 (9/10), D5 (10/10), D6 (9/10), D9 27/30), D14 (5/5) old tumors - Some mice in the vaccine control group did not develop tumor [154]</td>
</tr>
<tr>
<td>E7εβ-57 50µg+ VSSP 160µg (LPS 30µg/ml + OMPC 20-60µg/ml)+ IFA</td>
<td>Subcutaneous tumor model= 2x105 TC-1 cells Tumor inoculation= D0 Vaccination s.c. = D10, D24</td>
<td>- Eradication of 100% of tumors by day 45 after tumor challenge - Better survival compared to peptide+ IFA (0% v.s. 80%) - Higher numbers of antigen specific C8 T cells (2 folds higher than with IFA) [149]</td>
</tr>
<tr>
<td>E7εβ-37 50µg + CpG ODN 1826 50µg</td>
<td>Subcutaneous tumor model= 5x10⁵ TC-1 cells Tumor inoculation= D0 Vaccination s.c. = D10, D14</td>
<td>- Eradication of 8/10 tumors - Better anti tumor effect and survival compared to peptide plus IFA (0/10 vs. 8/10 tumor free mice) - Long peptide exerts better antitumor effect and survival than minimal epitope when combined with CpG - Activation of professional APC is necessary for generation of high numbers of antigen specific CD8 T cells [155]</td>
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</table>
Table 2. Clinical studies of peptide-based vaccines against HPV induced cancers: Clinical trials

<table>
<thead>
<tr>
<th>Phase / Vaccine</th>
<th>Experimental design</th>
<th>Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase II Nine HPV16 E6 and four HPV16 E7 peptides, 0.3mg each + Montanide ISA-51</td>
<td><strong>Objective</strong> = Test Immuno geneticity and efficacy of a synthetic long-peptide vaccine in women with HPV16+ HGVIN. N= 20 woman. Vaccinations s.c. = 3-4 times, at 3 week intervals, each time in a different arm or leg.</td>
<td>At 3 months after the last vaccination: - 60% had clinical responses and reported relief of symptoms.  - Complete regression of lesions= 5 women, no detectable HPV16 in 4 At 12 months of follow-up:  - Clinical responses= 79%, with a complete response in 47%, maintained at 24 months of follow-up.  - All patients had vaccine-induced T-cell responses, patients with a complete response at 3 months had a significantly stronger IFNγ associated proliferative CD4+ T cell response and a broad response of IFNγ CD8 T cells than did patients without a complete response [143]</td>
</tr>
<tr>
<td>Phase II / Long overlapping of HPV16 E6 and E7 300µg+ Montanide ISA-51</td>
<td><strong>Objective</strong> = to evaluate the effect of HPV16 E6 and E7 synthetic long peptides vaccine on the antigen-specific T-cell response in resected CC patients. N= 6 (4 completed, 2 no completed) Vaccination s.c. = 4 times at a 3 week intervals.</td>
<td>- T-cell responses= 6/6 (E6), 5/6 (E7)  - Expansion of CD4 and CD8 T cells, detected up to 12 months after the last vaccination.  - The vaccine-induced responses were dominated by effector type CD4 (+)CD25(+)/FOXP3(-) IFNγ producing T cells and CD4 (+)/CD25(+)/FOXP3 (+) T cells [156]</td>
</tr>
<tr>
<td>Phase I Nine HPV16 E6 +/- four E7 overlapping long peptides + Montanide ISA-51</td>
<td><strong>Objective</strong> = to determine the toxicity, safety, and immunogenicity of HPV16 E6 and E7 long peptide vaccine on end-stage cc patients. N= 35 Vaccination s.c. = 4 times, at 3 week intervals. G1= 300µg/peptide G2=100µg/E6 peptide + 300 µg/E7 peptide G3= separate injections of E6 and E7 50µg/peptide.</td>
<td>- No toxicity beyond grade 2  - Co-injection of E6+E7 induced a strong and broad T-cell response dominated by immunity against E6  - Injection of the E6 and E7 peptides at two different sites increased the E7 response but did not affect the magnitude of the E6-induced immune response [157]</td>
</tr>
<tr>
<td>Phase I / E712-20 (ascending doses)+ linker peptide (KSS) + PADRE+ E736-93 5mg/ml+ Montanide ISA 51</td>
<td><strong>Objective</strong> = to test toxicity and tolerability, immune, virological and clinical response to the vaccine. N= 18 women with HGCIN or HGVIN. HPV16+ and HLA-A*0201+ HPV16+ Vaccine s.c. = escalating doses: 207, 617, and 2057µg/ml, 3 weeks apart.</td>
<td>- 3/18 patients cleared dysplasia  - 6/6 patients had an increased S100+ dendritic cell infiltrate  - 10/16 patients had E7-specific immune responses  - No DTH E712-20 in any patient  - 12/18 patients cleared virus from cervical scrapings, but all biopsy had viral DNA  - 6 partial regression CIN lesions [158]</td>
</tr>
<tr>
<td>Phase III / E713-20+ E766-93+ PADRE+ Montanide ISA 51</td>
<td><strong>Objective</strong> = to determine clinical and immunologic parameters after vaccination. N= 15 HLA-A*0201+ HPV16+ patients with recurrent or residual CC. Vaccine s.c. = 4 times, at 3 week intervals in a dose escalation scheme: 100µg, 300µg and 1,000µg of each peptide.</td>
<td>- No signs of toxicity  - 2 patients had stable disease for more than 1 year after vaccination  - 3 patients died of the disease during or shortly after the vaccination period  - 10 patients maintained progressive CC  - No CTL against E7 peptide detected in any patient after vaccination  - Strong PADRE helper peptide-specific proliferation was detected in 4/12 patients [159]</td>
</tr>
<tr>
<td>Phase I / E766-93 lipopeptide (4 dosage groups: 0.1, 0.3, 1.0, 2.0µM)</td>
<td><strong>Objective</strong> = to test the effectiveness of an HLA-A*0201-restricted, HPV-16 E7 lipopeptide vaccine in eliciting cellular immune responses in vivo in women with refractory CC. N= 12. Vaccination s.c. = 4 times, at 3 week intervals.</td>
<td>- 4/10 patients had E766-93 specific CTLs before vaccination  - 5/7 evaluable HLA-A<em>0201 patients after 2 vaccinations  - 2/3 evaluable HLA-A</em>0201 cultures after 4 inoculations  - 2/3 converted from unreactive to reactive after administration of 4 inoculations  - No clinical responses or treatment toxicities [160]</td>
</tr>
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1.6 Ways of improving immunogenicity of peptide vaccines

1.6.1 TLRs as adjuvants

The cells of the innate immune system can recognize structures that are present in pathogens but not expressed by mammalian cells; these are called pathogen associated molecular patterns (PAMPs); the receptors capable to recognize these PAMPs are called pattern recognition receptors or PRRs.

There are three classes of PRRs: Toll like receptors (TLRs), retinoic acid inducible gene I (RIG-I) like RNA helicases (RLHs: include RIG-I and MDA5), and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs)[161]. TLRs are membrane bound PRRs located in the cytoplasmic and endosomal membranes, whereas RLHs and NLRs are localized to the cytoplasmic compartment [161].

TLRs allow the innate immune system to discriminate among different groups of pathogens, and because these receptors are expressed in cells of both the innate (DC, macrophages, NK cells and mast cells) and the adaptive immune system (antigen primed CD8 T cells), they serve as a bridge between these two arms or the immune system [162]. TLRs activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway, which regulates cytokine expression, through several adaptor molecules including Myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon β (TRIF). Activation of the NFκB pathway links innate and adaptive immune response by production of inflammatory cytokines such as IL1, IL6, IL8,
TNFα, IL12, chemokines and induction of costimulatory molecules such as CD80, CD86, and CD40.

Some of commonly used TLR ligands employed as adjuvants in cancer immunotherapy include CpG (TLR9 ligand), LPS (TLR4 ligand) and Poly I:C (TLR3 ligand) among others. The following paragraphs offer a brief description of each of these TLR agonists.

Unmethylated CG dinucleotides are present at high frequency in prokaryotic DNA but are rare in eukaryotic DNA and stimulate cells that express TLR 9 to mount an innate immune response characterized by the production of Th1 and proinflammatory cytokines improving the function of APCs and boosting the generation of humoral and cellular vaccine specific immune responses, maturation, differentiation and proliferation of NK cells, T cells, monocytes / macrophages and B cells [163-167]. Binding of CpG to TLR9 activates a signaling pathway that proceeds through the stimulation of MyD88, IL-1 receptor-associated kinase (IRAK) and TNF receptor associated factor (TRAF) 6 and subsequently, the recruitment of various MAP kinases and transcription factors (including NFκB, the activator protein 1 or AP-1 and Interferon regulatory factor 7) that up regulate the expression of pro inflammatory genes [168]. The net result is the stimulation of genes of the immune response (IL1α, IL1β, TLR9 and TNF), cell signaling (NFKB1A, MyD88, IRAK-M and A20) and cell movement (BCL2A1 BCL2-related protein A1, Nucleosome assembly protein 1, NAK and epidermal growth factor receptor) [169].
The effectiveness of CpG as adjuvant has been shown in pre clinical and clinical studies [170-176].

TLR4 activation by bacterial LPS is achieved by the coordinate and sequential action of three other proteins: LPS binding protein, CD14 and MD 2 receptors, that bind LPS and present it to TLR4 by forming the activated (TLR4-MD-2-LPS) 2 complex, this activation can cause an excessively potent host response that generates life-threatening syndromes such as acute sepsis and septic shock [177, 178]. For that reason some researchers prefer to use MPL a detoxified derivative of Salmonella minnesota lipid A, which has a strong safety profile while retaining the immunostimulatory properties of LPS [179]. MPL acts as a partial TLR4 agonist that is functionally biased to TRIF-related adaptor molecule (TRAM)/TRIF signaling pathway stimulating a protective immunity rather than activating a systemic inflammatory response and has been provided in several vaccine formulations, including AS01, AS02, and AS04 adjuvant systems [180-185]. Importantly, LPS causes up regulation of CD40 in human peripheral blood plasmacytoid DCs and myeloid DCs [186, 187]; the effects of the activation of the CD40-CD40L pathway are discussed below.

