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Perspectives for Preventive and Therapeutic HPV Vaccines

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Abstract

Cervical cancer is the second most common cause of female cancer death worldwide. Persistent infection with `high risk' HPV genotypes is the major etiological factor in cervical cancer and thus effective vaccination against HPV provides an opportunity to reduce the morbidity and mortality associated with HPV. The FDA has approved two preventive vaccines to limit the spread of HPV. However, these are unlikely to impact upon HPV prevalence and cervical cancer rates for many years. Furthermore, preventive vaccines do not exert therapeutic effects on pre-existing HPV infections and HPV-associated lesions. In order to further impact upon the burden of HPV infections worldwide, therapeutic vaccines are being developed. These vaccines aim to generate a cell-mediated immune response to infected cells. This review discusses current preventive and therapeutic HPV vaccines and their future directions.

Keywords

HPV; therapeutic vaccines; preventive vaccines; HPV L1; HPV L2; HPV E6; HPV E7; cervical cancer

Introduction

Despite widespread implementation of cytological screening in many countries, cervical cancer represents a major cause of morbidity and mortality. Worldwide, cervical cancer is the second most common female cancer, claiming around 270,000 lives annually (1). Persistent infection with human papillomavirus (HPV) is the most important etiological factor in cervical cancer and its precursor lesions (cervical intraepithelial neoplasia, CIN), with HPV DNA being identified in more than 99% of cervical cancers (2). Although over 100 genotypes of HPV have been identified, only several are considered “high risk” due to their oncogenic potential, notably HPV-16 and 18 (3).

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HPVs are non-enveloped viruses containing a circular double-stranded DNA genome of around 8,000 base pairs, which preferentially infect squamous epithelial cells. The genome encodes at least six early genes (E1, E2, E4, E5, E6, E7) and two late genes (L1, L2). The early genes regulate viral DNA replication while the late genes encode the viral capsid for packaging newly synthesized virions (Fig 1). HPV infects the basal cells of the cervical epithelium through microtrauma; however, the majority of HPV infections are self-limiting and transient (4). In persistent infection, the expression of the HPV genome is correlated to the maturation of the infected cell.

Immature epithelial cells in the basal layer allow expression of the HPV early genes whereas in terminally differentiated cells, transcription shifts to the late genes, allowing the newly assembled virions to be released away from the submucosa, the site of immune surveillance. The HPV genome is usually found in episomal form in productive infection. However, high risk HPVs may integrate into the host genome in some persistent infections. This integration causes deletion of some of the early genes (E2, E4, E5) as well as the late genes L1 and L2. E2 is a master regulator of the viral genome and notably a transcriptional repressor of the E6 and E7 genes. Loss of E2 through integration allows upregulation of E6 and E7 transcription. E6 and E7 are oncogenes, capable of inactivating tumor suppressors p53 and retinoblastoma (Rb), leading to genomic instability and repression of apoptosis (review:(5)). HPV utilizes several mechanisms to avoid and modulate the immune system, allowing HPV to freely proliferate within cells. An understanding of these defense mechanisms, HPV virology and its role in tumorigenesis has facilitated the development of preventive and therapeutic vaccines to stimulate the immune system into responding to HPV. While preventive vaccines aim to block initial HPV entry into epithelial cells, therapeutic vaccines generate a T-cell immune response to eliminate existing HPV infection and HPV-associated neoplasms.

Preventive Vaccines

Current strategy in preventive vaccines utilizes the capsid proteins L1 and L2 as target antigens, inducing antibodies to neutralize and prevent entry of HPV into cells. Expression of recombinant L1, the major component of the capsid, in various cell types results in spontaneous assembly of virus-like particles (VLPs), which are immunologically and morphologically similar to HPV virions (6–8). Vaccination of animal models with L1 VLPs protects them against subsequent exposure to the homologous virus. The main focus of preventive vaccines has been on HPV types 16 and 18 which together account for around 70% of cervical cancers (9). Clinical trials of L1 VLP vaccines in seronegative healthy volunteers have proven their immunogenicity and safety, producing high titers of neutralizing IgG antibodies, up to 40 times those found in natural infection with HPV-16 (review:(10)).

