HPV and Therapeutic Vaccines: Where are We in 2010?

Barbara Ma[1], Yijie Xu[1], Chien-Fu Hung[1,4] and T.-C. Wu[1,2,3,4,*]

Departments of [1]Pathology, [2]Obstetrics and Gynecology, [3]Molecular Microbiology and Immunology, [4]Oncology, The Johns Hopkins Medical Institutions, Baltimore, MD, USA

Abstract: The discovery of human papillomavirus (HPV) as a necessary etiological factor for cervical cancer has spurred the development of preventive and therapeutic HPV vaccines for the control of HPV-associated malignancies including cervical, vulvar, vaginal, and a subset of head and neck cancers. The commercial preventive HPV vaccines, Gardasil and Cervarix, use HPV virus-like particles to generate neutralizing antibodies against HPV major capsid protein L1. However, they do not exert therapeutic effects on existing lesions and are unlikely to have an immediate impact on the prevalence of cervical cancer due to their cost and limited availability in developing countries, which account for more than 80% of cervical cancers. Thus, there is an urgent need for therapeutic HPV vaccines. Therapeutic HPV vaccines can eliminate pre-existing lesions and infections by generating cellular immunity against HPV-infected cells. HPV E6 and E7 oncoproteins represent ideal targets for therapeutic intervention because of their constitutive expression in HPV-associated tumors and their crucial role in the induction and maintenance of HPV-associated disease. This review discusses the current progress of various therapeutic HPV vaccine approaches, including live vector-based, peptide/protein-based, nucleic acid-based and cell-based vaccines targeting E6 and/or E7 antigens, and their future prospects for the control of HPV-associated malignancies.

Key Words: Therapeutic vaccine, HPV, cervical cancer, clinical trials.

I. INTRODUCTION

A. HPV and Cervical Cancer

It is now firmly established that human papillomavirus (HPV) is the etiologic agent of cervical cancer and other HPV-associated malignancies, including anogenital cancers and a subset of head and neck cancers [1]. Cervical cancer represents the second most frequent gynecological malignancy in the world, with an estimated 493,000 new cases and approximately 274,000 deaths annually [2]. Over 100 types of HPV have been identified and can be classified by their oncogenic potential. High-risk types include 16, 18, 45, 31 while low-risk types include types 6, 11 [3]. While low-risk subtypes can cause low-grade benign lesions, high-risk subtypes are connected with the development of high-grade lesions and malignant tumors. The presence of premalignant squamous intraepithelial lesions (SIL), also known as cervical intraepithelial lesions (CIN), followed by persistent infection with high-risk type HPV, is a necessary trigger of cervical cancer [1]. As HPV-16 and HPV-18 are the types most commonly associated with cervical cancer, accounting for up to 75% of all cervical cancers, they have been the focal point of preventive and therapeutic HPV vaccine development.

B. Molecular Characterization of HPV

It is essential to understand the molecular biology of human papillomavirus in order to develop HPV vaccines (for review, see [4]). HPV is a non-enveloped, double-stranded, circular DNA virus with an icosahedral capsid. Its genome is approximately 8000 base pairs long and is composed of early proteins (E1, E2, E4, E5, E6, E7) and late proteins (L1, L2). The early proteins are regulators of the viral life cycle, as they control viral DNA replication (E1 and E2), viral RNA transcription (E2), cytoskeletal reorganization (E4) and cellular transformation (E5, E6 and E7). The late proteins are structural proteins that comprise the viral capsid. In some persistent infections with high-risk HPV, HPV DNA may integrate into the host genome, resulting in the deletion of non-essential, regulatory viral genes, such as E2, E4, E5, L1 and L2. As E2 is the transcriptional repressor protein of E6 and E7, the loss of E2 leaves E6 and E7 as the principal proteins expressed within the infected cell. The E6 and E7 proteins inactivate tumor suppressors p53 and retinoblastoma (Rb) respectively and render the breakdown of cell cycle regulation. Hence, high-risk HPV-infected cells develop genomic instability, which can lead to the progression of cancer (for a review, see [5]).

C. Preventive HPV Vaccines

To block viral entry, preventive HPV vaccines aim to generate protective humoral immune responses by stimulating the production of neutralizing antibodies against L1 and/or L2 HPV viral capsid proteins. It has been shown that cells transfected with the gene encoding L1 protein are capable of assembling into HPV virus-like particles (VLPs) that are morphologically similar to native HPV virions [6, 7]. The use of VLPs to generate protective immunity against HPV infection has been demonstrated and capitalized in the development of preventive HPV vaccines [8, 9]. This progress has led to two commercially available preventive HPV

*Address correspondence to this author at the Department of Pathology, The Johns Hopkins University School of Medicine, CRBII Room 309, 1550 Orleans Street, Baltimore, Maryland 21231, USA; Tel: (410) 614-3899; Fax: (443) 287-4295; E-mail: wutc@jhmi.edu

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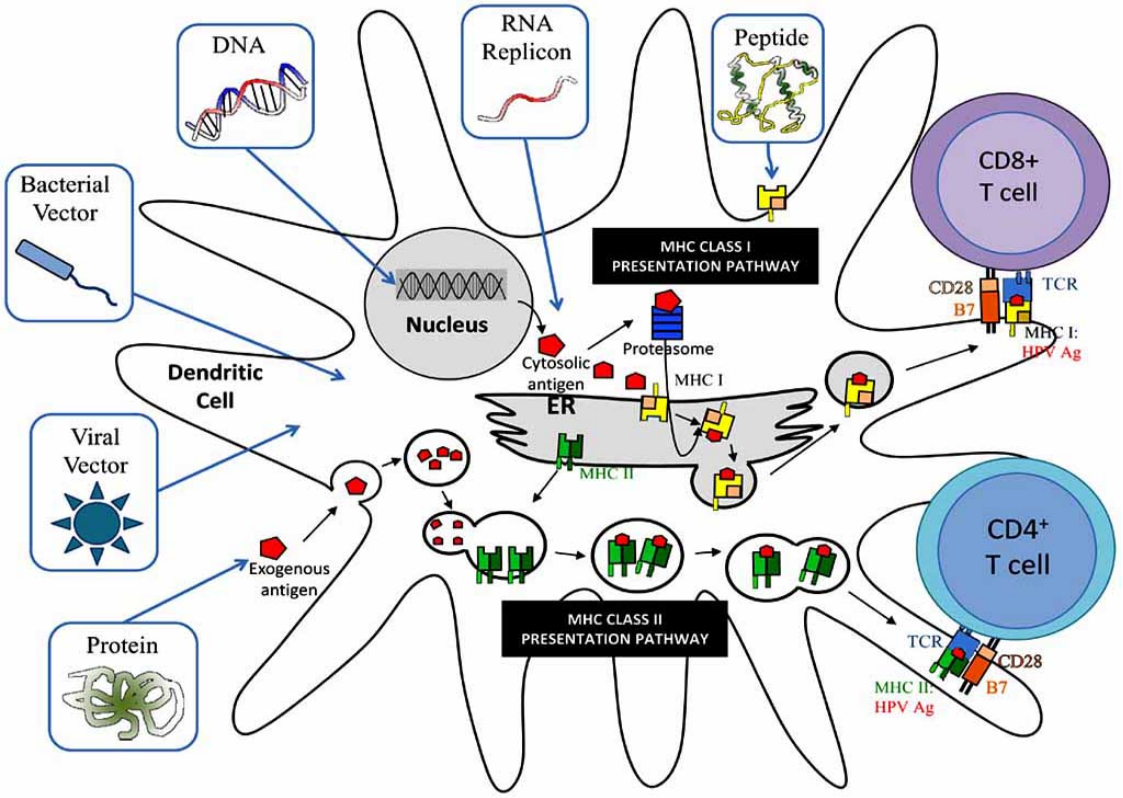

Fig. (1). Therapeutic HPV Vaccine Approaches. Various therapeutic HPV vaccines focus on interacting with professional antigen-presenting cells such as dendritic cells (DCs) to generate cell-mediated immunity for clearance of infection. The form of vaccination can influence antigen processing and presentation and the subsequent cellular immunity generated. In major histocompatibility complex (MHC) class I pathway, cytosolic antigen is broken up into smaller peptides via the proteasome, loaded onto MHC class I molecules in the endoplasmic reticulum (ER) and sent to the cell surface for presentation to CD8+ T cells. In the MHC class II presentation pathway, exogenous antigen is endocytosed into vesicles, loaded onto MHC class II molecules inside endosomal/lysosomal compartments and sent to the cell surface for presentation to CD4+ T cells. CD28 and B7 are co-stimulatory molecules required for T cell activation. DNA vaccines deliver DNA directly into the nucleus of DCs, where DNA is expressed and the cytosolic protein product is presented via MHC class I pathway to activate CD8+ T cells. RNA replicon-based, bacterial vector-based, viral vector-based deliver antigen of interest into the cytosol for presentation via MHC class I pathway to CD8+ T cells. Peptide-based vaccines can load antigenic peptides onto MHC class I molecules for presentation to CD8+ T cells. Protein-based vaccines can deliver proteins exogenously and usually activate CD4+ T helper cells.

vaccines - Gardasil and Cervarix. Merck's Gardasil is a quadrivalent L1 VLP recombinant vaccine that protects against four of the most medically relevant HPV genotypes: HPV-6 and HPV-11 for benign genital warts, and HPV-16 and HPV-18 for cervical cancer. GlaxoSmithKline's Cervarix is a bivalent L1 VLP recombinant vaccine derived from HPV types 16 and 18. They have been shown to be well tolerated, highly immunogenic, and able to induce the production of neutralizing antibodies and effectively prevent HPV-associated infection [10-12]. These vaccines, particularly Cervarix, have also exhibited partial cross-protection with other HPV types not included in the vaccine (HPV-31 and HPV-45) and hence the commercial preventive vaccines protect up to approximately 80% of cervical cancers [13].

D. Need for Therapeutic HPV Vaccines

While the successful commercialization of preventive vaccines is a significant milestone for the control of cervical cancer and possibly other HPV-associated malignancies, there is an urgent need for therapeutic HPV vaccines. First, there is a considerable population suffering from HPV infections worldwide. Since some of the HPV-associated tumor cells in which viral integration has occurred do not express detectable levels of capsid antigen (L1 and/or L2), preventive HPV vaccines are unlikely to be effective in the elimination of these HPV-associated lesions. Second, the high cost and need for appropriate storage of currently available preventive HPV vaccines may restrict their use in developing countries, which account for more than 80% of all cases of cervical cancer and have limited medical resources. Third, it is estimated that it would take approximately 20 years from the implementation of mass vaccination for preventive vaccines to impact the cervical cancer rates due to the prevalence of significant population with existing HPV infections and slow process of carcinogenesis. Thus, in order to facilitate the reduction on the mortality and morbidity of HPV-associated malignancies and its precursor lesions, it is important to continue the development of therapeutic vaccines against HPV.