1.6.1.1  Poly-IC (TLR3 Ligand)

We use of Poly-IC in the vaccines formulation because previous studies in our lab show that among several TLR agonists, Poly-IC is the most effective in the generation of long lasting CD8 T cell response probably because Poly-IC is the most potent inducer of Type I IFN [147, 188]. Poly-IC is a dsRNA synthetic
analog that binds to TLR3 and induces DC maturation via the adaptor molecule Toll interleukin 1 receptor domain 1 (TICAM1) and can enhance immune response against tumors [189]. Poly-IC has already proven to be beneficial as a mucosal adjuvant for influenza virus vaccine in a murine infection model. It is recognized through TLR3; in fact, TLR3 deficient mice showed reduced responses to Poly-IC and reduced production of inflammatory cytokines. Poly-IC has been used in the experimental treatment of melanoma, hepatoma, CC, breast, colon, urinary tract tumors, prostate cancer and hematopoietic tumors [190]. Interestingly, many tumor cells and tissues have elevated expression of some TLRs; TLR3 is expressed in Melanoma, breast cancer, colon cancer and Hepatocellular carcinoma. Zitvogel et al, found TLR3 positive tumor cells in 10% of patient samples and these cells responded well to Poly-IC treatment probably by inducing apoptosis [191].

In addition to TLR3, the RLHs RIG I and MDA5 may also act as sensors of viral infections through recognition of viral dsRNA and might up regulate Type I IFNs; MDA5 preferentially recognizes high molecular weight Poly-IC fragments while RIG I shows a preference for shorter RNA fragments and can also bind to ssRNA and recognizes paramyxoviruses, influenza virus and Japanese encephalitis virus [192, 193]. RIG I encodes a DExD/H box RNA helicase containing a caspase recruitment domain, the caspase recruitment domain transmits ‘downstream’ signals through the caspase recruiting domain containing IFNβ promoter stimulator (Cardif), resulting in the activation of the transcription factors NFκB and IRF-3 [194].
1.6.2 Targeting the CD40-CD40L pathway

We use an agonist αCD40 mAb in the formulation of one of our vaccines (TriVax) because CD40-CD40L interactions are important for the priming of CD8 T cell by APCs and DC maturation. CD4 T cells, which express CD40L, interact with DCs, which express CD40, and as a result the DCs become activated and are able to stimulate CD8 T cells. CD40-CD40L interactions up regulate the production of IL7, 12, and 15 on human mDCs, which contributes to CD8 T cell antigen specific expansion; thus, αCD40 mAb can directly activate APCs without the need of CD4 T cells and turn them into potent stimulators for CD8 T cells [195-198]. In addition to DCs, the activation of macrophages and NK cells appears to contribute to the in vivo antitumor effects of CD40 agonists [199, 200].

Several clinical trials using CD40 directed therapies for human cancers have been published and antibodies against CD40 have already been used as a therapeutic choice in an experimental model of autoimmune encephalitis, representing multiple sclerosis or in efforts to block atherosclerosis [201-208]. CD40 agonists have also been used to reinforce immune defense against tumors through CD40 system mediated pathways and CD40 ligation can overcome peptide induced peripheral CTL tolerance and increase antitumor vaccine efficacy [209-211].

C40-CD40L interaction stimulates the synthesis of many cytokines and adhesion molecules including IL1, IL2, IL6, IL8, IL10, TNFα, IFN, lymphotoxin A, TGFβ, GM-CSF and chemokines [212]. CD40 stimulation on endothelial cells up
regulates the expression of vascular cell adhesion protein 1 (VCAM1), intracellular adhesion molecule 1 (ICAM1) and E-selectin which facilitates lymphocyte attachment, rolling and transmigration into the inflamed site; as a result, CD40 mediated signaling may be crucial in promoting the migration of tumor reactive immune effector cells into the tumor tissue [213].

CD40 activation can induce the function of various downstream signaling pathways including both, pro apoptotic and anti apoptotic proteins. Indeed, signaling via CD40 may reactivate the antigen presenting functions of malignant cells and partly restore their in vitro recognition and killing by CTLs by upregulating the surface expression of ICAM1, MHC I, TAPs and tapasin, CD70, B7.1 and B7.2 and Fas and inducing immunostimulatory cytokine production on these cells [214, 215]. In contrast, CD40 expression in a wide variety of human cancers directly influence malignant cell proliferation and survival through the up-regulation of pro apoptotic gene products such as Bax and Fas, and the progressive loss of the survival protein cellular FLICE/caspase-8-inhibitory protein (cFLIP) protecting them from apoptosis [216-226].

1.6.3 Blockade of inhibitory/suppressor factors

Another way to improve immunogenicity of peptide vaccines is to administer (in addition to adjuvants) reagents that block inhibitory or suppressive factors either systemically or in the tumor microenvironment. To do so some researches use reagents capable of blocking the programmed death 1/
programmed cell death 1 ligand 1 (PD-1/PD-L1) pathway, the cytotoxic T lymphocyte antigen 4 (CTLA4) signaling or Treg's suppressive effect
Chapter 2: TriVax-HPV: An Improved Peptide-Based Therapeutic Vaccination Strategy Against Human Papillomavirus-Induced Cancers¹.

2.1 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. HPV is responsible for several malignancies such as cervical carcinoma. Vaccines targeting oncogenic viral proteins like HPV16-E6 and HPV16-E7 are ideal candidates to elicit strong immune responses without generating autoimmunity because: (1) these products are not expressed in normal cells and (2) 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

¹ Portions of these results have been previously published [227] and are utilized with permission of the publisher.
efficacy of TriVax prepared with the immunodominant CD8 T-cell epitope HPV16-E7\textsubscript{49-57}, mixed with Poly-IC adjuvant and costimulatory $\alpha$CD40 antibodies.

Results: TriVax using HPV16-E7\textsubscript{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 anti-tumor 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 [227].

2.2 Introduction

Cervical cancer is the second most prevalent cancer among women. HPV causes 99 % CC, and the HPV16 and HPV18 genotypes account for 80 % these cases [228]. Worldwide, CC is responsible for $\approx 250\,000$ deaths per year and for causing a huge economic burden in related health care costs [64, 229]. The current approved prophylactic vaccines for HPV are not useful for treating established malignant disease, nor can protect already infected individuals from
developing cancer [11-13]. Most importantly, a significant proportion of women, especially in the third world, will not receive the prophylactic vaccines and will continue to be at high risk of developing CC [230]. Because, conventional therapies for CC are usually devastating, invasive, toxic, and associated with 10 % recurrence [231], 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 [232-234]. The antigens recognized by CD8 T cells on their target cells are small peptides derived from viral or TAAs that associate with cell surface class I products of the 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 [143, 148, 150, 151, 235-237]. Synthetic peptides representing these TAAs have been tested in numerous ways in patients and mouse cancer models for their ability to generate anti-tumor T-cell responses capable of exhibiting anti-tumor effects [148, 150, 235, 238-240]. 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 [147, 241]. This vaccine called TriVax consists of a synthetic peptide corresponding to the minimal T-cell epitope, Poly-IC adjuvant, and costimulatory monoclonal αCD40 mAb, which are mixed together and administered intravenously. After two sequential TriVax immunizations (prime/boost) with the well-known Ova257-264 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 [147]. 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-E749-57 induced large and persistent T-cell responses that were effective against two different tumors expressing HPV16-E7. Interestingly, the anti-tumor 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.

2.3 Material and Methods

2.3.1 Mice

Six to nine week old female C57BL/6 (B6) mice were obtained from the National Cancer Institute/Charles River program (Wilmington, MA).
IFN\(\gamma\) 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.

2.3.2 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 [242]. 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 pRSV- neo-derived plasmid containing the complete HPV16 genome [238]. The EL4 cell line was purchased from the American Type Culture Collection (Manassas, VA).

2.3.3 Peptides, MHC-I tetramer, and antibodies

The synthetic peptide RAHYNIVTF from HPV16 E7 (E7\(_{49-57}\)), defined as an immunodominant H-2Db-restricted CD8 T-cell epitope [238, 243], and control
peptide NAYFKGL 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).

2.3.4 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 of 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.

2.3.5 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 percentage tetramer positive cells of the CD8+/MHC II negative population. To determine whether CD8 T cells 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 [244]. Briefly, CD8 T cells from spleens of vaccinated mice were purified by positive selection using antibody-coated magnetic beads (Miltenyi Biotec, Auburn, CA). Responder (CD8-purified) cells were incubated at 3x10^5, 1x10^5, and 3x10^4 per well, together with 1x10^5 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).

2.3.6 Evaluation of therapeutic anti-tumor effects

Mice received 3x10^5/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 was compared with B6 wild-type (WT) mice. For cell depletions, each mouse received 300 µg anti-NK1.1, 300 µg anti-CD4, or 500 µg αCD8 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 killed when the tumor area reached 400 mm². Results are presented as the mean tumor size (area in mm²) ± SD for every treatment group at various time points until the termination of the experiment.

2.3.7 Statistical analyses

Statistical significance of the numbers of antigen specific CD8 T cells (EliSpot) using 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).
2.4 Results

2.4.1 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 [238, 243]. We first determined the ability of synthetic peptide E7\textsubscript{49-57} representing this sequence to elicit an immune response when administered to mice in combination with Poly-IC and αCD40 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 αCD40 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 Fig. 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\textsubscript{49-57} peptide alone or in combination with αCD40 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\textsubscript{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).
spleen CD8 T cells from TriVax- and BiVax-immunized mice were shown to be very effective in recognizing E7\textsubscript{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 Fig. 2a, tumor growth was effectively controlled in those mice that received TriVax, BiVax, and, to some extent, peptide E7\textsubscript{49-57} with αCD40 mAb, as compared to mice vaccinated with E7\textsubscript{49-57} alone (where tumors grew at an accelerated rate and mice had to be euthanatized 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\textsubscript{49-57} with αCD40 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).