Two preventive vaccines have recently been licensed for use: Gardasil and Cervarix (Table 1). Gardasil is a quadrivalent vaccine containing recombinant L1 VLPs for HPV genotypes 6, 11, 16 and 18 whereas the bivalent vaccine Cervarix contains L1 VLPs for HPV-16 and 18. The FDA's advisory panel has recently voted that Gardasil be approved for use in males to reduce HPV-associated cancers in males, prevent genital warts and reduce transmission to uninfected women (http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/BloodVaccinesandOtherBiologics/VaccinesandRelatedBiologicalProductsAdvisoryCommittee/ucm181365.htm). The seroconversation rates for Cervarix and Gardasil is 97.5% or higher in women (11–14). The antibody response generated is unfortunately type-restricted to those HPV genotypes contained within the vaccine. However, there is some low-level cross-protection against

other closely related genotypes (15). Despite this partial cross-protection, a preventive vaccine would need to contain the eight most common HPV types found in cancer to create >90% protection against cervical cancer – a costly and complex processes (16). Ongoing studies show continued protection for up to 6.4 years post vaccination with HPV-16 and 18 L1 VLPs, as well as some cross-protection to HPV 45 and HPV 31 (17). One monovalent HPV-16 L1 vaccine with an aluminium hydroxyphosphate sulfate adjuvant shows 86% of volunteers are seropositive at an average 8.5 years, where mean HPV-16 antibody titers were 71.7 mMU/mL (milli-Merk units [mMU]/mL) in contrast to 150 mMU/mL at 4 years (18, 19). It is estimated that a reduction in cervical cancer rates will not be witnessed until at least 20 years of mass vaccination due to the high prevalence in the population and slow process of carcinogenesis.

Unfortunately, L1 VLP vaccines are expensive and require repeat vaccination and specific conditions for storage. For example, the vaccines must be refrigerated and delivered via intramuscular injection, introducing several hurdles to mass vaccination in developing countries, which carry the highest burden of HPV-related disease (20). Second generation vaccines are attempting to broaden HPV type coverage, be thermo-stable, inexpensive and have needle-free administration methods while maintaining long-term protection with a single dose.

Future Prospects of Preventive Vaccines

The future generations of preventive vaccines must address two main issues: (1) lowering the cost in order to increase availability of the vaccine to developing countries and (2) to increase the number of HPV types covered in order to maximize protection against HPV-associated malignancies. An attractive approach to substantially reduce the cost of producing L1 vaccines is the employment of L1 capsomers. The current L1 vaccines, Cervarix and Gardasil, are produced in insect cells and yeast respectively. Production of the vaccine in *Escherichia coli* may be a cheaper option. Use of recombinant *E. coli* to produce these L1 capsomers has demonstrated success in inducing protective antibodies in animal models (21–23). Additionally, L1 capsomer vaccines are stable at room temperature, negating the need for refrigeration. Trials with VLP vaccines have investigated needle-free administration routes such as transdermal application (24) and nasal inhalation (25), which could be of practical use in future capsomer vaccines.

To overcome the genotype restriction of L1 vaccines, the highly conserved and thus cross-reactive L2 can be employed. L2-based vaccines can also be produced using *E. coli* to reduce costs and increase availability to the developing world. However, L2 vaccines are less immunogenic than their L1 counterparts, creating comparatively lower titers of neutralizing antibodies. This may be overcome through the use of strong adjuvants, such as TLR 2 agonists, providing a promising future vaccine (26). Another method of creating broader protection is through polyvalent L1 vaccines containing VLPs for several HPV types, for example Merck is currently recruiting for Phase II clinical trials of a nine-valent vaccine, V503 (http://clinicaltrials.gov/ct2/show/NCT00943 722).