II. THERAPEUTIC HPV VACCINES

In order to eliminate existing lesions, a therapeutic HPV vaccine should target HPV antigens that are continuously expressed in the infected cells and cancer cells. HPV-

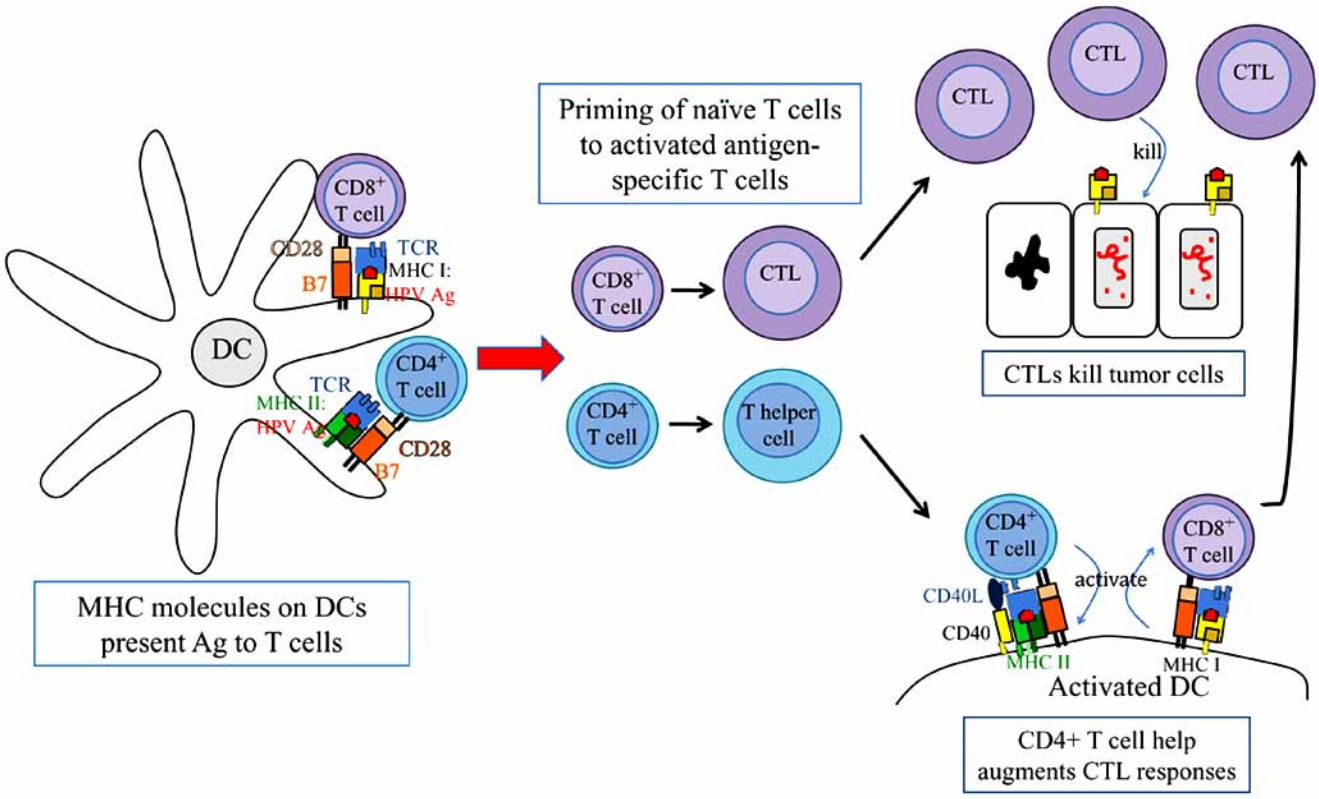

Fig. (2). Generation of Cellular Immunity. Upon HPV antigen (Ag) uptake, dendritic cells (DCs) undergo a maturation process and present the antigen on MHC class I and/or class II molecules on the cell surface. Dendritic cells prime naïve T cells to become antigen-specific effector T cells. This priming involves two signals: 1) peptide/MHC complex with T cell receptor and 2) co-stimulatory molecules B7 and CD28. Effector CD8+ T cells, also known as cytotoxic T lymphocytes (CTL), mediate antigen-specific killing of tumor cells, and effector CD4+ T cells differentiate into T helper cells to facilitate CD4+ T cell help, augmenting CTL immune responses. In CD4+ T cell help, CD40 ligand on the CD4+ T cells binds to CD40 on the dendritic cell, which in turn activates the naïve CD8+ T cell.

encoded proteins E6 and E7 represent ideal targets for therapeutic intervention because of several properties. Whereas L1 and L2 are expressed only in terminally differentiated keratinocytes, HPV E6 and E7 are constitutively expressed in all levels of the epithelium of HPV-infected cells. Furthermore, because E6 and E7 are critical for the induction and maintenance of cellular transformation in HPV-infected cells, it is unlikely that the tumor cells can escape immune attack through antigen loss. In addition, since E6 and E7 are foreign proteins, immunization against HPV-associated tumors circumvents some common cancer vaccine-associated issues such as immune tolerance.

Consequently, many therapeutic vaccine strategies have focused primarily on stimulating the production and activation of T cells that can recognize infected cells expressing the target antigens E6 and E7. By delivering antigens to professional antigen-presenting cells, particularly dendritic cells (DCs), therapeutic vaccines can generate antigen-specific CD8+ T cells and CD4+ T cells (see Fig. (**1**)). Type 1-helper CD4+ T cells particularly are able to efficiently stimulate and augment the immune response of cytotoxic CD8+ T cells (see Fig. (**2**)). Together, these two arms of the adaptive immune system have the specificity and potency to kill HPV-infected cells or HPV-associated tumor cells at multiple sites in the body without inflicting significant damage on normal tissues. Different therapeutic strategies have been developed including live vector-based vaccines, peptide- or protein-based vaccines, nucleic acid-based vaccines, cell-based vaccines and combinational approaches. Table **1** sums up the advantages and disadvantages of the each approach. Table **2** summarizes the various therapeutic HPV vaccine clinical trials. This review discusses the current status of therapeutic HPV vaccines for the control of HPV-associated malignancies, with emphasis on clinical trials.

A. Live Vector-Based Vaccines

Live vector-based vaccines are an attractive approach for therapeutic HPV vaccination. They are highly immunogenic as they replicate within host cells and can enable antigenic spread from cell to cell. Additionally, there are a wide variety of vectors available that can be tailored or engineered for a desired effect. This has been well utilized in the context of therapeutic HPV vaccines, in which live vectors are modified to express HPV E6 and/or E7 antigens. However, while live viral vectors are capable of inducing antigen-specific cytotoxic T cell immune responses, they raise significant concerns related to safety. They also have limited capacity for repeated administration due to the induction of neutralizing antibodies as well as the possibility of pre-existing vector-specific immunity. Live vector-based vaccines can be categorized into bacterial vectors and viral vectors.

1. Bacterial Vectors

Bacterial vectors can deliver desired genes or proteins to professional antigen-presenting cells such as DCs (Fig. (**1**)). Examples of bacterial vectors used in therapeutic HPV vaccines include *Lactococcus lactis* [14], *Lactobacillus plantarum* [15], *Salmonella enterica* [16], and *Listeria monocytogenes* (*Lm*) [17-19].

The vector that has generated most interest is *Listeria monocytogenes*. *Lm* is a gram-positive bacterium capable of escaping phagosomal lysis through listeriolysin O (LLO) secretion and replication within the cytosol of an antigen-presenting cell. As a result, antigens secreted by *Lm* can be presented by both major histocompatibility complex (MHC) class I and II pathways to stimulate antigen-specific $CD8^+$ and $CD4^+$ T-cell immune responses. Furthermore, the sensitivity of *Lm* to antibiotics means the vector can be easily killed if the patient shows severe adverse effects. Preclinical trials of *Lm* encoding a fusion protein of HPV-16 E7 and either ActA (Lm-ActA-E7) or a fragment of LLO (Lm-LLO-E7) both induced E7-specific $CD8^+$ T-cell responses [18, 19]. The qualitatively different T-cell immunity induced by both vaccines reflects their abilities to induce established tumor regression [20]. The success of *Lm* in preclinical trials has translated to clinical studies. Recently, the first clinical use of a therapeutic HPV vaccine candidate based on an attenuated *Lm* expressing HPV-16 E7 fused to LLO (Lovaxin C, recently renamed ADXS11-001) was reported in 15 patients with cervical cancer who failed previous modes of therapy [21]. In this Phase I safety study, patients were intravenously infused with 1 of 3 dose levels of ADXS11-001 (1×10^9 cfu, 3.3×10^9 cfu or 1×10^{10} cfu). At the highest dose, 3 patients experienced severe fever and dose-limiting hypotension. All patients showed self-limiting, transient flu-like symptoms, possibly attributed to the potent induction of innate immune responses, with 6 of 15 patients reporting grade 3 severe adverse events. At the end of the study, stable

Table 1. Characteristics of Therapeutic HPV Vaccine Approaches

Vaccine Approach	Advantages	Disadvantages
Live vector-based	• High Immunogenicity • Can facilitate intercellular antigen spreading • Wide variety of vectors available	• Safety concerns in using live vectors • Potential pre-existing immunity may inhibit repeated administration
Peptide-based	• Easy to produce, Stable, Safe • Can combine multiple epitopes • Can engineer peptides for enhanced MHC binding	• Low Immunogenicity • Need to define epitopes • Difficult to have one-fits-all peptide (unless using overlapping peptide)
Protein-based	• Stable, Safe • No HLA restriction	• Low Immunogenicity; requires adjuvant • Usually better induction of antibody response than CTL response
DNA-based	• Safe, simple, stable for storage and transportation • Capacity for repeated administration • Easy to prepare at high purity • Sustained antigen expression • Can be engineered to add targeting and/or co-stimulatory genes	• Inability to amplify and spread *in vivo*, leading to weak immunogenicity • Small risk of integration into genome or cellular transformation
RNA Replicon-based	• Non-infectious, no risk of chromosomal integration or cellular transformation • Capable of repeated immunization • Enhanced antigen expression • Multiple vectors are available	• Unstable compared to DNA • Difficulty in long-term storage • Labor-intensive preparation • Difficult to prepare large amounts
Dendritic cell-based	• High immunogenicity; uses the most potent APC • Efficient antigen presentation • Multiple methods of Ag loading available	• Labor intensive, costly, *ex vivo*, individualized cell processing • Variable quality control and a lack of standard criteria for quality of vaccines due to autologous nature • Difficult to produce on a large scale
Tumor cell-based	• Useful if tumor antigen unknown • Likely to express relevant tumor antigens • Potency can be enhanced by cytokine treatment	• Safety concerns • Labor-intensive procedure • Costly, difficult to produce on a large scale