**Fig. 1** Synergy for the potentiation of the immunogenicity of E7\textsubscript{49-57} peptide by Poly-IC and αCD40 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 IFNγ EliSpot assays. Stimulator cells: EL4 cells pulsed or not with E7\textsubscript{49-57} 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\textsuperscript{4} CD8 T cells and 1×10\textsuperscript{5} stimulator cells. Numbers represent the total spots present in each well. Representative results of data obtained from two different experiments.
2.4.2 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 Fig. 2a, tumor growth was effectively controlled in those mice that received TriVax, BiVax, and, to some extent, peptide E7\textsubscript{49-57} with αCD40 mAb, as compared to mice vaccinated with E7\textsubscript{49-57} alone (where tumors grew at an accelerated rate and mice had to be euthanatized 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\textsubscript{49-57} with αCD40 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\textsubscript{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\textsubscript{49-57} with αCD40 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\textsubscript{49-57} alone. The therapeutic effect of TriVax with E7\textsubscript{49-57} was antigen specific since TriVax prepared with an irrelevant peptide (Ova\textsubscript{176-183}) was not effective in reducing the rate of tumor growth (Fig. 2c). The therapeutic effects observed with E7\textsubscript{49-57} BiVax (Fig. 2a) were confirmed in an additional experiment using a larger number
of mice (6), where in this case, this therapeutic vaccination strategy resulted in 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).

![Therapeutic effects induced by TriVax and BiVax immunization against established TC-1 tumors.](image)

**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 x 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 x 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.
The impressive therapeutic anti-tumor effects observed with E7\textsubscript{49-57} TriVax was evaluated against larger and more established TC-1 tumors. The results shown in Fig. 3a demonstrate that therapeutic immunization with TriVax 11 days post-tumor inoculation resulted in 100% complete rejections and increased survival. The therapeutic effectiveness of E7\textsubscript{49-57} TriVax was also examined using a different tumor cell line called C3.43, which also expresses the HPV16-E7 antigen. As presented in Fig. 3b, TriVax using the E7\textsubscript{49-57} peptide resulted in complete rejection of 6-day established C3.43 tumors. Furthermore, at the conclusion of this experiment (day 30), considerable numbers of E7\textsubscript{49-57}-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 IFN\textgamma pretreatment of the tumor cells (Fig. 3d), which enhances the expression levels of MHC-I on both tumors (data not shown).
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 (IFNγ) pretreatment of the tumor cells (Fig. 3d), which enhances the expression levels of MHC-I on both tumors (data not shown).

**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 3x10⁵ 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 3x10⁵ 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 IFNγ ELiSpot assays. Stimulator cells were: EL4 cells loaded or not with E749-57 peptide, TC-1 and C3.43 tumor cells previously treated or not with IFNγ (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 3x10⁵ 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 3x10⁵ TC-1 on their right flanks and 3x10⁵ 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.
2.4.3 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 E7_{49-57} TriVax-treated mice that had successfully rejected TC-1 tumors would be able to respond to subsequent tumor re-challenges. The experiment shown in Fig. 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.

2.4.4 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 Fig. 4a, i.m. E7_{49-57} 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 82 (post prime), suggesting that the tumor had not been totally eradicated.

Fig. 4 Antitumor efficacy of intramuscularly administered TriVax. (a) Mice were inoculated s.c. with 3x10⁵ 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.
2.4.5 One TriVax injection is sufficient to elicit effective, long-lasting anti-tumor effect

Next we wanted to determine if administration of booster shots are necessary for TriVax immunogenicity and antitumor effect. To do so mice (5/group) were inoculated with $3 \times 10^5$ TC-1 cells s.c., 6 days later vaccinated with TriVax *i.v.*, and a booster was given on days 7, 13 or not at all and the percentages of tetramer positive cells measured in blood. As shown in Fig. 5, even though a single injection generated low numbers of antigen specific CD8 T cells (Fig. 5a) these mice were able to control tumor growth and ultimately completely reject tumor at the same level that mice that received a booster shot (Fig. 5b). To ensure that mice were completely cured from tumor, mice were depleted of CD8 cells by administration of 500 µg of αCD8 antibodies *i.p.* on days 43, 46, 49, 62 and 65 after prime, and continue to be followed for the presence of palpable tumors. Mice remained tumor free until the end of the experiment 90 days after prime. It is possible that the low number of antigen specific CD8 T cells detected in peripheral blood is due to the presence of the majority of these cell are in other tissues, it is also possible that the tumor itself as a booster for the antigen specific cells generated after prime leading to tumor clearance.
Fig. 5 One TriVax injection is sufficient to elicit effective, long-lasting anti tumor effect. (a) Mice (5/group) were inoculated with 3x10^5 TC-1 cells s.c., 6 days later mice were vaccinated with TriVax i.v., a booster was given on days 7, 13 or not at all and the percentages of tetramer positive cells measured in blood on day 39. (b) Tumor growth was measured 2 times per week with automatic calipers. To ensure tumor was completely rejected, mice were depleted of CD8 cells by administration of 500 µg of αCD8 antibodies IP on days 43, 46, 49, 62 and 65 after prime. A non-vaccinated group was included as control.

2.4.6 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 E7_{49-57} TriVax was examined in CD8-, CD4-, and NK-depleted mice and in IFNγ KO and IFNαβR KO mice. The results in Fig. 6a 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 IFNγKO and IFNαβR KO mice were able to control tumor growth to a great degree, and in some instances complete rejections were observed (Fig. 6b). When the intensity of the CD8 T-cell responses in immunized IFNγ and IFNαβR KO tumor-free mice was evaluated after TriVax immunization (prime/boost), it became evident that
absence of IFNγ had little effect, while the role of type-I IFN in generating high CD8 T-cell numbers was clearly more important (Fig. 6c).

**Fig. 6** 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 3x10^5 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 IFNγ KO and IFNαβR 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 IFNγ KO group and 2/4 mice from the IFNαβR KO group had complete tumor rejections. (c) Immune responses elicited by TriVax in naïve IFNγ KO and IFNαβR KO mice. In a separate experiment, non-tumor bearing wild type (WT), IFNγ KO and IFNαβR 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 (3 mice per group).
2.5 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 melanosomal 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 B16 melanomas in WT mice [147, 245].

Because the HPV16-E7 protein plays a critical role in maintaining the transformed phenotype of the tumor cells, the possible appearance of antigen-loss mutants, which is often seen in immunotherapy, should be diminished [233, 246, 247]. 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 αCD40 mAb (TriVax) resulted in extensive antigen-specific T-cell responses that were durable and capable of eradicating established tumors. Furthermore, administration of a single shot of TriVax was sufficient to eradicate TC-1 s.c.
tumor. 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 [248-250]. For this reason, some investigators advocate the use of long synthetic peptides that require antigen processing by professional APCs [239]. The present results and previous work by our group [147, 241] demonstrate that vaccines containing minimal CD8 T-cell epitopes can be highly immunogenic when provided together with Poly-IC and αCD40 mAb and in some cases with Poly-IC alone (Fig. 1). In fact, to the best of our knowledge, the magnitude of the responses we have observed with several minimal CD8 T-cell epitopes using TriVax (HPV16-E749-57, Trp1455/9M, Trp2180-188, Ova257-264, rNEU66-74) 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 whether 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 αCD40 mAb was also quite effective in eliciting CD8 T-cell responses that resulted in significant therapeutic benefit (Figs. 1, 2). These results indicate that the αCD40 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, 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 αCD40 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 αCD40 mAb, TriVax administered i.m. and TriVax prime only 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 or TriVax was administered i.v.

The rationale for the use of αCD40 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 [251]. 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 E7_{49-57} peptide appears to be somewhat unique, since other potent CD8 T-
cell epitopes such as Ova_{257-264} (SIINFEKL) and melanoma Trp_{1455-463/9M} (T
APDNLGYM) are ineffective and require αCD40 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 [252, 253], which is considered important for the
induction of effective T-cell responses [254]. Our results indicate that in the
absence of type-I IFN signaling (in IFNαβR KO mice), TriVax was less effective
in generating sufficient numbers of CD8 T cells that could be necessary for total
disease eradication (Fig. 6b, c). 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
IL12 generated by the combination of Poly-IC and αCD40 mAb. It was somewhat
unexpected to observe a significant anti-tumor effect of TriVax in the absence of
IFNγ (Fig. 6b), since this cytokine is considered to be critical for the anti-tumor
effects of CD8 T cells [255-257]. Nevertheless, our results indicate that IFNγ 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 IFNγ was shown to play a negative role in the anti-tumor effects of TriVax using Trp1455-463/9M and Trp2280-288, where complete rejections of established tumors were observed in IFNγ KO mice and not in WT mice [245]. Thus, it is possible that IFNγ plays an effector role with the TC-1 tumors, limiting cell proliferation or that the increase MHC-I expression induced by IFNγ 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 αCD40 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.
Chapter 3: BiVax: A Peptide/Poly-IC Subunit Vaccine That Mimics an Acute Infection Elicits Vast and Effective Anti-Tumor CD8 T Cell Responses

3.1 Abstract

Therapeutic vaccines for the treatment of cancer are an attractive alternative to some of the conventional therapies that are currently used. More importantly, vaccines could be very useful to prevent recurrences when applied after primary therapy. Unfortunately, most therapeutic vaccines for cancer have performed poorly due to the low level of immune responses that they induce.