Several factors highlight the need for a therapeutic, rather than preventive, vaccine. The most pressing of these factors is the high prevalence of existing HPV infection worldwide, on which preventive vaccines make little impact. Since over 80% of cervical cancer cases occur in the developing world, preventive vaccines would need to be in widespread use for many years to reduce this figure, which is currently improbable in the near future due to logistics and cost. Furthermore, in HPV-associated malignancies where genomic integration has occurred, infected cells may no longer express L1 or L2. To exert a therapeutic effect, a different vaccine target antigen is needed which is expressed constitutively in HPV-

associated tumor cells. Such a vaccine may exert an immediate effect on the mortality and morbidity of HPV-associated lesions.

Therapeutic Vaccines

The HPV E6 and E7 antigens represent ideal targets for therapeutic vaccines since these are constitutively expressed in HPV-infected cells and not healthy cells. E6 and E7 are essential to the induction and maintenance of cellular transformation, and thus are unlikely to be lost in an attempt to evade the immune system (for review, (see (5, 27)).

A number of therapeutic vaccines have been developed targeting E6 and E7 including live vector vaccines, peptide/protein-based vaccines, cell-based vaccines and nucleic acid-based vaccines, each with advantages and disadvantages (Table 2). These vaccines likely control HPV infection through cell-mediated immunity (Fig 2) and many have shown promise in both preclinical and clinical trials.

1. Live Vector-Based Vaccines

Live vector-based vaccines encompass both bacterial and viral vectors, many of which are available depending on the desired effect. These vectors are highly immunogenic as they replicate within host cells and facilitate spread of antigen. Vector-based vaccines can deliver the antigens E6 and E7 to the dendritic cells (DCs), stimulating antigen expression through MHC class I (to CD8+ cytotoxic T cells) and MHC class II (to CD4+ helper T cells). However, live vectors inherently pose a potential safety risk, especially to immunocompromised individuals. Another disadvantage is the generation of neutralizing antibodies, limiting the efficacy of repeat immunization, as well as the possibility of pre-existing immunity to the vector being employed.

Bacterial Vectors

Attenuated bacteria can deliver genes or proteins of interest, such as E6 and E7, to antigen-presenting cells. Various bacterial vectors have been explored in HPV therapeutic vaccines including *Listeria monocytogenes (28, 29)*, *Lactobacillus lactis (30, 31)*, and *Lactobacillus plantarum (32)*. *Listeria* is a promising vector due to its ability to infect monocytes and macrophages and secrete listeriolysin O, allowing evasion from phagosomes. Literiolysin O allows *Listeria* to be present in both the cell cytoplasm and endosomal compartments, resulting in antigen presentation through both MHC class I and II pathways, inducing CD4+ and CD8+ immune responses (33). In preclinical trials, *Listeria*-based E7 vaccines were shown to cause regression of implanted solid tumors in HPV-16 E6/E7 transgenic mice (29). Currently, a clinical trial is ongoing with a *Listeria*-based vaccine for HPV-16 E7 (Lovaxin C) in women with advanced cervical cancer (34). *Listeria*–based vaccine potency can be further enhanced by the means of encoding recombinant proteins composed of HPV E6/E7 antigen fused to immunostimulatory molecules, for example, through fusion of lysteriolysin O with E7 (35). Recently, Maciag et al. utilized this method and reported the first clinical use of a live-attenuated *Listeria monocytogenes* vaccine that secretes HPV-16 E7 antigen fused to a fragment of listeriolysin O (*Lm*-LLO-E7) (36). In this Phase I safety clinical trial, *Lm*-LLO-E7 infusion was found to be safe and well-tolerated in end stage cervical cancer patients who had failed prior chemotherapy, radiotherapy and/or surgery. Hence, there is potential for bacterial vectors to not only serve as vaccine vectors but possibly as cancer immunotherapeutics as well.

Viral Vectors

Highly efficient infection rates and expression of encoded antigen make viral vectors a feasible option in therapeutic HPV vaccines. Viruses that have been used include adenovirus

(37), adeno-associated virus (38), vaccinia virus (39) and alphaviruses, such as the Venezuelan equine encephalitis (VEE) virus (40, 41).