Table 2. Recent and Ongoing Therapeutic HPV Vaccine Clinical Trials

Type	Vaccine	Construct	Target Antigen(s)	Sponsor	Phase	Patient Population	Regimen	Ref.
Live vector	ADXS11-001 (Lovaxin C)	live *Listeria monocytogenes* secreting HPV-16 E7 fused to listeriolysin O	HPV-16 E7	Advaxis Inc.	I	15 patients with refractory or recurrent cervical cancer	Intravenous infusion of 1 of 3 dose levels (1 x 10^9 cfu, 3.3 x 10^9 or 1 x 10^{10} cfu), followed by second dose 3 weeks later	[21]
	TA-HPV	Live recombinant vaccinia virus expressing the E6 and E7 proteins of HPV-16 and HPV-18	HPV-16/18 E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I/II	8 patients with late-stage cervical cancer	Single dermal scarification of TA-HPV (2.5 x 10^5 pfu)	[30]
					I	29 patients with Stage Ib or IIa cervical cancer	Two vaccinations at least 4 weeks apart, using dermal scarification to deliver approximately 2.5 x 10^5 pfu	[31]
					II	12 patients with high-grade VIN or VAIN: (11 VIN 3, 1 VAIN 2)	Single dermal scarification of TA-HPV (2.5 x 10^5 pfu)	[32]
					II	18 patients with HPV-16-positive VIN 3	Single dermal scarification of TA-HPV (2.5 x 10^5 pfu)	[33]
				European Organization for Research and Treatment of Cancer	Ongoing, II	Patients with Stage Ib or IIa cervical cancer	2 vaccinations of TA-HPV, with 1st vaccination at least 2 weeks before surgery and 2nd vaccination at 8 weeks after 1st vaccination	[34]
	MVA E2	Recombinant vaccinia derived from Modified Vaccinia Ankara encoding E2 of bovine papillomavirus	HPV-16 E2	Instituto Mexicano del Seguro Social (IMSS)	I/II	36 patients with CIN 1-3	Intrauterine injection once a week over a 6 week period (10^7 virus particles/dose)	[35]
					II	34 patients with high-grade CIN	Intrauterine injection once a week over a 6 week period (10^7 virus particles/dose)	[36]
					I/II	50 men with flat condyloma lesions	Intraurethral injection once a week over a 4 week period (10^6 virus particles/dose)	[37]
	TG4001/R3484 (MVA-HPV-IL2)	Recombinant vaccinia virus derived from Modified Vaccinia Ankara expressing HPV-16 modified E6 and E7 proteins and IL-2	HPV-16 E6/E7	Transgene/Roche	IIa	21 women with CIN 2/3	3 subcutaneous injections of MVA-HPV-IL2 at 5 x 10^7 pfu/dose	[38]
					Plans for Phase IIb	200 women with CIN 2/3	Currently in planning	[38]

Table 2. contd….

Type	Vaccine	Construct	Target Antigen(s)	Sponsor	Phase	Patient Population	Regimen	Ref.
Peptide	Lipopeptide	Lipidated E7 (HLA-A*0201-restricted epitope, aa 86 - 93 lipopeptide) linked to PADRE helper peptide	HPV-16 E7	Cytel Corporation (later Epimmune, then aquired by IDM Pharma)	I	12 HLA-A2-positive patients with HPV-16-positive recurrent or refractory cervical cancer	4 subcutaneous injections at 1 of 4 dose levels (0.1, 0.3, 1.0 and 2.0 mg) of lipopeptide at 3-week intervals	[48]
	Peptide & Montanide ISA 51 adjuvant	HLA-A*0201-restricted HPV-16 E7 peptide (aa 11 - 20 & 86 - 93) and PADRE helper peptide, emulsified in Montanide ISA 51 adjuvant	HPV-16 E7	Cytel Corporation (later Epimmune, then aquired by IDM Pharma), Dutch Cancer Society	I/II	19 HLA-A*0201-positive patients with recurrent or residual cervical cancer	4 subcutaneous injections at 1 of 3 dose levels (100 ug, 300 ug, 1000 ug) at 3-week intervals	[49]
					I/II	15 HLA-A*0201-positive patients with recurrent or residual cervical cancer	4 subcutaneous injections at 1 of 3 dose levels (100 ug, 300 ug, 1000 ug) at 3-week intervals	[50]
	Peptide & Montanide ISA-51 adjuvant	HLA-A*0201-restricted HPV-16 E7 epitopes (aa 12 - 20 ± aa 86 - 93) linked to PADRE, emulsified in Montanide ISA 51 adjuvant	HPV-16 E7	NCI	I	18 patients with high-grade CIN or VIN: (16 with CIN 2/3, 2 with VIN 2/3)	4 subcutaneous injections (10 mg/vial of lipopeptide) at 3-week intervals	[51]
	Overlapping long peptide & Montanide ISA-51 adjuvant	13 peptides together (nine E6 and four E7 peptides of 25-35 aa long with an overlap of 10-14 aa) representing the entire sequence of HPV-16 E6 and E7, formulated in Montanide ISA 51 adjuvant	HPV-16 E6/E7	Dutch Cancer Society, ISA Pharmaceuticals	I	11 patients with HPV-16-positive VIN 3	4 subcutaneous injections at a 3 week interval (0.3 mg/each of 13 peptides)	[54]
					I	43 end-stage cervical cancer patients	4 subcutaneous injections at a 3 week interval (0.3 mg/each of 13 peptides)	[55]
					II	6 patients with resected HPV-16-positive stage 1B1 cervical cancer	4 subcutaneous injections at a 3 week interval after surgical dissection (0.3 mg/each of 13 peptides)	[56]
					II	20 patients with HPV-16-positive VIN 3	4 subcutaneous injections at 3 week intervals, each time in a different arm or leg (0.3 mg/each of 13 peptides)	[57]
Protein	Therapeutic Antigen-Genital Warts (TA-GW)	Recombinant HPV-6 L2/E7 fusion protein adjuvanted with 2% Alhydrogel	HPV-6 L2/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I	42 healthy male volunteers (32 immunized with TA-GW, 6 injected with placebo)	3 intramuscular injections at 1 of 4 dose levels (0, 3, 30 or 300 ug TA-GW) in accelerated scheme (weeks 0, 1, 4) or classical scheme (weeks 0, 4, 8)	[64]
					IIa	27 patients with genital warts	3 intramuscular injections at 4 week intervals	[65]
	TA-CIN	Recombinant HPV-16 L2/E6/E7 fusion protein	HPV-16 L2/E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I	40 healthy volunteers	3 intramuscular injections into same upper arm at 1 of 3 dose levels (26, 128, 533 ug) at 4 week intervals	[66]

Table 2. contd….

Type	Vaccine	Construct	Target Antigen(s)	Sponsor	Phase	Patient Population	Regimen	Ref.
	PD-E7	Fusion protein comprising mutated HPV-16 E7 linked to first 108 aa of *Haemophilus influenza* protein D, formulated in AS02B adjuvant	HPV-16 E7	GlaxoSmithKline	I/II	7 patients with CIN 1 or CIN 3: (5 with CIN3 and 2 with CIN 1)	3 intramuscular injections at 2 week intervals with 200 ug of PD-E7 formulated in 500 ul of AS02B adjuvant	[67]
	HPV16 Immunotherapeutic	Recombinant HPV-16 E6/E7 fusion protein adjuvanted with ISCOMATRIX	HPV-16 E6/E7	CSL Limited	I	31 patients with CIN 1-3	3 intramuscular injections at 3 weekly interval	[68]
	SGN-00101 (HspE7)	Fusion protein of HPV-16 E7 with *Mycobacterium bovis* variant bacille Calmette-Geurin heat shock protein Hsp65	HPV-16 E7	Nventa Biopharmaceuticals (formerly Stressgen, recently bought by Akela Pharma)	I	22 patients with anal HSIL	3 subcutaneous injections of either 100 ug of HspE7 or placebo at monthly intervals	[69]
					II	14 patients with anal HSIL	3 subcutaneous injections of 500 ug of HspE7 at monthly intervals	[69]
					II	27 pediatric patients with recurrent respiratory papillomatosis	After baseline debulking, patients received 3 subcutaneous injections of HspE7 (500 ug) at monthly intervals	[70]
					I/II	15 HIV patients with high-grade AIN	3 subcutaneous injections in alternating thighs at 1 of 3 dose levels (100, 500 or 1000 ug) at 4-week intervals	[71]
					II	20 women with high-grade CIN	4 subcutaneous injections at a dose of 500 ug at a 3 week interval followed by LLETZ	[72]
					II	58 patients with CIN 3	3 monthly subcutaneous vaccinations with 500 ug of HspE7 followed by colposcopic follow-up and cone biopsy of cervix	[73, 74]
					II	Women with CIN 3	3 subcutaneous injections at 4 week intervals	[75]
					Ongoing, II	Women with ASCUS/LSIL	3 subcutaneous injections at 4 week intervals	[76]
	SGN-00101 (HspE7) & Poly ICLC adjuvant	SGN-00101 adjuvanted in Poly ICLC	HPV-16 E7	Nventa Biopharmaceuticals (formerly Stressgen, recently bought by Akela Pharma)	Ongoing, I	Women with CIN 1,2, or 3	3 subcutaneous vaccinations at 4 week intervals	[81]

Table 2. contd….

Type	Vaccine	Construct	Target Antigen(s)	Sponsor	Phase	Patient Population	Regimen	Ref.
DNA	ZYC101	Plasmid DNA encoding HLA-A2-restricted epitopes derived from HPV-16 E7 protein (aa 83-95), encapsulated in 1-2 um biodegradable poly(lactide-co-glycolide) microparticles	HPV-16 E7	Eisai (formerly MGI Pharma, formerly Zycos)	I	12 HLA-A2-positive men with HPV-16-positive high grade AIN	4 intramuscular injections at a 3 week interval at 1 of 4 dose levels (50, 100, 200, 400 ug)	[94]
					I	15 patients with CIN 2/3	3 subcutaneous injections or intramuscular injections (depending on randomized group) every 3 weeks followed by surgical excision 4 weeks later	[100]
	ZYC101a (Amolimogene bepiplasmid)	Plasmid DNA encoding fragments derived from HPV-16 and 18 E6 and E7 proteins, encapsulated in 1-2 um biodegradable poly(D,L-lactide-co-glycolide) microparticles	HPV-16/18 E6/E7	Eisai (formerly MGI Pharma, formerly Zycos)	II	127 patients with CIN 2/3	3 intramuscular injections every 3 weeks followed by cervical conization 6 months after 1st injection	[101]
					Ongoing,II/III	251 patients with CIN 2/3	2 intramuscular injections at 100 ug/injection every 3 weeks	[102]
	pNGVL4a-Sig/E7(detox)/Hsp70	Plasmid DNA expressing HPV-16 E7 mutated to abolish Rb binding site linked to sequences coding for Sig and for heat shock protein 70	HPV-16 E7	NCI	I	15 patients with CIN 2/3	3 intramuscular injections at 1 of 3 dose levels (0.5, 1.0 or 3.0 mg/dose at 4 week interval	[122]
					I	Patients with advanced HNSCC	4 intramuscular injections at 1 of 4 dose levels (500 ug, 1mg, 2mg, 4mg) at weeks 1, 3, 5, 17	*
	pNGVL4a-CRT/E7(detox)	Plasmid DNA expressing HPV-16 E7 mutated to abolish Rb binding site linked to sequence coding for calreticulin	HPV-16 E7	NCI	Ongoing, I	Patients with CIN 2/3	Dose-escalating gene gun administration with ND10 gene gun device	**
	VGX-3100	Plasmid DNA expressing HPV-16 and HPV-18 E6 and E7 proteins	HPV-16/18 E6/E7	Inovio Biomedical Corp / VGX Pharmaceuticals	Ongoing, I	Adult females, post-surgical or ablative treatment of CIN 2/3	3 dose series (0.6, 2 or 6 mg of DNA/dose) administered by intramuscular injection with electroporation with CELLECTRA device	[88]
DC	DC + IL2	Autologous mature, monocyte-derived DCs pulsed with HPV-18 E7 protein	HPV-18 E7		Case report	1 patient with Stage IB2 cervical cancer	Subcutaneous injection 14 times with DCs (3 to 5 x 10^6/injection) combined with adoptive transfer of *in vitro* DC-primed and expanded HPV-17 E7-specific T cells and low-dose IL-2 treatment	[164]

Table 2. contd….