Previous work done in our laboratory in cancer mouse models demonstrated that vaccines consisting of synthetic peptides representing minimal CD8 T cell epitopes administered i.v. mixed with Poly-IC and αCD40 antibodies (TriVax) were capable of inducing massive T cell responses similar to those found during acute infections. We now report that some peptides are capable of inducing large T cell responses after vaccination with Poly-IC alone (BiVax). The results show that the differences between TriVax and BiVax immunogenicity and antitumor effect is most likely due to the numbers of antigen specific CD8 T cells generated after vaccination. Amphiphilic peptides are more likely to function as strong immunogens in BiVax and that systemic immunizations (i.v. or i.m.) were more effective than local (s.c.) vaccine administration. The immune responses induced
by BiVax were found to be effective against established tumors in two mouse cancer models. The role of various immune related pathways such as type-I IFN, CD40 costimulation, CD4 T cells TLRs and MDA5 was examined. The present findings could facilitate the development of simple and effective subunit vaccines for diseases where CD8 T cells may hold a therapeutic benefit.

### 3.2 Introduction

CD8 T cells play an important role in the control of intracellular infectious agents and have the potential to mitigate malignant diseases. The CD8 TCR recognizes small peptides bound to MHC class I (MHC-I) products on APCs. These peptides known as CD8 T cell epitopes are usually derived from processed proteins corresponding to microbial components or TAAs. The identification of these peptides has lead to developing epitope-based vaccines to induce antigen-specific CD8 T cell responses for the prevention or treatment of various infections and malignancies [258]. Synthetic peptides containing defined CD8 T cell epitopes constitute an attractive approach for vaccine development due to their ease of manufacturing and safety as compared to other vaccine types such as recombinant DNA-derived proteins, plasmids, viruses or genetically engineered cells [259]. However, most peptide vaccines generate minute CD8 T cell responses as compared to the T cell levels observed during acute infections. Without a doubt the vaccine’s poor immunogenicity results in suboptimal clinical benefit against an established/advanced disease and have enticed many clinical researchers to seek other immunotherapy alternatives.
These disappointing results could be explained in part by the use of weak immunological adjuvants (e.g., IFA), suboptimal peptide formulations and inappropriate routes of vaccine administration.

For some time our laboratory has been involved in the optimization of peptide vaccines for the induction of anti-tumor CD8 T cell responses [261, 262]. We have recently proposed that in order to have an impact against established tumors, the vaccines must elicit a CD8 T cell response resembling the magnitude and duration of the responses observed during acute viral infections, where more than one third of the circulating CD8 T cells show specificity for the offending microorganism [263]. We have reported that synthetic peptides corresponding to the minimal CD8 T cell epitope administered intravenously mixed with Poly-IC and costimulatory αCD40 antibodies resulted in the induction of vast numbers of antigen-specific CD8 T cells in mice, resembling the levels observed during acute infections [241]. Furthermore, experiments performed in several mouse cancer models demonstrated that this vaccination strategy (TriVax) was highly effective against established tumors resulting in many instances in complete disease eradication [147, 227]. Although these results were highly encouraging for developing therapeutic peptide vaccines for humans, there are serious concerns regarding the systemic use of agonistic αCD40 antibodies due to potential deleterious effects such as cytokine storm and or liver toxicity [264, 265].

We report here a novel strategy (BiVax) that allows synthetic peptides to induce high levels of antigen-specific CD8 T cells, when administered in
combination with Poly-IC without the use of costimulatory αCD40 antibodies. Immune responses produced by BiVax were highly dependent on the simultaneous administration of peptide and Poly-IC, on the peptide composition, vaccine formulation and route of administration. As expected, the magnitude of the response was dependent on the expression of the Poly-IC receptors TLR3 and MDA5. Peptide combinations with supposedly potent agonists to other TLRs (CpG, Pam3CSK4) were not able to generate the strong CD8 T cell responses. Interestingly, the magnitude and duration of the CD8 T cell responses generated by peptide and Poly-IC mixtures did not rely on the presence of CD4 T cells, scavenger receptor-A (SR-A) or type-I IFN signals and was minimally affected by the absence of CD40 signaling. The present findings may help to clarify some of the mechanisms involved in the generation of massive and lasting CD8 T cell responses by peptide epitope vaccines and could facilitate the development of more effective immunotherapies for cancer.

3.3 Materials and Methods

3.3.1 Mice and cell lines

Six- to eight-week-old female C57BL/6 (B6) mice were obtained from the National Cancer Institute/Charles River Program (Wilmington, MA). CD40-deficient (B6.129P2-Tnfrsf5<sup>tm1Kik</sup>/J), TLR3-deficient (B6;129S1-<i>Tlr3<sup>tm1Flv</sup></i>/J) mice in a B6 genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeder mice deficient on MDA5 (<i>Ifih1</i>) and interferon alpha/beta
receptor (IFNαβR,) both on a B6 background were kindly provided by Dr. Marco Colonna (Washington University School of Medicine, St. Louis MO) and Philippa Marrack (National Jewish Medical and Research Center, Denver CO), respectively. Mice deficient for the expression of both scavenger receptors-A (SR-A) and MARCO (double knockouts on a B6 background) were a gift from Drs. Jim Mulé and Shari Pilon-Thomas (Moffitt Cancer Center). EL4 thymoma cells were purchased from the American Type Culture Collection (Manassas, VA). The TC-1 tumor cell line, expressing HPV16-E7 antigen was obtained from Dr. T-C Wu (Johns Hopkins University, Baltimore, MD). The B16-F10 mouse melanoma cell line was provided by Dr. Alan Houghton (Memorial Sloan Kettering Cancer Center, New York, NY).

3.3.2 Reagents

All synthetic peptides were purchased from A&A Labs (San Diego, CA). Lipopeptides were synthesized by attachment of 2 palmitic acid (Pam) chains via the 2 amino groups of an N-terminal lysine. The purity and identity of peptides were determined by high-performance liquid chromatography and mass spectrometry analysis by the vendor. Amino acid sequences of the peptides used in these study are shown throughout the text and in the corresponding figures. All peptides were solubilized at 20 mg/ml in DMSO-TFA (99.9%/0.1%) and stored in aliquots at -80 °C.

Polyinosinic:polycytidylic acid (Poly-IC) stabilized with poly-lysine and carboxy-methylcellulose (Poly-ICLC/Hiltonol) was kindly provided by Dr. Andres
Salazar (Oncovir, Inc., Washington, DC). Synthetic oligodeoxynucleotides containing CpG motifs, CpG-1826 (Class-B; 5’-tccatgacgtctgtcagtt-3’) and CpG-1585 (Class-A; 5’-ggGGTCAAAGTTGAgggggg-3’) were prepared at the Mayo Clinic Molecular Biology Core and were kindly provided by Dr. R. Vile (Mayo Clinic, Rochester, MN). Poly-AU and Pam3CSK4 were purchased from InvivoGen (San Diego, CA). Antibodies for in vivo use in mice, anti-PD-L1 (clone 10F.9G2) and anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) were purchased from BioXCell (West Lebanon, NH).

Fluorochrome-labeled antibodies were obtained from eBioscience, Inc (San Diego, CA). Fluorescence-labeled MHC-I/peptide tetramers were kindly provided by the National Institute of Allergy and Infectious Disease Tetramer Facility at the Emory University (Atlanta, GA NIH).

3.3.3. Immunizations and assessment of immune responses

Vaccines were freshly prepared by diluting and mixing the peptides and TLR agonists in PBS to the appropriate concentration in order to inject 30-200 µg peptide (depending on the peptide) and 50-100 µg TLR agonist in 50-200 µl per mouse (depending in the route of administration). In most instances vaccines were administered intravenously (200 µl/injection in the tail vein). In some experiments mice were vaccinated subcutaneously or intramuscularly (50 µl/injection in 2 sites per mouse). Mice received 1-3 booster immunizations every 5-7 days. In some experiments, mice were depleted of CD4 T cells using αCD4
monoclonal antibodies administered \textit{i.p.} 300 \textmu g twice, on days -2 and 0 before each immunization. Immune responses were mostly assessed by tetramer staining using peripheral blood samples obtained at various time points or in spleen cells at the termination of the experiments. Fluorescence was measured using either a FACSCalibur or an LSRII flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Ashland, OR). Immune responses were also determined in spleens with EliSpot assays as described [147].

\subsection{3.3.4 Evaluation of vaccine therapeutic anti-tumor effect}

Mice received $3 \times 10^5$/mouse tumor cells (TC-1 or B16-F10) s.c. in a shaved rear flank 5-6 days (as noted) before their primary immunization. In some instances, mice received 200 \textmu g anti-PD-L1 antibodies \textit{i.p.} on days 1 and 3 post-immunization. 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 mm$^2$. Results are presented as the mean tumor size (area in mm$^2$) \textpm SD for every treatment group at various time points until the termination of the experiment.

\subsection{3.3.5 Peptide curves}

As a mean to gauge the avidity of CD8 T cells from TriVax and BiVax vaccinated mice for their cognate peptide, CD8 T cells from spleens of vaccinated mice were co-cultured in triplicated for 36-48 hours with irradiated
EL4 cells that were pulsed for 3 hours with decreasing amounts of the $E_{749-57}$ peptide. Then the supernatants were collected and the amount of IFN$_{\gamma}$ produced assessed by means of ELISA. Briefly, ELISA plates were coated with $\alpha$IFN$_{\gamma}$ capture antibody at 4°C overnight, blocked with blocking solution for 2.5 hours at room temperature and the 100 $\mu$l of supernatant added to the plate and incubated for 24 hours at 37°C. Then plates were washed and incubated at room temperature with biotinilated $\alpha$IFN$_{\gamma}$ for 2.5 hours and for 1 hour with horseradish peroxidase (HRP)-Avidin. The plates were revealed with the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate and read within 20 minutes at 420nm in an automatic ELISA plate reader. Results are expressed as total amount of IFN$_{\gamma}$ produced and as percentage of maximum response.