Vaccinia's large genome and highly infectious nature make it a promising vector. Vaccinia-based vaccines include vaccinia encoding fusion of E7 and calreticulin (CRT) to enhance MHC I processing in DCs (39, 42), and vaccinia encoding E7 and listeriolysin O (42), to facilitate MHC I and II presentation. Phase I/II clinical trials have shown that a recombinant vaccinia virus expressing the HPV-16 and 18 E6 and E7 fusion protein, TA-HPV, induces potent antigen-directed antibody and cytotoxic responses in patients with CIN III and cervical cancer (43–45).

Adenoviruses have also shown promising results. A recent study demonstrated that vaccination with replication-deficient adenoviruses encoding CRT/E7 fusion protein conferred immunity to an E7-expressing tumor challenge, and eradicated established tumors in mice (37). Creation of an adenovirus vaccine encoding a fusion protein of Hepatitis B surface antigen (HBsAg) and HPV-16 E7 has been described. Vaccination with adenovirus vector encoding HBsAg/E7 fusion protein stimulated E7-specific antibody and CD8+ T cell immune responses in vaccinated mice (46). The adeno-associated viruses can also be engineered to express E7 linked to *Mycobacterium tuberculosis* heat shock protein 70 (hsp70) enhancing MHC class I antigen processing in a number of ways, such as through translocation of E7 to sub-cellular compartments, inducing CD8+ T cell immune responses (47, 48).

RNA viruses, such as the Semliki Forest virus (SFV) can also be utilized as live vectors. SFV expressing HPV-16 E7 can induce E7-specific cytotoxic T cells in HPV-transgenic mice (49). This response can be enhanced through the co-administration of interleukin-12, inhibiting tumor angiogenesis (50, 51).

The future of therapeutic live vector-based vaccines requires identification of efficient bacterial and viral vectors and enhancement of the immunogenicity of these vector-based vaccines through expression of cytokines or co-stimulatory molecules. A further hurdle to overcome is the generation of neutralizing antibodies upon first exposure to the vaccine; a means to prevent this is required to allow effective repeated administration. A recent study showed that COX-2 inhibitors, such as Celecoxib, can prevent the generation of neutralizing antibodies to vaccinia, allowing repeated administration without losing infectivity, a promising advance (52).

2. Peptide/Protein-based Vaccines

Administered peptides and proteins derived from HPV antigens are taken up by DCs, processed and expressed via MHC II and/or I to the appropriate CD4+/CD8+ T cell.

Peptide-based Vaccines

Peptide vaccines are safe, stable and easy to produce. However, widespread use is restricted by the necessity to identify immunogenic epitopes corresponding to the polymorphic MHC molecules within the population. This can be partially overcome through the use of overlapping long peptides that contain several epitopes of E6/E7. Preclinical studies in mice and rabbits have shown that long E6/E7 peptides are able to induce antigen-specific T cells in all animal subjects (53, 54).

Due to the poor immunogenicity of peptide vaccines, adjuvants such as chemokines, cytokines and Toll-like receptor ligands, must be simultaneously administered. Examples of these include GM-CSF to activate DCs (54), the co-stimulatory 4-IBB ligand (55), mutant

cholera toxin (56) and CpG oligodeoxynucleotides (CpG ODN), which mimic bacterial danger signals (57, 58).

Despite these limitations, several peptide vaccines have advanced to clinical trials. Phase I trials in end-stage cervical cancer patients employing HPV-16 E6 and/or E7 long peptides with the adjuvant Montanide ISA-51 show a significant E6-specific T cell response (59). A similar study using HPV-16 E6 and E7 long peptides with Montanide ISA-51 in women with HPV-16+ cervical cancer induced E6-specific CD4+ and CD8+ immune responses in all 6 patients, and E7-specific CD4+ and CD8+ immune responses in 5 out of 6 patients, an encouraging result (60).

The future of peptide-based vaccine relies on augmentation of their immunogenicity and epitope enhancement to prevent degradation and thus prolong antigen presentation. Furthermore, targeting delivery specifically to DCs will additionally increase antigen presentation, for example, using liposomal vehicles, which also act as a potent adjuvant. A recent study utilizing this method created an E7 lipopeptide vaccine, which greatly enhanced the E7-specific CD8+ activity in TC-1 tumor-bearing mice compared to E7 peptide alone (61).