Type	Vaccine	Construct	Target Antigen(s)	Sponsor	Phase	Patient Population	Regimen	Ref.
	DC	Autologous, mature, monocyte-derived DCs loaded with recombinant HPV-16 E7 or HPV-18 E7	HPV-16 E7 or HPV-18 E7	Deutsche Forschungsgemeinschaft, BMBF	Pilot study	15 patients with progressive or recurrent cervical cancer	Subcutaneous injection ($2x10^4$ or $2x10^6$ DCs/injection) into upper arm 1 to 4 times	[165]
	DC + IL2	Autologous, mature, monocyte-derived DCs pulsed with recombinant HPV-16 E7 or HPV-18 E7	HPV-16 E7 or HPV-18 E7	Italian Institute of Health (ISS)	Case series	4 cervical cancer patients with recurrent disease refractory to standard treatment	Subcutaneous injection (1×10^7 DCs/dose) into anterior mid-thigh. Vaccinations 1-5: 2 week intervals. Vaccinations 6-10: 30 day intervals. Vaccinations 11-13: 60 day intervals. All injections followed by twice daily subcutaneous injections of IL-2 from day 3 to day 7 postvaccination	[166]
	DC + KLH	Autologous, mature, monocyte-derived DCs pulsed with recombinant HPV-16/18 E7 antigens and keyhole limpet hemocyanin	HPV-16/18 E7	NIH	I	10 patients with Stage Ib or IIa cervical cancer	Escalating doses (5, 10, 15×10^6 cells for injection) of 5 subcutaneous injections at 21-day intervals	[167]
	DC	Autologous mature, monocyte-derived DCs pulsed with HPV-16 E7 peptide	HPV-16 E7	National Taiwan University Hospital	Ongoing, I	12 patients with recurrent cervical cancer	4 injections of DCs into inguinal lymph nodes under guidance of real-time sonography	[168]
Prime-boost	Prime with TA-CIN, boost with TA-HPV	TA-CIN: (HPV-16 L2 E6/E7 fusion protein) TA-HPV: (vaccinia expressing HPV-16 and -18 E6 and E7)	HPV-16/18 E6/E7 + HPV-16 L2/E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	II	29 patients with high-grade AGIN	3 intramuscular doses of TA-CIN at 4 week intervals followed by a single dermal scarification of TA-HPV	[77]
					II	29 patients with AGIN 3	3 intramuscular doses of TA-CIN on days 0, 28 and 52 followed by single dermal scarification of TA-HPV on day 72	[78]
	Prime with TA-HPV, boost with TA-CIN	TA-HPV: (vaccinia expressing HPV-16 and -18 E6 and E7) TA-CIN: (HPV-16 L2 E6/E7 fusion protein)	HPV-16 L2/E6/E7 + HPV-16/18 E6/E7	Xenova/Cantab (now acquired by Celtic Pharma)	I/II	10 patients with high-grade AGIN	Single dermal scarification of TA-HPV followed by 3 intramuscular injections of TA-CIN 1 month apart at 24 weeks after TA-HPV vaccination	[79]
	Prime with pNGVL4a-Sig/E7 (detox)/Hsp70, boost with TA-HPV ± imiquimod	pNGVL4a-Sig/E7(detox)Hsp 70 plasmid DNA + recombinant vaccinia ± topical agent	HPV-16 E7 + HPV-16/18 E6/E7	NCI	I	Patients with HPV infection and CIN3	Intramuscular injection of DNA vaccine on days 1 and 29 and intramuscular TA-HPV on day 57 (with or without topical imiquimod)	[123]

*M Gillison, personal communication.
** C Trimble and W Huh, personal communication.

aa, amino acid; ASCUS, atypical squamous cells of undetermined significance; AGIN, anogenital intraepithelial neoplasia; CIN, cervical intraepithelial neoplasia; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma; HPV, Human papillomavirus; HSIL, high-grade squamous intraepithelial lesions; Hsp, heat shock protein; IL, interleukin; KLH, keyhole limpet hemocyanin; LLETZ, loop electrosurgical excision of the transformation zone; LSIL, low-grade squamous intraepithelial lesions; NCI, National Cancer Institute; NIH, National Institutes of Health; PADRE, Pan-DR binding T helper epitope; Rb, Retinoblastoma; VAIN, Vaginal intraepithelial neoplasia; VIN, Vulvar intraepithelial neoplasia

disease was reported in 7, progressive disease in 5 and 1 qualified as a partial responder of 13 evaluable patients, with tumor size reduction observed in 4 patients [21]. While ADXS11-001 shows more severe adverse events compared to other therapeutic HPV vaccines, it is significantly milder when compared to the adverse effects attributed to chemotherapy. ADXS11-001 is currently in plans for evaluation in Phase II trials (www.advaxis.com). This report of the first bacterial vector used in clinical trials in HPV-associated cancer patients opens up the arena for further development of bacterial vectors for clinical translation. Efforts have been made to further increase bacterial vectors' immunogenicity, especially in the case of *Lm*, through fusion with the PEST protein sequence that target proteins for degradation [22]. Continued progress in increasing the immunogenicity of the vector and limiting vector-associated toxicity is needed for further bacterial vector-based therapeutic HPV vaccine development.

2. Viral Vectors

Viral vectors are attractive vaccines for therapeutic HPV vaccination due to their high immunogenicity. There are many preclinical studies on the efficacy of live viral vectors, such as vaccinia viruses [23, 24], adenoviruses [25-27], vesicular stomatitis viruses (VSV) [28, 29], and alphaviruses. One viral vector of interest is the adenovirus. One study found that using adenovirus to deliver calreticulin and HPV E7 fusion protein (Ad-CRT/E7) protected mice against E7-expressing tumors. Rechallenge with live tumor cells showed that the mice developed immunological memory against E7-expressing TC-1 cells [25]. Recent studies have also shown that vesicular stomatitis virus (VSV) functions as an effective vector for HPV antigens. In murine models, single doses of recombinant attenuated VSV expressing HPV E7 elicited a significant E7-specific $CD8^+$ T cell response and reduced established tumor size [28].

Clinical trials have focused on the vaccinia virus as a viral vector due to its high efficiency of infection and large genome. One therapeutic HPV vaccine candidate is TA-HPV, a recombinant vaccinia virus expressing HPV-16/18 E6 and E7. TA-HPV was first evaluated in an open label Phase I/II trial in 8 patients with therapy-unresponsive late-stage cervical cancer. Patients were vaccinated with a single dose (2×10^6 pfu) of TA-HPV using a scarification technique. Three of 8 patients developed HPV-18 E7-specific antibodies and HPV-specific CD8+ T cells were detected in 1 of 3 evaluable patients, showing that TA-HPV was capable of eliciting immune responses [30]. In a subsequent Phase I safety and immunogenicity study, 29 patients with stage Ib or IIa cervical cancer were vaccinated at least 4 weeks apart, using a scarification technique estimated to deliver approximately 2.5×10^5 pfu of TA-HPV[31]. Likewise, no significant clinical side effects were observed and a serological response to vaccinia was observed in 18 of 29 patients. HPV-specific CTL response was demonstrated in 4 patients, confirming the safety and immunogenicity of TA-HPV [31]. Two additional Phase II trials have also examined TA-HPV in patients with vulvar intraepithelial neoplasia (VIN). In one of the Phase II trials, single dermal scarification of 2.5×10^5 pfu of TA-HPV administered to 11 women with VIN 3 and 1 woman with vaginal intraepithelial lesions (VAIN) grade 2 elicited increased antibody and T-cell responses in 11 out of 12 patients. Additionally, lesion reduction was observed in ten of 12 patients. One patient's VAIN cleared completely after vaccination and HPV-16 was no longer detected in the previous area of the lesion [32]. Similar clinical and immune responses were reported in a separate trial evaluating the same regimen in 18 women with HPV-16-positive VIN 3, with 8 patients demonstrating reduction in lesion diameter, reduced viral load or viral clearance and increased antigen-specific immune responses [33]. An ongoing multicenter Phase II clinical trial is investigating TA-HPV in combination with surgery to treat women with Stage Ib or IIa cervical cancer [34].

Another viral vector-based therapeutic HPV vaccine candidate evaluated in clinical trials is MVA E2, a recombinant vaccinia virus derived from Modified Vaccinia Ankara (MVA) encoding E2 protein of bovine papillomavirus [35-37]. MVA E2 expresses E2, the transcriptional repressor of E6 and E7 oncogenic proteins. Although MVA E2 was designed for gene therapy, it has applications for vaccination in the induction of E2-specific immune responses. CIN lesions may or may not experience viral integration into host genome. Therefore, for CIN lesions that have no viral integration into host genome and have subsequent expression of HPV E2, MVA E2 can bind to HPV genome and prevent the upregulation of E6 and E7 oncogenic proteins for the potential control of HPV-associated CIN lesions. MVA E2 has demonstrated efficacy in a Phase II trial in 34 CIN 2/3 patients intrauterinely injected once a week (10^7 virus particles/dose of MVA E2) over a 6 week period, inducing lesion regression in all patients, with 14 of 34 patients presenting up to 60% reduction in lesion size, and eliminating all papillomavirus DNA in 12 out of 34 patients. MVA E2 was also well-tolerated and increased antibody levels against both MVA virus and E2 protein [36]. It has demonstrated similar safety and immunogenicity profiles in a Phase I/II study of 50 men with intraurethral flat condyloma [37]. 30 men treated with MVA E2 were compared to a control group of 20 men treated with 5-fluorouracil. The 30 men administered MVA E2 by intraurethral injection once a week (10^6 virus particles/dose) over a 4-week period developed antibodies against MVA virus and E2 protein and had no detectable viral DNA after treatment. 28 out of 30 patients showed no lesion or presence of papillomavirus after 4 weeks of MVA E2 treatment. Patients treated with MVA E2 did not show any recurrence of lesions after 1 year of treatment whereas 3 control patients had recurrence of lesions after 3 months of treatment [37]. While MVA E2 has demonstrated efficacy in controlling HPV-associated lesions, it is not clear how much of these effects are attributed to immunotherapy.