### 3.3.6 Statistical analyses

Unpaired Student’s $t$ test was used to determine statistical significance of differences in numbers of antigen specific CD8 T cells (EliSpot, tetramer) and cytokine levels (ELISA). 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).
3.4 Results

3.4.1 TriVax and BiVax differential effects are due to the quantity of antigen specific CD8 T cells generated after vaccination

We recently reported that 2 sequential peptide immunizations (prime and boost, 2 weeks apart) using the CD8 T cell epitope HPV16-E7\textsubscript{49-57} (RANYNIVTF) combined with Poly-IC and αCD40 monoclonal antibodies (TriVax) resulted in huge CD8 T cell responses capable of eradicating established TC1 tumors in 100% the mice (Chapter 2, published in Cancer Immunology Immunotherapy [227]). Interestingly, vaccination with a mixture of HPV16-E7\textsubscript{49-57} and Poly-IC, without the αCD40 mAb (BiVax) was able to induce a CD8 T cell response, but the magnitude was substantially lower (~30%) as compared to TriVax and only 30-50% of the vaccinated mice were able to completely reject their tumors.

We wanted to examine the source of these differential effects. To do so we vaccinated mice with either TriVax or BiVax \textit{i.v.} and checked the number of antigen specific cells generated in blood and spleen after the booster shot given 13 or 6 days after prime, respectively (Fig. 7a). As shown before BiVax generated approximately half of the numbers of tetramer positive cells both in blood and spleens. Next we wanted to know if these levels of antigen specific cells co-related with the ability of the CD8 T cells to recognized tumor cells \textit{in vitro}. Indeed CD8 T cells from BiVax vaccinated mice were less efficient in recognizing tumor cells in an EliSpot assay (Fig. 7b). These results might be due
to the fact that (i) CD8 T cells generated by TriVax have a higher avidity for the
tumor cells or (ii) the sheer number of CD8 T cells are responsible for these
differential effect antigen and/or (iii) somehow CD8 T cells from TriVax
vaccinated mice persist for a longer period of time than those generated by
BiVax immunization. To explore these options CD8 T cells from spleen of
vaccinated mice (from mice on figure 7a) were positive selected and co-culture
with irradiated EL4 cells pulsed with decreasing amount of the E7\textsubscript{49-57} peptide
then the amount of IFN\textsubscript{γ} produced was measured using ELISA. As shown on
figure 7c and d, even though CD8 T cells from TriVax vaccinated mice produced
double the amount of IFN\textsubscript{γ} at any given dilution of stimulating peptide, the curve
of percentage of maximum response are almost identical, indicating that CD8 T
cells from both TriVax and BiVax vaccinated mice have the same avidity for the
E7\textsubscript{49-57} peptide. Lastly, we followed the persistency of antigen specific CD8 T
cells in the blood of mice vaccinated with BiVax of TriVax, to do so mice were
immunized with TriVax or BiVax using either the E7\textsubscript{49-57} peptide (Fig. 7e) or the
Pam Trp1 peptide (Fig. 7f); a booster shot was given 9 days later and the
percentage of tetramer positive CD8 T cells determined at different time points
for two months. We found that antigen specific CD8 T cells from BiVax-HPV and
TriVax-HPV vaccinated mice follow the same kinetics and the same hold true for
another tumor model antigen (Pam Trp1).
Fig 7. TriVax and BiVax differential effects are due to the quantity of antigen specific CD8 T cells generated after vaccination. (a) Mice (3 per group) were vaccinated with TriVax or BiVax i.v. a booster shot was given 13 and 6 days after the prime (respectively) and the percentage of tetramer positive CD8 T cells determine in blood and spleens 7 days after boost. (b) CD8 T cells from spleen of vaccinated mice (from mice on figure a) were positive selected and a fraction of them co-cultured with target cells in an EliSpot assay as described in "Materials and Methods"; the other fraction was used to measure IFNγ production after co-culture the CD8 T with irradiated EL4 cells pulsed with decreasing amount of the E7 49-57 peptide using ELISA, results are expressed as total amount of IFNγ produced (c) and as percentage of maximum response (d). Mice (3 per group) were immunized with TriVax or BiVax using either the E7 49-57 peptide (e) or the Pam Trp1 (f) peptide; a booster shot was given 9 days later and the percentage of tetramer positive CD8 T cells determined at different time points for two months.
3.4.2 Additional BiVax boosters improve vaccine immunogenicity and correlates with enhance anti tumor effect

To optimize BiVax in the aforementioned tumor model we first assessed the possibility of reducing the time interval between prime and booster immunizations and adding another booster to increase the speed and intensity of immune response and enhance the anti-tumor effect. As shown in Fig. 7a, a time interval between prime and boost of 7 or 5 days resulted in ~40% tetramer positive CD8 T cells, which is identical to the levels previously observed when prime and boost were administered 13-21 days apart [227]. On the other hand, a substantially lower response was observed when the booster immunization was given 3 days after the prime. Next, we assessed whether an additional booster immunization (prime and 2 boosts, 6-7 days apart), would further augment the numbers of antigen-specific CD8 T cells and would improve the outcome in TC-1 tumor-bearing mice. Indeed the additional BiVax booster immunization increased the levels of circulating tetramer positive CD8 T cells to ~60% in both tumor-free and tumor bearing mice (Fig. 8b). Most importantly, all the mice that received the prime+2 booster BiVax therapy rejected their tumors (Fig. 8c).
Additional BiVax boosters improve vaccine immunogenicity and correlates with enhanced anti tumor effect. (a) B6 mice were given a boost 3, 5 and 7 days after prime; five days later the percentage of tetramer positive CD8 T cells was determined in blood \((n=3 \text{ mice per group})\). (b) Mice were inoculated with \(3 \times 10^5\) TC-1 cells s.c.; six days later they were primed with BiVax E7\(_{49-57}\) and boosted 6 and 14 days later. The percentage of tetramer positive CD8 T cells was determined in blood 7 or 8 days after boost. A tumor bearing non vaccinated group and a tumor free vaccinated group were included as controls. *No statistical significant differences between 1 and 2 boosts in tumor free mice were observed. **Statistical significance between 1 and 2 boosts \((P<.001)\) in tumor bearing mice was observed. (c) Tumor sizes were assessed 2-3 times a week using automatic calipers. Mice were euthanized when tumors reached 2cm in diameter on either side. After tumor rejection mice were CD8 T cell depleted by i.p. inoculation with 500 \(\mu\)g of αCD8 antibody (days 43, 46, 49, 62 and 65 after prime) and no tumors appeared. CD8 T cell depletion was monitored by flow cytometry \((n=6 \text{ mice treatment group, 3 mice in the control groups})\).

3.4.3 Effect of peptide residue substitution on BiVax immunogenicity

Since variation on peptide residue that augment peptide binding to the MHC molecule can augment immunogenicity of peptides use in vaccination, we set out to test the effect of modifying the E7\(_{49-57}\) peptide on BiVax.
immunogenicity. To do so mice were vaccinated \textit{i.v.} with the AAHYNIVTF (AF), AAHYNIVTL (AL), RAHYNIVTF (RF= E7\textsubscript{49-57}) or RAHYNIVTL (RL) peptide; seven days later mice received a booster shoot and the percentages of tetramer positive CD8 T cells was measured at different time points. At the end of the experiment (day 27 after the prime) CD8 T cells from the aforementioned groups were positive selected from spleen and assayed for IFN\textgamma production by means of EliSpot as described in “Materials and Methods”. As shown on Figure 9, even though binding prediction analysis indicates that mouse TCR should have higher binding affinity for the AL peptide followed by RL, AF and finally the RF peptide, our results show that the RF is actually the optimal peptide in our vaccine formulation. Not only the RF peptide generates the strongest immune response (Fig. 9a) but they also are more efficient in recognizing tumor cells in vitro (Fig. 9b).

![Fig 9. Effect of peptide residue substitution on BiVax immunogenicity. (a) Mice (3 per group) were immunized with AAHYNIVTF (AF), AAHYNIVTL (AL), RAHYNIVTF (RF= E7\textsubscript{49-57}) or RAHYNIVTL (RL) peptide \textit{i.v.} seven days later mice received a booster shoot and the percentages of tetramer positive CD8 T cells was measured at different time points. (b) At the end of the experiment (day 27 after the prime) CD8 T cells from the aforementioned groups were positive selected from spleen and assayed for IFN\textgamma production by means of EliSpot as described in “Materials and Methods”.](image-url)
3.4.4 Effect of peptide size on BiVax E7\textsubscript{49-57} immunogenicity.

It has been suggested that vaccines using of long synthetic peptides containing within their sequence a defined CD8 T cell epitope will result in superior immune responses as compared to the use of peptides corresponding to the minimal epitope. The reasons proposed for the use of long peptides versus minimal peptides is that the former would need to be presented to the CD8 T cells by professional APCs such as DCs that are capable of internalizing and processing the long peptides, while the short minimal peptides could be presented by any MHC I expressing cell, potentially resulting in immune tolerance. In addition, some long peptides may contain MHC II binding sequences, which may stimulate CD4 T helper cells, further enhancing CD8 T cell activation end expansion. Thus, we assessed the immunogenicity using the BiVax approach of two long peptides of 13 and 35 residues, HPV16-E7\textsubscript{45-57} (AEPDRAHYNIVTF) and HPV16-E7\textsubscript{43-77} (GQAEPDRAHYNIVTFCCCKCDSTLRLCVQSTHVDIR), containing within their sequences the minimal 9-residue HPV16-E7\textsubscript{49-57} CD8 T cell epitope (underlined). Both long peptides contain a CD4 T cell epitope, HPV16-E7\textsubscript{48-57} (DRAHYNIVTF) that could potentially increase the extent of the CD8 T cell responses. Contrary to what was predicted, the magnitude of the CD8 T cell responses observed in blood generated by the long peptides was considerably lower as compared to the result obtained using the minimal CD8 T cell epitope (Fig. 10a). The differences in immunogenicity between short and long peptides were even more evident when quantifying of the total numbers of antigen-specific CD8 T cells in spleens,
where the minimal epitope HPV16-E7\textsubscript{49-57} was found to generate vastly more antigen-specific CD8 T cells as compared to the long peptides (Fig. 10b).