Protein-based Vaccines

Protein-based vaccines contain all antigenic epitopes, circumventing the MHC-restriction limitation associated with peptide vaccines. However, while protein-based vaccines are safe, they suffer low immunogenicity. Strategies to improve their potency are similar to those employed in peptide-based vaccines. Unfortunately, due to their exogenous nature, protein-based vaccines are presented via MHC class II pathway and thus predominantly generate an antibody response, rather than a T cell response, necessitating strategies to create a predisposition for the MHC I presentation pathway.

Adjuvants trialled include liposome-polycationic-DNA (LPD) carrier particles (62) and the saponin-based ISCOMATRIX (63), both which enhance endogenous processing and thus MHC I expression of antigen. Creation of fusion proteins to target the antigen to DCs can increase MHC I presentation and thus CD8+ responses. Examples of these include HPV-16 E7 linked to *Bordetella pertussis* adenylyl cyclase (CyaA), which interacts with the DC's integrin receptors (64), and fusion of the translocation domain of *Pseudomonas aeruginosa* exotoxin A with HPV-16 E7 to target E7 to the MHC class I presentation pathway (65). A similar strategy includes fusion of antigen to heat shock proteins (hsp) that act as molecular chaperones to target antigen to DCs for cross-priming, stimulate DC maturation and induce cytokines (66). Such examples include a fusion of *Mycobacterium* hsp65 with HPV-16 E7 (HspE7) (67) and a fusion of hsp 70, CRT and HPV-16 E7 (68).

A clinical trial of HspE7 in 58 women with CIN III generated a complete pathologic response (defined as a LEEP specimen being negative for CIN) in 13 and a partial clinical response (defined as a colposcopic lesion regression of >50% based on measurements made on a grid form) in 32 women. However, it is difficult to determine if this observed regression was contributed by spontaneous regression (69).

Another protein-based vaccine that has progressed to clinical trials is TA-CIN, containing a fusion protein composed of HPV-16 L2, E6 and E7. Injection of the vaccine into 40 healthy volunteers induced antibody response to L2 in all, and T cell-mediated responses to HPV-16 E6 and E7 in 8 of the 11 patients receiving the highest dose (70). The inclusion of L2 within this vaccine offers a new step in the evolution of HPV vaccines by combining both preventive and therapeutic vaccines. In general, the future of protein-based vaccines relies

upon enhancement of immunogenicity and CD8+ T cell response through adjuvant and fusion protein strategies.

3. Whole Cell Vaccines

Dendritic Cell-based Vaccines

Circumvention of HPV-induced immunosuppression can be achieved by delivering antigenic peptides directly to DCs in those with HPV-associated lesions. In such a setting, DCs act as natural adjuvants (review:(71)). Unfortunately the preparation involved is costly and time-consuming and consequently widespread use is currently impractical. A lack of agreed standards and culturing techniques for the generation of such vaccines adds a further challenge. Various methods employed in preparing DCs *ex vivo* include the usage of viral vectors (72, 73), transfection with DNA or RNA encoding antigen (74, 75) and pulsation of DCs with antigenic protein, peptide or tumor cell lysates (76–79).

T-cell mediated apoptosis limits the lifespan of DCs and their ability to prime T cells. Therefore, methods to prolong DC survival enhance antigen-specific responses. One way to achieve this is through transfection of DCs with siRNAs intended to interfere with the expression of pro-apoptotic molecules. DCs loaded with E7 and transfected with siRNA targeting the pro-apoptotic Bak and Bax proteins generate enhanced E7-specific CD8+ activation and antitumor effects in mice (77). Similarly, E7-presenting DCs transfected with siRNA to Bim, Bid, Bak, Bax and caspase 8 found that siRNA to Bim generated the strongest E7-specific CTL response in mice (78).