A third candidate is TG4001/R3484 (MVA-HPV-IL2), which also uses a modified vaccinia Ankara viral vector. TG4001/R3484 contains DNA encoding HPV-16 E6 and E7 and the immunoregulatory cytokine IL-2. Early Phase I trials established the safety profile of TG4001/R3484 and led to Phase II trials to evaluate vaccine efficacy. In a 6-month Phase IIa trial, 21 patients with HPV-16-related CIN 2/3 were administered 3 subcutaneous injections of MVA-HPV-IL2 at 5×10^7 pfu/dose. Ten out of 21 women cleared the

pre-cancerous lesions completely, with levels of E6 and E7 antigen difficult to detect after six months [38]. Currently, placebo-controlled Phase III trials evaluating TG4001/R3484 antigen-specific therapy in 200 patients suffering from high-grade pre-cancerous cervical lesions (CIN 2/3) caused by HPV-16 are about to be initiated [38].

Currently, efforts are made to further improve the immunogenicity of various viral vectors, including coexpression of soluble cytokines such as the hFlt3 ligand, which induces DC maturation [23], linkage with calreticulin-encoding gene to improve major histocompatibility complex (MHC) class I presentation [24], or fusion of HPV-16/18 E7 with heat shock protein to prevent cellular transformation and enhance immune response [27]. However, preexisting immunity to the viral vector remains a major limitation for vector-based therapeutic HPV vaccines. Researchers are testing prime-boost regimens using recombinant viral vectors in combination with other vaccines and more recently, it has been shown that the use of Cox-2 inhibitors may enable repeated administration of vaccinia virus [39]. Combinations of recombinant viral vectors with other forms of vaccines such as inactivated viruses, proteins, peptides, DNA, or RNA-based vaccines may synergistically promote stronger pathogen-specific immune responses, while limiting vector-specific immunity.

B. Peptide/Protein Vaccines

1. Peptide-Based Vaccines

Peptide-based therapeutic HPV vaccination involves the direct administration of peptides derived from HPV antigens for uptake by DCs (see Fig. (**1**)). While peptide vaccines are easy to produce, stable and safe, they suffer from low immunogenicity. Furthermore, developing a peptide vaccine that is effective in all patients may be hindered by the polymorphic nature of human leukocyte antigen (HLA) molecules. The need to identify specific immunogenic epitopes of HPV antigens for many different HLA haplotypes may make it difficult to produce a peptide-based vaccine that will cover the whole population, making it impractical for large-scale vaccination treatments. Yet, once the epitopes are defined, it is possible to control which peptide epitopes are present in the vaccine. This has implications for engineering peptide vaccines to contain particularly immunogenic peptides or peptides that direct CD4+ T-helper or CD8+ cytotoxic immune responses.

Development of peptide vaccines for cervical cancer have been made possible by the identification of various MHC-restricted CD4+ and CD8+ T cell epitopes of HPV early proteins such as murine (H-2Db) and human (HLA-A.2) CTL epitopes for HPV-16 [40, 41]. Preclinical studies have focused on boosting the immunogenicity of peptide vaccines through the use of adjuvants such as 4-1BB ligand [42], CpG oligodeoxynucelotide [43-45], and mutant cholera toxin [46] to enhance vaccine potency (for review, see [9]). Other potential strategies to enhance peptide vaccine potency include epitope enhancement to increase peptide affinity for MHC molecule and linkage of peptides to lipids (lipopeptides) [47].

Several peptide-based therapeutic HPV vaccines have been found to be safe and well tolerated in early phase clinical trials [48-51]. In a non-randomized Phase I dose escalating study, 12 HLA-A2 positive patients with HPV-16-positive recurrent or refractory cervical cancer were vaccinated with a lipopeptide consisting of HLA-A*0201-restricted lipidated HPV-16 E7 (aa 86-93) peptide linked to PADRE, a non-specific helper peptide. The lipopeptide-based vaccine elicited no significant adverse effects, and generated E7-specific cytotoxic T lymphocyte immune responses in 5 of 7 who received two vaccinations, and 2 of 3 who received all four injections [48]. Phase I/II trials have also examined HLA-A*0201-restricted HPV-16 E7 peptide (aa 11-20 and aa 86-93) and PADRE emulsified in Montanide ISA 51 adjuvant in HLA-A*0201-positive patients with HPV-16 positive recurrent or refractory cervical cancer [49, 50]. Low count of lymphocytes before and after vaccination in patients in both trials, suggesting reduced immunocompetence in patients with advanced cervical cancer. A Phase I trial evaluated HLA-A*0201-restricted, lipidated HPV-16 E7 (aa 12-20 ± 86-93) peptide linked to PADRE emulsified in Montanide ISA 51 adjuvant in 18 HLA-A2-positive patients with HPV-16-positive high-grade CIN or VIN. This lipopeptide generated increase in HPV E7-specific T cell immunity for 10 of 16 patients tested and also generated partial or complete regression of CIN lesions in 9 out of 17 evaluable patients. Overall, these peptide-based vaccines have been found to be well tolerated but only generated detectable clinical responses in immunocompetent individuals with pre-invasive diseases [51].

The future of peptide vaccines may tend towards the use of long overlapping peptides. Overlapping long peptides may broaden the range of antigenic epitopes and limit the obstacle of MHC restriction. Overlapping peptides have proven to be effective in generating antigen-specific T cell immune responses in preclinical animal models such as mice [52] and rabbits [53]. In particular, an overlapping peptide-based vaccine comprising 13 peptides (9 E6 and 4 E7 epitopes 25-35 aa long with an overlap of 10-14 aa) representing HPV-16 E6 and E7, formulated in Montanide ISA 51 adjuvant, has been the focus of several clinical trials. In a phase I trial with 11 patients with HPV-16-positive VIN 3, patients were subcutaneously injected 4 times at a 3 week interval with the overlapping peptide-based vaccine. Vaccination induced HPV-16 E6 and E7-specific CD4+ and CD8+ T cells and generated complete clearance of lesions in 4 out of 11 patients [54]. Kenter *et al.* conducted a Phase I trial with the same vaccine in 43 end-stage cervical cancer patients and showed that the vaccine was safe, well tolerated, and elicited a broad IFN-γ-associated T cell response in patients [55]. Furthermore, the same vaccine regimen was also tested in stage 1B1 HPV-16+ cervical cancer patients and demonstrated increased HPV-16-specific CD4+ and CD8+ T cell responses to a broad array of epitopes in all 6 patients [56]. Recently, this vaccine was evaluated in a Phase II clinical trial in 20 HPV-16+ grade 3 VIN patients. The overlapping peptide vaccine generated T cell responses in all patients and led to objective clinical responses in 15 of 19 evaluable patients at 12 months of follow-up, including 9 patients with complete responses and continuing to be disease-free at 24 months of follow-up [57]. Generally, the promising data for

the use of overlapping peptides has renewed enthusiasm for therapeutic HPV E6/E7 peptide-based vaccines.

2. Protein-Based Vaccines

Protein-based vaccines have several advantageous properties for vaccine development. They are safe compared to live-vector based vaccines. Furthermore, they can overcome the limited specificity of MHC responses associated with peptide vaccines. Proteins contain all possible HLA epitopes and are processed into peptide epitopes by the patient's own cells, eliminating the need to first determine a patient's HLA type and immunogenic epitopes of an antigen. However, like peptide vaccines, protein vaccines also suffer from low immunogenicity. Another concern for the development of protein-based vaccines is that proteins may elicit better antibody responses than CTL responses. Since proteins are often administered exogenously, they may have limited efficacy in generating cytotoxic T lymphocyte (CTL) responses.

Preclinical trials have shown that use of adjuvants, such as the liposome-polycation-DNA (LPD) [58] or the saponin-based ISCOMATRIX [59], and fusion with other immunostimulatory molecules, such as those responsible for targeting antigens to antigen-presenting cells (APCs) and thus improving APCs' antigen uptake and presentation, can improve CTL responses. Examples include fusion proteins of HPV-16 E7 with bacterial proteins such as the *Bordetella pertussis* adenylate cyclase (CyaA), which targets DCs through interaction with $\alpha_M ß_2$ integrin to induce E7-specific CTL responses [60], or the *Mycobacteria*-derived heat shock proteins (Hsp) [61, 62], which enhance CTL responses and inhibit angiogenesis in tumors. Fusion of HPV-16 E7 and truncated *Pseudomonas aeruginosa* exotoxin A, which enhances protein translocation and thus MHC class I presentation, also increased E7-specific T-cell and antibody responses [63].

Protein-based therapeutic HPV vaccines are vaccine candidates commonly tested in clinical trials, compared to other forms of therapeutic HPV vaccines (see Table **2**). Several clinical trials have tested the safety and efficacy of HPV protein-based vaccines [64-76]. One vaccine candidate is TA-CIN, a genetically engineered fusion of L2, E6 and E7 proteins from HPV-16. In a Phase I trial, vaccination with TA-CIN was shown to induce antibody responses against L2 in all patients and T cell immunity against HPV-16 E6 and E7 oncoproteins in 8 out of 11 healthy patients receiving the highest dose, proving to be safe and immunogenic [66]. Phase II trials have focused on using TA-CIN as part of a prime-boost regimen with TA-HPV [77-79] since preclinical studies suggested this was a more effective regimen [80]. For example, in a Phase II clinical trial, HPV protein-based vaccine, TA-CIN, was used for priming and a recombinant vaccinia virus encoding HPV-16/18, E6/E7 fusion protein (TA-HPV) was used for boosting in 29 patients with anogenital intraepithelial neoplasia. No serious adverse effects were observed in all patients; in addition, 5 out of 29 patients showed increased HPV-16-specific T cell mediated immune responses. However, the result does not show significant advantage over single TA-HPV vaccination [78]. In another prime-boost regimen, ten patients with HPV-16+ high-grade vulvar intraepithelial neoplasia were primed with TA-HPV and boosted with TA-CIN. Among all the patients, 9 patients developed HPV-16-specific T cell responses, and 3 showed significant reduction in the size of the lesion. However, the result does not show direct correlation between clinical and immunological responses [79].

The protein vaccine candidate that has garnered significant interest is SGN-00101, also known as HspE7, which is a fusion of *Mycobacterium bovis* variant bacille Calmette-Guerin heat shock protein (Hsp65) and HPV-16 E7. In Phase I and II clinical trials, HspE7 has been found to be well tolerated and to generate lesion regression in several HPV-associated diseases including genital warts [69], recurrent respiratory papillomatosis [70], anal intraepithelial neoplasia (AIN) [71] and high-grade cervical intraepithelial neoplasia (CIN) [72-74]. In a Phase II trial, 58 patients with CIN 3 received 3 monthly subcutaneously injections of 500 ug of HspE7 followed by colposcopic follow-up before undergoing cone biopsy of the cervix [73]. Of the 58 evaluable patients, 13 had a complete response, in which post-vaccination biopsy specimen tested negative for CIN, 32 had a partial response (> 50% reduction of lesion size) and 11 had stable disease (persistent CIN 3 with less than 50% reduction of lesion size). Serological assessment of these patients found that increased HPV-16 E7-specific IgG levels appeared to be correlated with a positive therapeutic effect [74]. Clinical trials are ongoing in patients with CIN 3 [75] and atypical squamous cells of undetermined significance (ASCUS)/low-grade squamous intraepithelial lesions (LSIL) [76] to further evaluate the efficacy of HspE7. It has been found that HspE7 potency could be increased significantly with the adjuvant Poly-ICLC and provide the rationale for further pursuit of clinical development of a new formulation of HspE7 incorporating Poly-ICLC [81, 82]. More studies are needed to better define the clinical outcomes of the vaccine.