**Fig. 10** Effect of peptide size on BiVax E7\textsubscript{49-57} immunogenicity. Mice were immunized with equimolar amounts of the E7\textsubscript{49-57}, E7\textsubscript{45-57} and E7\textsubscript{43-77} peptides, a booster shot was given 7 days later and the percentage of tetramer positive CD8 T cells was determined in blood (a) and spleens (b) 14 and 22 days after prime, respectively. (c) Mice were primed (p) with either BiVax E7\textsubscript{49-57} or BiVax E7\textsubscript{44-77} (as indicated), 7 days later received a boost (b) with BiVax E7\textsubscript{49-57} and 7 days after the boost the % tetramer positive cells were measured in blood. *No statistical significant difference was observed (n= 3 mice per group for all experiments).

### 3.4.5 Applicability of BiVax to other CD8 T cell epitopes

Next we wished to extend the observations of the BiVax immunization strategy to other MHC-I binding peptides known to function as strong CD8 T cell epitopes. For these experiments we selected the well-known H-2Kb-restricted epitope Ova\textsubscript{257-264} (SIINFEKL) and 2 epitopes derived from the melanosomal antigens tyrosine-related proteins 1 and 2 (Trp1 and Trp2), which function as
tumor-associated antigens for the mouse B16 melanoma. While Trp2_{180-188} (SVYDFFVWL) is restricted by H-2Kb [266], the heteroclitic epitope Trp1_{455-463/9M} (TAPDNLGYM) is restricted by H-2Db and has a substitution at position 9 (M for A) to improve MHC-I binding and immunogenicity [245, 267]. After a prime-boost BiVax with Trp2_{180-188}, mice generated approximately 25% tetramer positive CD8 T cells in blood (data not shown). In contrast, mice that received BiVax using Ova_{257-264} or Trp1_{455-463/9M} produced a much lower T cell response (< 5%; data not shown). Comparing the sequences of the 2 epitopes that performed relatively well in BiVax (HPV16-E7_{49-57} and Trp2_{180-188}) with the 2 peptides that did not functioned well (Ova_{257-264} and Trp1_{455-463/9M}) it became apparent that immunogenicity could not be clearly attributed to the overall hydrophobicity/hydrophilicity nature of the peptides, which could affect pharmacokinetics and antigen persistence after immunization. Ranking the peptides by the Kyte-Doolittle hydrophobicity scale [268] showed that the two most immunogenic peptides ranked 1st (Trp2_{180-188}) and 3rd (HPV16-E7_{49-57}). However, when examining the hydrophobic and hydrophilic regions within each peptide it became evident that the 2 immunogenic peptides had an amphiphilic feature, where approximately one half of the molecule was highly hydrophobic while the other half was relatively hydrophilic.
3.4.6 Enhancing immunogenicity of Trp1-BiVax by increasing peptide amphiphilicity

In view of the above, we examined whether the immunogenicity of Trp1\textsubscript{455-463/9M} using BiVax, could be improved by increasing the peptide’s amphiphilic nature, which was achieved by the addition of 2 palmitic acid chains (Pam2) to the amino terminus end using a KMFV linker (the positive charges of K are eliminated with Pam conjugation). The results in Figure 11a demonstrate that peptide Pam2KMFVTAPDNLGYM was substantially more immunogenic than the minimal epitope TAPDNLGYM after a BiVax prime-boost protocol. Interestingly, the minimal epitope was highly effective in boosting responses in mice that were generated by priming with Pam2KMFVTAPDNLGYM (Fig. 11b). The enhancement of immunogenicity was also achieved with the addition of 6-7 hydrophobic residues to the amino terminus end of the peptide (Fig. 11c). On the other hand, adding only 3 hydrophobic residues did not affect the immunogenicity of the peptide. Extending the natural sequence of the epitope at the amino end by 7 residues (TNTEMFV) or adding the palmitic acid chains directly to the minimal T cell epitope (without the hydrophobic MFV linker) somewhat increased the immunogenicity as compared to the minimal epitope but to a much lower extent as compared to the peptide constructs that contained 6-7 hydrophobic residues or the Pam2KMFV extension. Lastly, extending the Trp1 peptide using charged or hydrophilic residues did not substantially improve the immunogenicity of the peptides using BiVax (data not shown). The strategy to enhance the
peptide immunogenicity by generating an amphiphilic construct also worked well with Ova<sub>257-264</sub> (data not shown).

![Fig. 11](image)

**Fig. 11** Increasing peptide hydrophobicity enhances immunogenicity of BiVax. B6 mice were vaccinated *i.v.* on days 0 and 12 with BiVax composed of 150 µg of peptide and 50 µg of Poly-IC. (a) Mice (4 per group) received identical BiVax immunizations (prime/boost) with either minimal peptide (TAPDNLGYM) or palmitilated peptide (Pam2KMFVTAPDNLGYM). Immune responses were measured in blood 7 days after the boost. **Statistical significance (P<.001) between the 2 peptides was observed after the boost.** (b) Mice (3 per group) were primed with palmitilated peptide and boosted with either the palmitilated peptide or the minimal peptide. On day 7 and 19, antigen-specific CD8 T cells were evaluated in blood by tetramer analysis. *No statistical significant difference was observed between the 2 peptides after the boost.** (c) Mice (3 per group) received two identical BiVax immunizations (prime-boost) with indicated peptides as described above. 7 days after the boost the antigen-specific CD8 T cells in blood were evaluated by tetramer analysis. Results represent the average percentage of tetramer positive CD8 T cells from 3 mice per group with SD (bars) of the means. (d) Purified spleen CD8 T cells from the experiment presented in panel (A) from the mice vaccinated with palmitilated peptide were evaluated for their ability to recognize tumor cells using an IFNγ secretion EliSpot assay. APCs used: Trp<sub>1455-463</sub>-pulsed EL4 (EL4 + pep), B16F10 melanoma, and Un-pulsed EL4 cells (negative control). Results represent the average number of spots from triplicate wells with SD (bars) of the means.
3.4.7 Therapeutic effects of BiVax-Trp1 against established melanomas

A significant concern raised with the use of synthetic peptides as immunogens is that these vaccines will mainly generate low avidity T cells incapable of recognizing tumor cells that may express low density of peptide/MHC complexes on their surface. Nevertheless, as shown in Figure 11D, the CD8 T cells induced by BiVax with Pam2KMFVTAPDNLGYM (from hereafter simply referred to as “Pam2-Trp1”) were highly efficient in vitro in recognizing B16 melanoma cells. The therapeutic effect of BiVax immunization was evaluated against 5-day established subcutaneous B16-F10 melanoma tumors using Pam2-Trp1. Tumors grew at a significantly lower rate in mice that received 2 sequential BiVax immunizations as compared to the untreated group (Fig. 12a). Moreover, the inclusion of 2 additional booster immunizations further increased the therapeutic effect of BiVax with Pam2-Trp1 (Fig. 12b) and the addition of PD1 blockade (with αPD-L1 antibodies) to resulted in a remarkable therapeutic effect, where the majority of the mice (4/5) were able to completely reject their tumors (Fig. 12c). Interestingly, the control groups that received an irrelevant peptide (Pam2-Ctrl) and Poly-IC also showed a decrease in the rate of tumor growth but the therapeutic benefit was not as effective as compared to the use of the Pam2-Trp1 peptide. The therapeutic effects of Pam2-Trp1 BiVax and the control Pam-Ctrl BiVax disappeared when mice were depleted of CD8 T cells (data not presented).
Fig. 12 Therapeutic effects of BiVax against established B16 melanoma. B6 mice (4 per group) were inoculated s.c. on day 0 with $3 \times 10^5$ live B16-F10 cells and vaccinated i.v. with Pam-Trp1 or control Pam2-Ctrl BiVax. Tumor-bearing mice received 2 (a), or 4 (b) immunizations (arrows). (c) PD1 blockade was included in an experiment using 4 BiVax immunizations. Anti-PD-L1 mAb (200 µg/dose) was administered i.p. on days 1 and 3 post-immunization, and two more antibody injections (on days 5 and 7) were added after the 4th BiVax immunization. Non-vaccinated mice (No Vax) and palmitilated irrelevant peptide-BiVax (Pam2-Ctrl) were included as controls. Tumor sizes were determined in individual mice by measuring 2 opposing diameters and are presented as tumor areas in square millimeters. Points, mean for each group of mice; gray bars, period of anti-PD-L1 mAb treatment; bars, SD. $P$ values were calculated using 2-way ANOVA tests. *, 4 of 5 mice in this group rejected their tumors.
3.4.8 Mechanisms involved in the immunogenicity of BiVax

Next, we examined some of the mechanisms that could play a role in the generation of the strong CD8 T cell responses observed with BiVax. First we assessed whether other TLR agonists also being considered as immune adjuvants performed with Pam2-Trp1. The results in Figure 13a demonstrate that only Poly-IC was capable of generating strong CD8 T cell responses with Pam2-Trp1. The use of poly-AU (another TLR3 agonist) did not lead to the generation of the large CD8 T cell response observed with Poly-IC. Furthermore, CpG containing oligodeoxynucleotides of either type-A (CpG-1885) or type-B (CpG-1826), which function as TLR9 agonists or the TLR2/TLR1 agonist Pam3CSK4 failed to generate substantial T cell responses when administered mixed with Pam2-Trp1. Interestingly, combining CpG-1826 with Poly-IC reduced the magnitude of the CD8 T cell response as compared to the use of Poly-IC alone. On the other hand, the low T cell response generated by the administration of Pam2-Trp1 peptide with CpG-1826 could be dramatically increased with a BiVax booster containing Poly-IC (Fig. 13b). The results so far presented were obtained with Poly-IC formulation, known as poly-ICLC containing poly-lysine and carboxymethyl cellulose to stabilize the compound and protect it from RNAse degradation, which occurs mostly in primates [269]. Nevertheless, identical results were obtained using several commercially available non-stabilized Poly-IC formulations (data not presented).
**Fig. 13** Effect of various TLR agonists on BiVax immunization. (a) Mice (3 per group) were immunized *i.v.* on days 0 and 12 with Pam2-Trp1 peptide and one of the following TLR agonists: 50 µg Poly-IC, 100 µg CpG-1826, 100 µg CpG-1585, 50 µg poly-AU, 100 µg Pam3CSK4, and a mixture of Poly-IC + CpG-1826. On day 7 (post prime) and 19 (post boost), blood samples were evaluated by tetramer analysis. (b) Mice (3 per group) were immunized *i.v.* on days 0 and 12 with BiVax/CpG-1826, and boosted one more time with BiVax/poly-IC on day 24. The presence of antigen-specific CD8 T cells in blood was evaluated by tetramer analysis on days 7 (post prime), 19 (post boost), and 31 (post 2nd boost). Points, the value for each individual mouse; horizontal line, the average value of the group; bars, SD.