In clinical trials, autologous DCs loaded with HPV-16 or HPV-18 E7 antigen were administered to women with HPV-16+ or HPV-18+ late-stage cervical cancer respectively. E7-specific T cell responses were present in 4 out of 11 patients (80). A similar study of DCs loaded with HPV-16 or HPV-18 E7 co-administered with IL-2 in HPV-16/18+ refractory cervical cancer patients showed E7-specific CD4+ responses in 2 of 4 patients and E7-specific CD8+ responses in all 4 patients (81). An ongoing clinical study using DC-based vaccines with HPV-16 E7 is currently underway in patients with HPV-16+ recurrent cervical cancer at the National Taiwan University Hospital (http://clinicaltrials.gov/ct2/show/NCT00155766?term=HPV%2C+DC&rank=1).

Antigen-loaded DCs must travel to lymphoid organs in order to prime T cells and as a result, the route of administration of DC-based vaccines is an important issue. Methods previously used include intramuscular, subcutaneous, intravenous and intranodal delivery. Improvement strategies for future generations of DC-based vaccines include elucidating the most effective delivery route and developing methods to enhance antigen loading and prolong DC survival.

Tumor Cell-based Vaccines

Isolating and manipulating tumor cells *ex vivo* to express immunomodulatory proteins can enhance their immunogenicity *in vivo*. The cytokines IL-2 (82), IL-12 (83, 84) and GM-CSF (84, 85) have been trialled in mice with HPV-16 induced tumors. Clinical studies have not yet begun for HPV-associated tumor-based vaccines, although tumor-based vaccines have undergone clinical trials in melanoma, pancreatic cancer and renal cell carcinoma (review: (86)). These vaccines are advantageous in that tumour antigens do not have to be identified. However, we already hold this knowledge for cervical cancers, limiting the usefulness of this approach for the development of cervical cancer vaccines.

Some reluctance surrounds tumor-based vaccination due to the risk of seeding new cancers in patients, preventing clinical trials in healthy individuals or those with mild CIN. Due to

the nature of these vaccines, their potency and purity may be inconsistent and must be individualized, creating additional problems for clinical studies.

4. Nucleic Acid-Based Vaccines

DNA-based Vaccines

Naked DNA is safe, stable, relatively easy to manufacture on a large scale at high purity and capable of sustaining antigen expression in cells longer than RNA or protein vaccines. DNA vaccines can be repeatedly administered, as they do not generate neutralizing antibodies. Although no supportive evidence currently exists, there is a risk that DNA vaccines could integrate into the host genome. Furthermore, administering HPV E6 and E7 DNA may cause cellular transformation as they are oncogenes. However, this is addressed through modification of E6 and E7 DNA into proteins incapable of oncogenic transformation.

An important limitation of DNA vaccines is their intrinsic low immunogenicity due to an inability to amplify or spread from transfected cells into surrounding cells *in vivo*. To overcome this, strategies to enhance DNA vaccine potency have been developed, taking into consideration the central role that DCs play in vaccine-mediated immunity. Strategies include i) increasing numbers of DCs expressing antigen, ii) enhancing antigen processing and presentation in DCs and iii) improving the interaction between DCs and T cells (for review, see (87, 88)).

i) Increasing the antigen-expressing/antigen-loaded DC population

Delivery methods targeting DNA directly to areas rich in DCs increase the population of DCs presenting the antigen. Intradermal administration via gene gun ballistically delivers gold particles coated in DNA directly to the immature DCs of the skin, the Langerhan cells. This route of administration is convenient and a potent method of DNA delivery. Head-to-head comparison shows that the gene gun requires the lowest dose to generate a comparable antigen-specific CD8+ T cell immune response, compared to the biojector or intramuscular injection (89). Unfortunately, the DNA dose that can be delivered with each shot of the gene gun is limited (90), which may necessitate multiple administration sites, risking local side effects. Another effective administration method is the combination of intramuscular injection with electroporation. Electroporation enhances DNA uptake through the application of a small electric current, creating large numbers of muscle cells expressing the desired antigen and increasing release of antigen, which local DCs can then process and present through the MHC class I pathway. Electroporation can also induce cytokine release, creating a favourable environment for the DCs. A Phase I trial is currently underway delivering VGX-3100, a DNA vaccine targeting HPV-16 and 18 E6 and E7, via intramuscular injection and electroporation in patients with a diagnosis of CINII/III (http://clinicaltrials.gov/ct2/show/NCT00685412?term=VGX-3100&rank=1). A recent study comparing several methods of DNA administration found the highest numbers of E7-specific CD8+ were produced through intramuscular injection with electroporation (91). Other novel methods to enhance DNA delivery include laser (92) and microencapsulation of DNA (93).