C. Nucleic Acid Vaccines

1. DNA Vaccines

DNA vaccination represents an appealing vaccination approach. Since DNA vaccines do not elicit anti-vector immune responses in the vaccinated patient, they have the capacity for repeated administration that may be needed to achieve and maintain target immune responses. DNA vaccines are also simple, safe and stable and are easy to manufacture on a large scale. DNA vaccines are also able to provide a sustained release of antigenic proteins, thereby enhancing immunological memory. Furthermore, they can be engineered to express HPV antigenic peptides or proteins and have a variety of delivery methods, enabling DNA vaccines to deliver HPV antigens to APCs and stimulate development of both CD4+ and CD8+ antigen-specific T cell responses *in vivo*. However, DNA suffers from low immunogenicity due to insufficient intrinsic specificity for APCs and an inability to amplify and spread between cells *in vivo*. Strategies to enhance therapeutic HPV DNA vaccines have focused on targeting DNA encoding antigens to DCs to boost vaccine-induced immune responses.

a. Strategies to Enhance DNA Vaccine Potency

Dendritic cells are key players in the initiation of the adaptive immune response. Thus, numerous efforts have

been made to enhance the immunogenicity of these vaccines by focusing on 1) increasing the number of DCs transfected with HPV DNA plasmids, 2) improving HPV antigen expression, processing and presentation by DCs and 3) enhancing the ability of HPV DNA transfected DCs to prime E6/E7-specific T cells in order to generate therapeutic effects against established HPV infections and HPV-associated lesions.

i. Increase Number of HPV Antigen-Expressing/HPV Antigen-Loaded DCs

Efficient routes of administration to delivery DNA directly to DCs may increase the number of antigen-expressing/antigen-loaded DCs. Gene gun technology uses compressed helium to propel a stream of gold particles coated with DNA into the skin, where Langerhans cells (immature DCs) are located. Gold particle-mediated gene gun delivery was shown to be the most dose-efficient method of vaccine administration in comparison to routine intramuscular and biojector injection [83]. Recently, gene gun has also demonstrated the capability to deliver noncarrier naked DNA under a low-pressure system, generating antitumor effects comparable to those of gold particle-mediated gene gun delivery [84]. Another method is electroporation, which permeabilizes the cell membrane with an electric current in order to increase cellular uptake of intramuscularly injected DNA. Electroporation also leads to local inflammation and cytokine recruitment, generating a favorable environment for maintenance of vaccine-elicited immune responses. In a direct comparison of electroporation, intramuscular injection and gene gun delivery for HPV DNA vaccination in preclinical models, electroporation was shown to elicit the highest number of E7-specific cytotoxic CD8+ T cells and greatest antitumor immune response against E7-expressing tumors [85]. Several preclinical studies have also demonstrated improved antigen expression and enhanced DNA vaccine-elicited antigen-specific immune responses by electroporation [86, 87]. Recently, it has been shown that co-administration of a viral fusogenic membrane glycoprotein can couple concentrated antigen transfer to DCs, facilitating antigen uptake, and also locally induce acute inflammatory response to enhance DNA vaccine potency [88]. Vesicular Stomatitis Virus G protein (VSV-G) was shown to induce extensive cell fusion and nonapoptotic death, with concomitant release of antigen. The intramuscular administration by electroporation of DNA encoding VSV-G combined with CRT/E7 DNA generated robust and amplified E7-specific CD8+ T cell responses and demonstrated therapeutic control of E7-expressing tumors in vaccinated mice [88]. Electroporation has also been used in clinical trials. VGX-3100, a DNA vaccine targeting HPV-16 and HPV-18 E6 and E7, delivered via electroporation has been investigated for treatment of patients with CIN 2 or 3 lesions in a Phase I clinical trial [89]. Interim safety and immunogenicity data indicate that the vaccine was found to be generally safe and well tolerated, generated antigen-specific cytotoxic T-lymphocyte responses against all four antigens and achieved significant cellular (3 out of 6) and humoral (5 out of 6) immune responses in vaccinated patients [90].

Other routes of administration with potential applications for HPV DNA vaccines include intradermal vaccination followed by pulses of laser to increase transfection efficiency [91, 92], intramuscular administration of plasmid DNA microencapsulated in a biopolymer to protect DNA from nuclease degradation [93-95], intradermal vaccination by tattooing [96, 97], skin patches to deliver DNA [98], and microneedles [99]. In particular, microencapsulation of DNA has been investigated in clinical trials. Amolimogene bepiplasmid (ZYC101a) is a DNA-based therapeutic vaccine encoding HPV-16 and HPV-18 E6- and E7-derived epitopes encapsulated into 1 to 2 μm poly-lactic-co-glycolic acid microparticles to prevent DNA from nuclease degradation [100]. Early-phase clinical trials had been completed with ZYC101, the precursor of amolimogene bepiplasmid, which encodes multiple HLA-A2-restricted HPV-16 E7 epitopes. In a Phase I/II clinical trial evaluating ZYC101, 4 intramuscular injections (at either 50, 100, 200, 400 ug) at 3-week intervals in 12 men with high-grade anal dysplasia produced 3 partial responses (regression to low-grade), including 1 patient at the 200 ug dose and 2 patients at the 400 ug dose level. Ten patients demonstrated antigen-specific activated T cells for up to 6 months after vaccination [95]. However, these clinical responses did not correlate to immune responses. In a separate dose-escalating Phase I trial evaluating ZYC101, 15 patients with CIN 2/3 were assigned to subcutaneous injection or intramuscular administration of 3 doses (50 ug, 100 ug, or 200 ug) at 3 week intervals. At the highest dose, intramuscular injection generated 4 of the 5 complete responses observed, defined by no cytologic or histologic evidence of residual pre-invasive disease, indicating that intramuscular injection was more effective than subcutaneous administration of ZYC101. Furthermore, 11 out of the 15 patients developed measurable HPV-16-specific CTL responses [101]. The development of ZYC101a spurred its evaluation in Phase II trials. In one Phase II trial, vaccination with amolimogene bepiplasmid was shown to promote the resolution of CIN 2/3 in most patients younger than 25 years compared to the placebo group of the same age [102]. A Phase II/III randomized clinical trial of intramuscular administration of amolimogene in patients under 25 years of age with HPV-associated CIN 2/3 is currently ongoing [103].

Another way to increase the number of antigen-loaded/antigen-expressing DCs is to facilitate intercellular spreading of HPV antigens. Since naked DNA is limited in its capacity to spread encoded antigen to surrounding cells *in vivo*, strategies to help antigen spreading may enhance DNA vaccine potency. Intercellular spreading of E7 is facilitated by linking E7 to HSV-1 VP22 or one of its homologues (bovine herpes virus VP22 or Marek's disease virus VP22), tegument proteins that can mobilize antigen for intercellular transport to neighboring cells. Several preclinical studies have evaluated the significance of VP22 in intercellular spreading by linking VP22 to molecules such as p53 [104], thymidine kinase [105], cytosine deaminase [106] and green fluorescent protein (GFP) [107]. Mice vaccinated with VP22/E7 DNA generated a significantly greater number of E7-specific CD8+ T cells [108] and a stronger antitumor effect than wild-type E7 DNA [109]. MVP22/E7 DNA [110] has shown similar potent antigen-specific immune responses.

It is also possible to engineer DNA encoding antigens linked to molecules that preferentially bind to dendritic cells in order to increase the number of antigen-expressing DCs.

Molecules commonly employed in this strategy are DC receptor ligands such as FMS-like tyrosine kinase 3 (Flt3) ligands, which bind with Flt3 receptors on DCs, and heat shock proteins (Hsp), which bind with scavenger receptors on DCs such as CD91. For example, a HPV DNA vaccine encoding a recombinant chimera consisting of extracellular domain of Flt3 ligand linked to HPV-16 E7 has been shown to generate significantly higher levels of E7-specific cytotoxic immunity against E7-expressing tumors and reduce the size of established pulmonary metastases compared to wild-type E7 DNA [111]. In another example, HPV antigen linked to Hsp70 was presented in the context of MHC I and induced antigen-specific CD8+ T cell immune responses [112, 113]. Additionally, Hsp70 may activate the innate immune system, which can provide signals for DC maturation and result in more effective antigen cross-presentation.

ii. Improve HPV Antigen Expression, Processing and Presentation in DCs

Efficient antigen expression, processing and presentation in dendritic cells will lead to increased priming of antigen-specific T cells for more potent antigen-specific immune responses. Codon optimization and demethylating agents are strategies to increase antigen expression in DCs. Codon optimization enhances translation of genes coding for HPV antigens in cells transfected by HPV DNA vaccines by replacing rarely used codons with more commonly recognized codons. Transfection with codon optimized versions of the E6 [114] and/or E7 DNA [86, 115, 116] strongly enhanced E6 and/or E7 protein expression and enhanced antigen-specific CD8+ T cell immune responses in vaccinated mice. Since methylated CpG motifs may silence gene expression [117], demethylating agents may also enhance the level of expression of antigen encoded in the DNA vaccine. Demethylating agent nucleoside analogue 5-aza-2'-deoxycystidine (DAC) used in the context of a HPV DNA vaccine encoding calreticulin linked to HPV 16-E7 (CRT/E7) increased CRT/E7 expression and enhanced E7-specific CD8+ T cell immune responses in vaccinated mice [118].

To enhance antigen processing and presentation, it is necessary to understand the biology of antigen processing pathways via MHC class I and MHC class II (Fig. (**1**)). In the MHC class I pathway, antigens are translocated from the cytosol to the lumen of the endoplasmic reticulum. Antigenic proteins are degraded to peptide fragments by the proteasome and transported to the ER. Therefore, linkage of antigen to proteins that target antigen to the proteasome [119] or the endoplasmic reticulum [120-122] may enhance MHC class I antigen presentation. In particular, calreticulin (CRT) is a chaperone molecule located in the ER that is involved in the loading of antigenic peptide to MHC class I. Vaccination with CRT/E7 DNA has been shown to significantly increase E7-specific CD8+ T cell precursors and exhibit an impressive antitumor effect in E7-expressing tumors in vaccinated mice compared to vaccination with wild-type E7 DNA or CRT DNA [120]. In a direct comparison of HPV-16 E7 DNA vaccines employing intracellular targeting strategies, DNA vaccine encoding CRT/E7 generated the greatest E7-specific CD8+ T cell immune responses and antitumor effects against E7-expressing tumors in vaccinated mice [24]. These MHC class I pathway targeting strategies have been applied in clinical studies. In a Phase I clinical trial, a DNA vaccine encoding a signal sequence for the endoplasmic reticulum (Sig) linked to an attenuated form of HPV-16 E7 (with a mutation that abolishes the Rb binding site; E7(detox)) and fused to Hsp70 (Sig/E7(detox)/Hsp70) was found to be well tolerated in patients with CIN 2 and 3 lesions [123]. Fifteen patients were given 3 intramuscular injections (at 0.5, 1.0 or 3.0 mg/dose) at 4 week intervals and complete histologic regression occurred in 3 of 9 individuals at the highest dose. Although HPV-E7-specific and HPV-E6-specific T cell responses were detected, they were low in frequency and in magnitude and did not correlate with clinical outcome. This DNA vaccine (Sig/E7(detox)/Hsp70) has also been tested in HPV-16 positive patients with advanced head and neck squamous cell carcinoma in a Phase I trial that has recently been completed (M Gillison, personal communication). A clinical trial employing the same DNA vaccine encoding Sig/E7(detox)/HSP70 boosted with recombinant vaccinia virus encoding HPV-16/18 E6/E7 fusion protein (TA-HPV) with or without imiquimod is in progress in patients with CIN 2/3 lesions [124]. Additionally, a Phase I trial with a DNA vaccine encoding the modified HPV-16 E7 linked to CRT (CRT/E7(detox)) in patients with high grade intraepithelial cervical lesion using a clinical-grade gene gun device has recently begun (C Trimble and W Huh, personal communication).