CD4 T cells may be important in the generation and long-term maintenance of CD8 T cell responses by functioning as helper T cells. On the other hand, another CD4 T cell subset known as T regulatory cells are known to inhibit CD8 T cell responses, in particular those directed against self-antigens such as Trp1. Thus, we assessed whether depletion of CD4 T cells prior to each immunization would impact the magnitude and duration of the CD8 T cell response generated by BiVax with the Pam2-Trp1 peptide. As shown in Figure 14a, the level of the CD8 T cell response after the BiVax prime (day 6-post...
vaccine priming) was almost 2-fold higher when CD4 T cells were depleted as compared to the untreated mice. The magnitude of the immune response increased after the booster immunization regardless of whether CD4 T cells had been depleted or not. Also, the duration of the Trp1 CD8 T cell response to BiVax was not affected by the absence of CD4 T cells during the immunizations.

Next, using several genetically deficient mouse strains, we evaluated the role that various immune-related receptors could play in the generation of CD8 T cell responses by BiVax. The absence of CD40 and TLR3 resulted in a marked reduction in the levels of antigen-specific CD8 T cells observed after the vaccine prime (Fig. 14b). On the other hand, in the absence of IFNαβR, MDA5, and scavenger receptors SR-A and MARCO, the primary immune responses to BiVax were comparable as those observed in wild type (WT) mice. Strong secondary immune responses, that more than doubled the levels of antigen-specific CD8 T cells as compared to the prime, were observed in all instances. However the absence of TLR3 resulted in severe toxicity where 3/4 vaccinated mice died by what appeared to be cytokine storm syndrome. In addition the fold-increase of the secondary response was lower in mice deficient of the MDA5 RNA helicase, as compared to the WT and other genetically deficient mice. Although the CD40 and the TLR3 deficient mice exhibited lower primary CD8 T cell responses, the booster immunization generated strong secondary responses when considered as the fold-increase, compared to the primary response for each mouse strain. Since the adjuvant effect of Poly-IC is considered to depend in great part by its ability to induce high levels of type-I IFN, it was somewhat puzzling that the CD8
T cell responses observed in IFNαβR deficient mice were similar, if not identical to those observed in WT mice (Fig. 14b). However, very different results were observed when the minimal Trp1 peptide was used to boost the T cell response induced by priming with Pam2-Trp1. While as previously noted, the minimal Trp1 peptide was efficient in boosting the responses in WT mice (Fig. 11b), this was not the case with the IFNαβR deficient mice where only the Pam2-Trp1 was capable of boosting the responses (Fig. 14c). These results indicate that type-I IFN may be critical only when antigen is presented by non-professional APCs, which occurs when the minimal peptide is used to expand the primed CD8 T cells.
Fig. 14 Immunological mechanisms involved in BiVax CD8 T cell responses. (a) Effect of CD4 cells in immune responses to BiVax. Untreated or CD4 T cell depleted mice (3 per group) were immunized i.v. on days 0 and 14 with Pam2-Trp1-Bivax (arrows), and the presence of antigen-specific CD8 T cells in blood were measured at various time points using tetramer analysis. CD4 depletion was initiated 3 and 1 d before the BiVax prime. CD4 T-cell depletion was confirmed by flow cytometry. (b) Role of immune receptors in the immunogenicity of BiVax. WT B6 mice (WT), IFNαβR−/−, CD40−/−, TLR3−/−, MDA5−/−, and scavenger receptor SR-A and MARCO double knockout (SR-A−/−MARCO−/−) mice were evaluated for their immune responses to Pam2-Trp1 7 d after prime and boost. *, 3/4 TLR3−/− mice died after the boost. (c) IFNαβR−/− mice were primed with Pam2-Trp1 and boosted with either the Pam2-Trp1 or the minimal peptide. On day 7 (post prime) and 19 (post boost), antigen-specific CD8 T cells were evaluated in blood by tetramer analysis. **Statistical significance (P<.01) between the 2 peptide responses was observed after the boost.

3.4.9 Effects of the mode of administration in the immunogenicity of BiVax

So far, the results presented above were obtained using i.v. Immunizations, which could be considered somewhat unconventional. Thus, we
compared BiVax immunogenicity using three different vaccination routes. While both the *i.v.* and *i.m.* routes were highly effective for the generation of strong responses, administration of BiVax via the *s.c.* route was clearly less effective (Fig. 15a). Lastly, we assessed whether the 2 components of BiVax, peptide and Poly-IC needed to be administered simultaneously, or whether separate injections given at different times would provide similar results. Injecting Pam2-Trp1 5 h before Poly-IC had no deleterious effect as compared to the simultaneous administration of both BiVax components (Fig. 15b). On the other hand, the level of the immune response was dramatically reduced when Poly-IC was injected 5 h prior to the administration of the Pam2-Trp1 antigen. A somewhat different pattern of responses was observed with BiVax using the HPV16-E7_{49-57} peptide. As with the previous result, the simultaneous administration of peptide and Poly-IC resulted in generating the strongest immune response and administration of the Poly-IC before peptide reduced to a great extent this response (Fig. 15c). However, in this case HPV16-E7_{49-57} injection before Poly-IC reduced the response by ~50%.
Fig. 15 Effects of the mode of administration in BiVax vaccines. (a) Mice (3 per group) were immunized through different routes and the presence of antigen-specific CD8 T cells in blood was analyzed by tetramer analyses 7 d after the prime and the boost. **Statistical significance (P<.01) between i.v. and s.c. induced responses was observed after the boost. *No statistical significant difference was observed between i.v. and i.m. after the boost. (b) Mice (3 per group) were pretreated i.v. with either PBS (control), Pam2-Trp1 or Poly-IC, and 5 h later received i.v. BiVax (Poly-IC and Pam2-Trp1) Poly-IC or Pam2-Trp1 (as indicated for each group). CD8 T cell responses were measured by tetramer analysis on days 7 (post prime) and 19 (post boost). (c) Mice were injected i.v. with PBS, E7_{49-57}, or Poly-IC and 5 h later received a second i.v. injection with BiVax (E7_{49-57} + Poly-IC), Poly-IC or the E7_{49-57} (as indicated for each group) and were boosted the same way 16 days later. The percentage of tetramer positive CD8 T cells was determined in blood 6 days after prime and boost (n=3 mice per group).

3.5 Discussion

There is little doubt that the effectiveness of a vaccine will depend in great part on its ability to elicit high levels of long-lasting antigen-specific, pathogen-reactive CD8 T cells. Our group has proposed that these levels and the duration of the response should resemble those observed during acute infections [263]. The failure of many vaccination strategies can be attributed in many cases to the inability of the vaccines to generate potent and persistent immune responses. Other instances of vaccine failures, when strong immune responses have been observed, could be related to the inability of the T cells to recognize the
pathogen-infected or tumor cells due to low avidity of the T cells for their antigen. Particularly, in the case of TAAs that are also expressed by normal cells such as Trp1, it is likely that the highest avidity T cells are eliminated via immunological tolerance and that vaccines would recruit T cells of insufficient avidity to recognize tumor cells. Additional concerns have been brought up on the use of vaccines that utilize synthetic peptides corresponding to the minimal CD8 T cell epitopes, including the induction of low avidity T cells due to the generation of supraoptimal levels of peptide/MHC-I complexes on APCs. In addition, some vaccines prepared with short peptide epitopes have been reported to induce T cell deletion [270], presumably because these peptides can be presented by non-professional APCs. In view of this, it has been advocated that peptide vaccines should be prepared using long peptides that would require capture and antigen processing by professional APCs [239]. Indeed, when long peptides were administered s.c. using suboptimal adjuvants such as IFA they were found to be more immunogenic than their short peptide counterparts [271].