Since DNA vaccines are unable to spread between cells, the linkage of HPV antigen with proteins capable of intercellular transport in the context of DNA vaccination allows this spread of antigen in cells transfected with DNA. One example is the use of DNA encoding both HPV-16 E7 and herpes simple virus type 1 VP22 (HSV-1 VP22), which has been shown to have intercellular trafficking properties. Although questions have been raised as to whether VP22 transports DNA between cells, or if this is a fixation artifact, vaccinated mice unequivocally generate around a 50-fold increase in the number of E7-specific CD8+ compared to vaccination with wild-type E7 DNA (94). Further strategies to enhance DNA-

encoded antigen uptake by DCs include linkage of HPV antigen to molecules that target the antigen to the DC surface, such as FMS-like tyrosine kinase 3 (flt3) ligands (95) and heat shock protein (hsp), which binds with scavenger receptors on DCs, such as CD91 (89, 96).

ii) Improving antigen expression, processing and presentation in DCs

Antigen expression can be increased through codon optimization, which replaces codons infrequently used by the host cells with more commonly used codons to enhance translation of the encoded antigens in cells transfected with DNA. In preclinical mouse models, CD8+ T cell immune responses and antitumor effects are enhanced through codon optimization of HPV DNA vaccines (97–100).

A second strategy to enhance antigen expression is the application of demethylation agents. There is reduced expression of DNA when methylated, and thus demethylating agents upregulate gene expression. A DNA vaccine encoding calreticulin (CRT) plus E7 combined with the demethylation agent 5-aza-2'- deoxycytidine (DAC) upregulates CRT/E7 expression in mice, enhancing anti-tumor effects against an E7-expressing tumor (101).

Antigen-specific CD8+ T cell responses rely upon presentation of antigen via MHC class I on DCs. To increase MHC class I processing, HPV E7 DNA can be linked with molecules that localise antigen to the endoplasmic reticulum (102) or that facilitate proteasome degradation (103). Other MHC I targeting proteins trialled in HPV DNA vaccines to enhance cross-priming include *M. tuberculosis* hsp70 (104), calreticulin (105–107), the heat shock protein Gp96 (108), the translocation domain of *Pseudomonas aeruginosa* exotoxin A (109) and γ-tubulin which targets HPV antigen to the centrosomal compartment, rich in proteasomes (110). All of these show improvement in MHC I presentation of the HPV E6/E7 antigen, inducing potent CD8+ T cell immune responses to the DNA vaccines.

MHC I single-chain trimer (SCT) technology can be utilized to circumvent antigen processing and presentation altogether. DNA vaccines encoding antigenic peptide is linked to β2-microglobulin and MHC class I heavy chain genes. The gene encoding the SCT is transcribed and expressed on the DC surface as MHC I molecules already loaded with the desired peptide. HPV-16 E6 SCT vaccines greatly increase E6-specific CD8+ T cell immune response in vaccinated mice, protecting them from a lethal challenge of E6-specific TC-1 tumor cells (111).

MHC class II processing can also be enhanced, resulting in greater CD4+ T cell responses to augment CD8+ T cell responses. DNA vaccine encoding E7 antigen linked to the sorting signal peptide of lysosomal-associated membrane protein 1 (LAMP-1) has been shown to generate greater numbers of E7-specific CD4+ and CD8+ cells and antitumor effects in vaccinated mice than wild type E7 (112). A second way to target antigen through the MHC class II processing pathway is through the use of the invariant chain (Ii). Substitution of the CLIP region of Ii, which normally occupies the antigen peptide-binding groove of MHC II to prevent premature binding of MHC class II molecules to antigenic peptides, with a T helper epitope such as the pan-DR helper T lymphocyte epitope (PADRE), allows presentation of PADRE via MHC II (113). A DNA vaccine encoding this (Ii-PADRE) generates significant PADRE-specific CD4+ responses in vaccinated mice and co-administration of Il-PADRE DNA with HPV E7 DNA elicits potent E7-specific CD8+ responses compared to E7 DNA co-administered with unmodified Ii (113). Recent advances show that both MHC I and II expression are regulated by CIITA, and thus administering DNA for CIITA with CRT/E6 DNA leads to enhanced E6-specific CD8+ T cell immune responses in vaccinated mice, which can be further improved with co-administration of Ii-PADRE DNA (114).