Antigen processing and presentation through the MHC class II pathway is important in the priming of CD4+ T cell helps to provide T cell help. In the MHC class II pathway, extracellular pathogens and proteins are internalized and degraded by proteases into antigenic peptides within endocytic vesicles, bound to MHC class II molecules and delivered to the cell surface (Fig. (**1**)). To enhance antigen processing by the MHC class II pathway, Ji *et al.* developed a DNA vaccine encoding HPV-16 E7 protein linked to the sorting signal of lysosomal-associated membrane protein type 1 (LAMP-1) [125]. Transfected cells were shown to reroute E7 antigen from the cytoplasm/nucleus to the endosomal/lysosomal compartments for more efficient antigen presentation. Mice vaccinated with E7/LAMP-1 generated greater E7-specific CD4+ and CD8+ effector cells compared to those vaccinated with DNA vaccine encoding wild-type E7 alone [126].

The MHC class II-associated invariant chain (Ii) has also been employed in the context of DNA vaccine to enhance MHC class II antigen presentation [127]. CLIP (Class II-associated peptide) is a region of the invariant chain that occupies the MHC II peptide-binding groove to prevent premature binding of the antigenic peptide. The substitution of CLIP with Pan-DR helper T lymphocyte epitope (PADRE) in the invariant chain (Ii-PADRE) can enable more efficient antigen presentation for activation of a strong PADRE-specific CD4+ T cell immune response. Mice vaccinated intradermally via gene gun with DNA coding for Ii-PADRE in conjunction with HPV-16 E7 DNA have shown to generate significantly greater E7-specific CD8+ T cell response compared to those immunized with E7 DNA in conjunction with DNA coding for unmodified Ii [127]. Furthermore, CD4+ T cells activated by PADRE were shown to secrete IL-2, a cytokine important for T cell proliferation, and hence facilitate CD4+ T cell help [128]. Ii-PADRE DNA

has been used synergistically with other intracellular targeting strategies to further enhance antigen-specific CD8+ T cell immune responses generated by HPV DNA vaccines [129]. It has also been demonstrated that co-administration of Ii-PADRE DNA with CRT/E6 DNA vaccination in mice pretreated with doxorubicin reversed doxorubicin-mediated immunosuppression of antigen-specific immune responses [130].

Cross-presentation, in which extracellular protein is presented through the MHC class I pathway, is also utilized in enhancing MHC class I antigen processing and presentation. Linkage of HPV E7 to various cross presentation pathway-targeting proteins, including HSP 70, GP96 [131], and domain II of *P. Aeruginosa* [132], in the context of DNA vaccines have been shown to result in enhanced E7-specific CD8+ T cell immune responses. Additionally, intramuscular injection of DNA vaccines can generate antigen-specific immune responses predominantly through cross-priming mechanisms. It has been recently demonstrated that intramuscular administration of DNA encoding xenogenic MHC class I molecules with HPV E7 DNA vaccine enhanced E7-specific CD8+ T cell immune responses and antitumor effects against E7-expressing tumors in tumor-bearing mice. It was also shown to lead to an increase in the number of infiltrating CD8+ T lymphocytes and activated APCs at the injection site, increasing the rate of apoptosis, leading to increased release of antigen for cross-priming in local muscle tissue to enhance MHC class I antigen processing and presentation [133].

Another strategy to enhance antigen processing and presentation is to bypass the MHC processing. For example, single chain trimer (SCT) technology involves the linkage of the genes encoding E6 antigenic peptide to ß2 microglobulin and MHC I heavy chain to generate a stable single-chain construct encoding antigenic peptide fused to an MHC class I molecule. Mice vaccinated intradermally via gene gun with DNA vaccine encoding SCT of MHC class I linked to HPV-16 E6 CTL epitope exhibited complete protection against a lethal challenge of E6-expressing tumor cells and also generated greater E6-specific CD8+ T cell immune responses compared to mice administered with wild-type HPV-16 E6 DNA [134].

Another potential strategy to increase the MHC class I and II expression in order to activate more T cells is the intradermal administration via gene gun of DNA encoding transcriptional activator MHC CIITA with HPV DNA vaccines. MHC CIITA is a known master regulator for MHC II expression and has also been shown to upregulate the expression of MHC I molecules on the surface of DCs. Therefore, co-administration of CIITA with HPV DNA vaccines represents a potential strategy to enhance antigen presentation through both MHC class I and MHC class II pathways. Kim *et al.* have shown that this strategy was able to potentiate a stronger anti-tumor CD4+ and CD8+ T cell immune responses, prolonging the survival of mice better than the vaccine given without DNA encoding CIITA [135].

iii. Enhance DC Function and Interaction with T Cells

After antigens are processed and presented, the interactions between DCs and T cells are essential for T-cell activation. Since DC cells are susceptible to T cell mediated apoptosis after T cell priming, prolonging DC survival will enhance the long-term ability of DCs to prime T cells. Approaches that have been used often center on the intradermal administration of therapeutic HPV vaccine using DNA encoding anti-apoptotic proteins such as Bcl-xL, Bcl-2, X-linked inhibitor of apoptosis protein and dominant negative mutants (dn) of caspase-9, dn caspase-8 [136] or connective tissue growth factor [137]. However, this combination raises concerns for possible cellular transformations, limiting its clinical utility. Therefore, the employment of small interfering RNA (siRNA) may alleviate concerns of oncogenicity. The co-administration of DNA vaccine encoding HPV-16 E7 with siRNA targeting pro-apoptotic proteins Bak and Bax intradermally via gene gun has been shown to extend DC survival in the draining lymph nodes, stimulating stronger E7-specific CD8+ T cell responses and eliciting more potent antitumor effects compared to mice administered HPV-16 E7 DNA alone [138].

Another approach to improve DC and T cell interaction is to prevent apoptotic signaling to T cells in order to increase the number of activated T cells. DCs produce Fas Ligand (FasL), which induce T cell apoptosis upon binding to Fas, a cognate death receptor on T cells. Intradermal administration of therapeutic HPV DNA vaccine with DNA encoding short hairpin RNA (shRNA) targeting FasL on the HPV-16 E7 peptide-loaded DCs was shown to reduce the apoptosis of E7-specific CD8+ T cells, generating stronger E7- specific CD8+ T cell response in mice in comparison to HPV DNA vaccine alone and resulting in a more potent cytotoxic response against E7-expressing tumors [139]. Therefore, HPV DNA vaccine potency may be enhanced through anti-apoptotic signals to APCs as well as inhibition of apoptosis in T cells.

Activating DC expansion can also enhance T cell priming. Co-administration of HPV DNA vaccines with DNA encoding immunostimulatory cytokines such as GM-CSF [140], Il-2 [141], IL-12 [142], or codon-optimized IL-2 and IL-12 [143] have been shown to enhance antigen-specific cytotoxic T cell-mediated immune responses. An alternative way to promote DC activation is to block negative regulators of DC activation, such as SOCS-1, which suppresses DC function by inhibiting signaling through Jak-STAT pathway. Therefore, using siRNA to target and inactivate SOCS-1 in DCs could potentially enhance CD8+ immune responses elicited by therapeutic HPV DNA vaccines [144].

Strategies to overcome negative regulation of the immune system may also be used to optimize vaccine-induced immune responses. The function of regulatory T cells (T_{reg}) is to maintain immune tolerance by inhibiting DC and CD8+ T cell responses. Hence, T_{reg} depletion may enhance DC and T cell. Several agents including cyclophosphamide, COX-2 inhibitors, Foxp3-transfected DC and anti-CD25 monoclonal antibody have been used to deplete T_{reg} cells [145-147] and enhance antigen-specific CD8+ T cell responses and antitumor effects.

2. RNA Replicon-Based Vaccines

RNA replicon-based vaccines utilize naked RNA molecules, termed RNA replicons that can replicate in a self-

limiting fashion within the transfected cell but are modified to lack structural sequences necessary to form viral particles. These vaccines can be administered as RNA or DNA, which is transcribed by the cell into RNA replicons. RNA replicons may be derived from alphaviruses, such as Venezuelan Equine Encephalitis [148, 149], Semliki Forest virus [150, 151] and Sindbis virus [152, 153] and can replicate within a variety of cells, allowing them to produce more proteins of interest than conventional DNA vaccines. The lack of structural genes prevents the formation of viral particles and neutralizing antibodies against the viral capsid, thus enabling repeated administration. Furthermore, RNA replicons bypass the risks of integration with host genome and cellular transformation associated with DNA vaccines. However, the instability of RNA compared to DNA leads to difficulty in clinical translation of the vaccine.

Preclinical models have attempted to address this disadvantage by combining the stability of DNA with high expression of the encoded antigen by RNA replicons. A DNA-launched RNA replicon vaccine administers the easily prepared 'suicidal' DNA, which is transcribed into RNA replicons for abundant expression of encoded antigens. Since the cells transfected with the suicidal DNA eventually undergo apoptosis, there are no concerns for genomic integration or cellular transformation. Hsu *et al.* demonstrated that the use of 'suicidal' DNA vector generated a significant HPV antigen-specific $CD8^+$ T-cell immune response and antitumor effects in mice vaccinated with HPV suicidal DNA [154]. However, the suicidal vector also leads to poor immunogenicity because the transfected cells, such as DCs in gene gun delivery, undergo apoptosis. Fusion of E7 with BCL-xL, an antiapoptotic protein, delivered by pSCA1, a suicidal DNA vector, improves immunogenicity by enhancing antigen-presenting cell survival. The pSCA1 encoding E7 linked to BCL-xL generates stronger E7-specific $CD8^+$ T-cell immune responses and antitumor effects than the pSCA1 encoding wild type E7 [155].

Another strategy to prevent apoptosis involves delivering antigens of interest to target cells via a flavivirus, Kunjin (KUN). Since KUN does not induce apoptosis in transfected cells, KUN-based vaccines prolong antigen presentation and lead to improved immunogenicity compared to other RNA replicon vectors [156]. In murine models, vaccination with DNA-launched KUN replicons encoding HPV-16 E7 induced E7-specific T-cell responses and protected vaccinated mice against subsequent tumor challenges [157]. However, naked RNA replicon vaccines have not been tested in clinical trials yet despite their preclinical successes. Further research is needed to evaluate the efficacy and safety of RNA replicon-based vaccines before clinical translation.