We present here a novel vaccination strategy called BiVax that was designed to mimic a viral infection, inducing high levels of CD8 T cells with sufficient avidity to recognize foreign and self-TAAs and capable of persisting for a long time period. Our results show that a synthetic peptide corresponding to a minimal CD8 T cell epitope, HPV16-E7_{49-57}, mixed with Poly-IC and administered systemically (i.v.) in an aqueous formulation generated after a short prime-boost protocol (5-7 days apart), huge numbers of antigen-specific, tumor-reactive T cells capable of rejecting established tumors (Fig. 8). In contrast to results from
others obtained with vaccines administered s.c. in oil:water emulsions, the long peptides bearing the HPV16-E7<sub>49-57</sub> epitope were markedly less immunogenic than the minimal epitope, when administered using the BiVax strategy (Fig. 10). It remains unknown the degree of participation of professional APCs in the response to BiVax with the minimal HPV16-E7<sub>49-57</sub> epitope and whether non-professional APCs could be presenting antigen to the CD8 T cells to facilitate clonal expansion. Also, we do not know the reason(s) why the elongated HPV peptides were not as effective as the short peptide in generating the vast CD8 T cell responses observed with BiVax. One could assume that the large CD8 T cell responses observed in BiVax with HPV16-E7<sub>49-57</sub> (and hydrophobic Trp1 peptides) require the participation of both professional APCs such as DCs (for the initial priming), and non-professional APCs (for facilitating clonal expansion of the activated T cells). Thus, the long HPV peptides would be inefficient because they rely solely in professional APCs, which exist at much lower frequencies than non-professional APCs. Our results agree with the above-stated assumption since a long HPV16-E7 peptide was found to be effective in priming but not boosting the CD8 T cell response, while the short HPV16-E7 peptide was effective in boosting responses induced by priming with either the short or the long peptide (Fig. 10). Also, the minimal Trp1 peptide was found to be inefficient in priming CD8 T cells (Fig. 11a) but was highly effective in expanding the T cells generated by the Pam2-Trp1 peptide (Fig. 11b). Nevertheless, the ability of the Pam2-Trp1 and elongated hydrophobic Trp1 peptide constructs not only to effectively prime the CD8 T cells, but also to successfully expand these
responses needs to be addressed since these peptides presumably would require to be presented by non-professional APCs. The possibility exists that lipidated and hydrophobic elongated peptides may be able to be captured and undergo processing by both professional and non-professional APCs, while other long, not-so-hydrophobic peptides such as HPV16-E745-57 and HPV16-E743-77 may not be so effective in being captured by non-professional APCs. The possibility also exists that short peptides such as HPV16-E749-57 and elongated hydrophobic Trp1 peptides may form complexes with Poly-IC, which are more effectively internalized by APCs via some type of nucleic acid-binding scavenger receptor (SR). Once inside of the cell, the peptide/ Poly-IC complexes within endosomal compartments will stimulate TLR3 and may spill the contents to the cytoplasm due to the proton sponge effect generated by the endosomal nucleic acid content. Although all these possibilities remain to be studied, the necessity for the formation of peptide/ Poly-IC complexes is somewhat supported by the observations that administration of Poly-IC prior to the peptide did not lead to the generation of the strong immune responses (Fig 15b-c). One would expect that Poly-IC would quickly be cleared from circulation by binding to the numerous SRs whose function is to rapidly eliminate foreign and self-free nucleic acids. The injection of the peptide before Poly-IC could work if the persistence of the peptide in circulation allows the in vivo formation of complexes with Poly-IC. Members of the SR class A (SR-A) family are known to bind nucleic acids. Our results indicate that BiVax generated levels of CD8 T cells in mice deficient in SR-Al/II/III and MARCO that were equivalent to those observed in WT mice. Thus, it is likely
that other class of SRs may be capable of interacting with Poly-IC. The redundancy in the specificity of SRs makes it difficult to pinpoint exactly those receptors in professional and non-professional APCs that would be involved in the capture of peptide/ Poly-IC complexes.

With respect to the differences observed with the various routes of administration, we initially reasoned that a systemic administration (i.v.) of the vaccine would be able to deliver antigen throughout most lymphoid organs recruiting larger numbers of antigen-specific T cells as compared to a local (s.c.) immunization, where only draining lymph nodes would be involved. Indeed, BiVax given i.v. was far superior as compared to BiVax s.c. (Fig. 15a). Interestingly, BiVax i.m. was as effective as BiVax i.v.. Thus, it is possible that the peptide, Poly-IC mixture (or complexes) can effectively diffuse from the muscle into the general circulation to reach proximal and distal lymphoid organs. It has been advocated that most vaccines should be administered i.m. and not s.c. because of higher vascularity of the muscle as compared to the subcutaneous fat layers, which will influence antigen diffusion into the bloodstream [272].

Poly-IC has been considered to function as a potent immune adjuvant due to its ability to stimulate endosomal TLR3 and cytoplasmic retinoic acid-inducible gene (RIG)-1-like receptor (RLR) family member, MDA5. As a result of TLR3 and MDA5 activation, cells produce high amounts of IFN-I and other proinflammatory cytokines such as IL-1, TNFα, IL-6 and IL-12. Our results showed that the levels
of immune responses induced by BiVax were lower in mice deficient for either TLR3 or MDA5 as compared to WT mice (Fig. 14b). The levels of CD8 T cells induced after priming were substantially lower in TLR3-KO but not in MDA5-KO as compared to the WT mice. On the other hand, the fold-increase in the level of the immune response observed in MDA5-KO was decreased as compared to what was observed in WT mice. There was some indication that the secondary response induced by booster immunization in TLR3-KO mice was effective, as evident by the large difference between primary and secondary responses, but this could only be verified in 1/4 mice because of severe toxicity of the vaccine in these mice. Mice deficient in CD40 responded poorly to the primary immunization but exhibited a large secondary response after the booster. Putting together all of these results, one could speculate that during the primary immune response in which professional APCs may play a more critical role, that TLR3 activation and CD40 costimulation play an important role in the activation of the naïve T cells. On the other hand, during the secondary response, the stimulation of the previously activated T cells can be effectively carried out by non-professional APCs that are activated by Poly-IC mostly through MDA5 and that do not require the participation of TLR3 and CD40.

The elimination of CD4 T cells resulted in an increased primary CD8 T cell response as compared to the normal mice controls. These results suggest the possible elimination of a CD4 T regulatory cell population that somewhat decreases the effectiveness of priming with BiVax. More importantly, the results demonstrating that CD40 was required for effective priming, suggest that other
cells besides CD4 T cells that express CD40L (CD154) such as platelets, granulocytes, subsets of CD8 or gamma-delta T cells may function as helpers during the BiVax primary response. As mentioned above, Poly-IC is known to induce the production of high levels of IFN-I, which has been demonstrated to function as “signal 3” for the activation and expansion of CD8 T cells [273]. In view of this, we assumed that the adjuvant effect of Poly-IC in BiVax could be due to the signal 3 effects of IFN-I on the peptide-stimulated CD8 T cells. Thus, we were surprised that BiVax using Pam2-Trp1 functioned well in IFNαβR deficient mice (Fig. 14b), indicating that IFN-I signals are not critical for the activation and expansion of CD8 T cells in this vaccination strategy. Interestingly, in contrast to what was observed in WT mice, that the minimal Trp1 peptide was effective in boosting the CD8 T cell responses of Pam2-Trp1 primed mice (Fig. 11b), in IFNαβR deficient mice only the Pam2-Trp1 was able to boost while the minimal Trp1 peptide did not (Fig. 14c). These findings suggest that IFN-I may play a critical role when non-professional APCs present antigen during the expansion phase to the previously activated CD8 T cells. Thus, IFN-I may serve as an endogenous danger signal that would allow proliferation of T cells to antigens presented by infected cells that are not professional APCs. On the other hand, T cells activated by professional APCs reactive to self-antigens, when presented by antigen by non-professional APCs in the absence of IFN-I would not continue to proliferate, limiting an autoimmune response. These results indicate that Poly-IC stimulation of professional APCs results in a TLR3/CD40-dependent and IFN-I/MDA5-independent primary activation of CD8 T cells where
alternative cytokines such as IL2, IL12 and IL15 may serve as signal 3 leading to activation and an initial somewhat restrained clonal T cell expansion. However, soon after (5-7 days) the initial activation of the CD8 T cells by professional APCs when non-professional APCs present antigen to the T cells in circumstances where IFN-I is being produced via MDA5 stimulation by Poly-IC (or viral dsRNA), a massive T cell expansion will be generated. We believe that many of vaccines designed to elicit CD8 T cell responses such as peptide (long or short) vaccines formulated in IFA, peptide-pulsed DCs and plasmid DNA immunization are probably effective in accomplishing the primary T cell activation and initial limited T cell expansion but fail to induce the secondary massive T cell expansion, observed in natural immune responses during acute infections, which may be necessary to obtain effective anti-tumor effects. In contrast, BiVax using amphiphilic peptides and Poly-IC is highly effective in both the priming (activation/initial expansion) and massive expansion after the secondary immunization.
Chapter 4: General Conclusions

The results of my doctoral work show that immunization with a synthetic peptide representing an exact CD8 T-cell epitope from the HPV 16 E7 protein in combination with Poly-IC (BiVax) and αCD40 mAb (TriVax) resulted in extensive antigen-specific T-cell responses that were durable and capable of eradicating established tumors. In addition, TriVax generates immunological memory that prevents tumor recurrences and although the optimal route of administration is the i.v. route, the i.m. route was almost as effective (it generated less number of tetramer cells and recurrence 1/6 of mice). TriVax mechanism of action is CD8 T cell dependent and IFNγ and type I IFN absence had little effect in early stages of tumor growth control. Interestingly, while one single TriVax vaccination induces tumor clearance in 100% mice, 2 vaccinations with BiVax induce clearance in just 50% of mice. These differential effects are due to the quantity of antigen specific CD8 T cells generated after vaccination and additional BiVax boosters improved vaccine immunogenicity and correlated with enhance anti tumor effect generating clearance of tumor in all vaccinated mice.

In the search for improved immunogenicity we tested several HPV peptides including modification of the E7\textsubscript{49-57} (minimal peptide) and extensions of the minimal peptide (HPV16-E7\textsubscript{45-57} and HPV16-E7\textsubscript{43-77}); none of the peptide
assayed surpasses the minimal peptide immunogenicity in BiVax’s formulation. The results showed that BiVax can be used in the context of other TAAs (Trp2), furthermore the immunogenicity of peptides in the BiVax formulation can be enhanced by generating an amphiphilic construct, such improvement in immunogenicity correlated with enhanced antitumor effect. BiVax anti tumor effect was CD8 dependent and CD4 depletion affected priming with amphiphilic Pam2-Trp1 but not the response after boost and duration of the immune response. In addition, the absence of CD40 and TLR3 resulted in a marked reduction in the levels of antigen-specific CD8 T cells observed after the prime with BiVax. And while both the i.v. and i.m. routes were highly effective for the generation of strong responses, administration of BiVax via the s.c. route was clearly less effective. Finally, BiVax’s components have to be administrated simultaneously to obtain optimal immune response, interestingly administration of Poly IC 5 hours before the administration of the peptide (but not the other way around) reduces immunogenicity considerably.

The results of the work shown here indicate that the TriVax and BiVax strategies are appealing immunotherapeutic approaches 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.
Chapter 5: List of References


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Chapter 6: Appendix (Permission)

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