D. Cell-Based Vaccines

1. Dendritic Cell-Based

Use of DC-based therapeutic vaccines can potentially target HPV-associated malignancies (for review, see [158]). Dendritic cell-based HPV vaccines are created by loading DCs with viral antigens *ex vivo* and delivering them to patients. Potential preparations include physically loading MHC class I and class II molecules of DCs with antigenic peptides or proteins, or transfecting DCs with viral DNA or RNA. The reintroduction of mature DCs bearing HPV antigens allows for more effective antigen presentation and thus a stronger immune response [159]. However, effective DC-based vaccines are expensive and difficult to produce on a large scale due to their autologous nature. Variant culturing techniques may lead to inconsistencies in vaccine efficacy and a lack of standard in vaccine evaluation. Furthermore, since antigen-presenting DCs must home to the lymphoid organs where the naïve T cells are located, delivery is crucial to maximize the effects of a DC-based vaccine.

Preclinical models have tried to determine the optimal conditions for preparation and delivery of DC-based vaccines. Effective loading of tumor antigens unto DCs can be achieved through gene delivery to DCs by targeting adenoviral vectors to CD40 with bispecific antibodies [160]. These DCs can then be delivered via the intramuscular, subcutaneous, or intravenous route. Intramuscular delivery has been shown to generate the most potent E7-specific adaptive immune response and the greatest anti-tumor effect [161]. Furthermore, methods such as downregulation of proapoptotic protein expression on DC cells [162, 163], targeting of antigen to endoplasmic reticulum [164] and increased MHC class I/II presentation [135] can further enhance DC-based vaccine efficacy.

Clinical studies have mainly evaluated DCs as a compassionate, salvage therapy and therefore have focused on feasibility and safety of DC vaccines in patients and immunological responses elicited [165-169]. For example, in a Phase I trial in 10 patients with Stage Ib or IIa cervical cancer, subcutaneous injections of autologous DCs pulsed with HPV-16/18 E7 protein and keyhole limpet hemocyanin (KLH) elicited antigen-specific responses, showing significant increases in E7-specific $CD4^+$ T cell responses in all ten vaccinated patients and E7-specific $CD8^+$ T cell response in six out of ten patients [168]. Overall, while immunologic responses have been observed, there have been a lack of objective clinical responses in terms of tumor regression or metastatic lesion regression, possibly due to the advanced stage of disease. Still, DCs have a promising future in that they play a vital role in stimulating a strong immune response. However, their labor-intensive procedure makes them inconvenient for mass production. Hence, strategies must focus on improving efficient loading of antigen to DCs and also to use adjuvant and fusion protein strategies to augment immunogenicity of DC-based vaccines.

2. Tumor Cell-Based Vaccines

Through *ex vivo* manipulation, tumor cells can enhance their immunogenicity by expressing immunomodulatory proteins, especially cytokines such as IL-2 [170, 171], IL-12 [172], and GM-CSF [171, 173]. Since tumor-cell-based vaccines do not require a clear identification of tumor antigens, they are more useful for cancers without well-defined tumor-specific antigens than cervical cancer, which has well-defined antigens.

Several tumor-cell-based HPV vaccines have been tested in preclinical models. Vaccination of mice with irradiated E6/E7-positive tumor cells expressing IL-12 significantly decreased the size of E6/E7-expressing tumors [174]. While

autologous and allogeneic tumor-cell-based vaccines have been tested in clinical trials of other cancers such as colon cancer, melanoma, and prostate cancer [175], there have been no such clinical trials against HPV-associated cancers. In addition, the tumor-based vaccine most likely will not be used in HPV-associated precursor lesions. For relatively healthy HPV patients with mild neoplasia, the risk of introducing new cancers via tumor cell-based vaccines is a major concern. Furthermore, individual autologous vaccines are costly and difficult to produce on a large scale without introducing variations in purity and efficacy [175]. As a result, tumor-cell-based vaccines have a limited future in HPV vaccine development.

III. COMBINED APPROACHES

A. Prime-Boost Regimens

Combinatorial vaccination using a prime-boost regimen is an attractive approach for cancer immunotherapy. It involves priming the immune system, then ideally augmenting and maintaining a long-term immune response with a booster vaccine. A preclinical trial showed that priming with a DNA vaccine encoding HPV-16 E7 and LAMP-1 (Sig/E7/LAMP1) followed by vaccinia boost of Sig/E7/LAMP1 significantly increases E7-specific T-cell responses compared to DNA vaccination alone [176]. DNA vaccines can also be boosted with recombinant adenovirus [177] or tumor cells expressing HPV 16 E6/E7 [178] to increase HPV antigen-specific CTL response in vaccinated mice compared to DNA vaccination alone. A prime-boost regimen of Sindbis virus RNA replicon expressing HPV-16 E7 and *M. tuberculosis* HSP70 (E7/HSP70) followed by a recombinant vaccinia virus encoding E7/HSP70 elicited significantly higher E7-specific CTL responses than other combinations, such as DNA prime-vaccinia boost regimens [179]. Other successful preclinical prime-boost studies include HPV-16 E7 protein prime followed by vaccinia boost [180] and HPV E6/E7 encoded by both the Venezuelan equine encephalitis virus replicon particles (VRP) prime and the recombinant vesicular stomatitis virus (VSV) boost [181].

Due to the success of prime-boost regimens in preclinical studies, several clinical trials have evaluated the safety and efficacy of these heterologous strategies [77-79]. In a Phase II clinical trial, priming with HPV-16 L2/E6/E7 protein (TA-CIN) vaccine was followed by boosting with vaccinia expressing HPV-16/18 E6/E7 (TA-HPV) in women with high-grade anogenital intraepithelial neoplasia (AGIN). The regimen induced E6-specific T-cell responses in 11 out 25 patients but an association between vaccine efficacy and clinical outcome remains inconclusive [77]. In another clinical study, women with high-grade, HPV-16 positive, vulvar intraepithelial neoplasia (VIN) were primed with TA-HPV and boosted with three TA-CIN booster immunizations. The prime-boost regimen elicited increased antigen-specific antibody response in 9 out of 10 patients and induced lesion regression in 2 out of 10 patients but showed no significant effect on CTL response [79]. A Phase I trial is currently underway studying the effect of a topical adjuvant, imiquimod, in addition to prime-boosting with pNGVL4a/Sig/E7(detox)/HSP70 DNA and TA-HPV, in patients with high grade cervical intraepithelial neoplasia lesions [124].

B. Immunomodulatory Therapy

Certain factors in the tumor microenvironment can inhibit antigen presentation and T-cell activation, and thus limit vaccine efficacy. Therefore, targeting negative regulators of T-cell activation, such as CTLA-4 and PD-1, may enhance the antitumor T-cell responses (for reviews, see [182, 183]). A combination of HPV therapeutic vaccine with such immunomodulatory agents can potentially enhance therapeutic effects against HPV-associated malignancies. Agents that inhibit immunosuppressive factors such as immunosuppressive cytokines such as IL-10 [184] and TGF-β [185], T-cell expression of PD-1 [186], T regulatory cells [187], myeloid-derived suppressor cells [188], constitutive STAT3 activation [189], and tumor cell expression of MIC-A and B [190], indoleamine 2,3-dioxygenase (IDO) [191], and galectin-1 [192] can potentially enhance therapeutic effects of individual HPV vaccines.

C. Combination with other Therapeutic Modalities

Therapeutic vaccines may be further enhanced through a combination with chemotherapeutic agents, topical adjuvants, or radiation. Apigenin is a chemotherapeutic agent that contains anti-carcinogenic properties and is abundant in common fruits and vegetables (for review, see [193]). Apigenin administered with therapeutic HPV DNA vaccine enhanced tumor cell apoptosis in a dose-dependent manner and E7-specific cytotoxic T cell activity, thus improving vaccine potency against E7-expressing tumors [194]. Another study also has shown epigallocatechin-3-Gallate (EGCG), derived from green tea, as an effective chemotherapeutic agent capable of inducing tumor cell apoptosis. A combination of EGCG and HPV-16 E7 DNA vaccine elicited stronger E7-specific T-cell responses and antitumor effects than either modality alone [195]. Combination of chemotherapy and adoptive E7-specific CTL transfer has also been shown to augment antitumor therapeutic activity *in vivo* [196].

The topical agent cidofovir, an acyclic phosphonate nucleoside, inhibits CXCR4 gene expression, E6/E7, cell invasion, and Rho/ROCK signaling in HPV tumor cells and enhanced anti-metastasis and therapeutic vaccine efficacy [197, 198] (for review, see [198]). A preclinical trial has demonstrated cidofovir's efficacy in preventing HPV tumor cell homing and invasion to inhibit metastasis [197]. A clinical study involving women with high grade cervical intraepithelial neoplasia showed lesion disappearance in 14 out of 23 patients after topical application of cidofovir with no significant reported adverse side effects [198]. Such agents can potentially be combined with therapeutic HPV vaccines.

Radiotherapy has also been considered as a method to improve therapeutic vaccine potency. In preclinical models, low dose radiation rendered tumor cells more susceptible to E7-specific $CD8^+$ T-cell activity and enhanced the antitumor effects of the combined HPV DNA vaccine [200]. Additional studies involving death receptor 5-specific antibodies [201] or proteasome inhibitor such as bortezomib [202] have also demonstrated improved therapeutic vaccine efficacy. The existence of a wide variety of methods to enhance immunogenicity of individual therapeutic vaccines and the general success of combined approached compared to mono-

therapy render multiple treatment regimens a promising therapeutic approach for treatment of HPV-related malignancies.

IV. PERSPECTIVES

The discovery of HPV as the etiologic agent of cervical cancer has been a major driving force in the development of HPV vaccines for cancer immunotherapy. While the commercial development of preventive HPV vaccines has been hailed as a major breakthrough, the critical challenge for public health remains in implementing cost-effective HPV vaccine programs in developing countries, where women are deprived of access to effective screening and treatment programs. The next generation of preventive vaccines and new generation of therapeutic HPV vaccines must aim to be cost-effective, stable for transport and storage, suitable for mass immunization, safe and highly effective in clearing existing HPV infection.

Significant progress has been made in the preclinical models, which have resulted in sevepral early phase clinical trials. However, it is important to pave the way for advanced phases of clinical trials to see the true efficacy of these vaccines. In advanced stages of cervical cancer, it is more difficult to treat with immunotherapy alone due to the potential immunosuppressive condition of patients. Therefore, the optimal approach for vaccination against cervical cancer will most likely involve the use of combinatorial strategies, including prime-boost regimens, immunomodulatory agents or other therapeutic modalities such as chemoradiotherapy and surgical debulking. Continuing progress into advanced phases of clinical trials is critical for the success of the therapeutic HPV vaccine. With ongoing advances in the development of HPV vaccines, we may some day accomplish the successful treatment of established lesions with therapeutic vaccines for the control of cervical cancer.

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