iii) Enhancing DC and T cell interaction

Strategies to enhance DC and T cell interaction may rely upon prolonging DC survival, increasing DC expression of cytokines and blocking negative regulation of DC activation.

Once DCs have primed naive T cells, they become the targets of these effectors cells and undergo apoptosis. Inhibiting this T cell-mediated apoptosis allows DCs to prime a greater number of T cells and can be achieved through the use of anti-apoptotic proteins (Fig 3A). For example, DNA encoding E7 co-administered with DNA for inhibitors of apoptosis, such as BCL-xL, BCL-2, X-linked inhibitor of apoptosis protein (XIAP) and dominant-negative capsases has been shown to enhance E7-specific CD8+ responses in mice (115). However, introduction of DNA encoding anti-apoptotic proteins raises concerns of oncogenicity. This may be alleviated through the use of short interfering RNA (siRNA) to instead transiently silence the expression of pro-apoptotic proteins. SiRNA targeting the key proapoptotic proteins Bak and Bax with the E7 DNA vaccine improves DNA-transfected DC resistance to apoptosis and enhances CD8+ antitumor effects in mice (116). A recent study established that DNA encoding connective tissue growth factor (CTGF) linked to E7 can prolong survival of DCs, generating potent E7-specific antitumor responses without any oncogenic risk (117).

Preventing activated T cell apoptosis is another way to enhance overall CD8+ responses (Fig 3B). The Fas ligand (FasL) found on the surface of DCs is a pro-apoptotic signalling protein that binds to the Fas receptor on T cells causing them to undergo apoptosis. Creation of DNA encoding small hairpin RNA (shRNA) to block FasL allows co-administered E7 DNA to generate significant E7-specific CD8+ responses and antitumor effects in vaccinated mice (118).

Enhancing stimulatory cytokine release from DCs further improves DC and T cell interaction. DNA-encoded cytokines can be included within the E6/E7 DNA vaccine, for example GM-CSF (119), IL-2 (120) and IL-12 (121). Combining HPV E7 DNA vaccines with DNA encoding sequence-optimized (as opposed to wild type) IL-2 and IL-12 as adjuvants caused tumor regression in mice through E7-specific CD8+ responses (100).

Several DNA vaccines have translated to clinical trials. A microencapsulated DNA vaccine encoding several HLA-A2-restricted HPV-16 E7 epitopes (ZYC-101) has been tested in patients with HPV-16+ CIN II/III, causing complete histological response in 5 of the 15 women and E7-specific T cell responses in 11 of the 15 patients (122). An updated version of this, ZYC-101a, contains HPV-16 and HPV-18 E6 and E7-derived epitopes and was studied in a Phase II trial in patients with CIN II/III lesions providing resolution in 70% of those patients younger than 25, although this may be attributable to spontaneous resolution (123). One DNA vaccine encoding HPV-16 E7, modified through the abolition of the Rb-binding site, was linked to *M tuberculosis* hsp 70 and administered to women with CIN II/III. Results revealed that those receiving the maximum dose had detectable E7-specific CD8+ T cell responses and complete histological regression was observed in 3 of the 9 women receiving the highest dose (124). Plans are underway to initiate a Phase I trial with a DNA vaccine encoding this modified E7 linked with CRT (CRT/E7 detox) in patients with high grade CIN through use of a clinical-grade gene gun (Huh and Trimble, personal communication).

Naked RNA replicon vaccines

Naked RNA replicon vaccines provide a new and interesting approach to HPV vaccination. RNA replicons can be derived from alphaviruses, such as the Sindbis virus (125, 126), Semliki Forest virus (127) and Venezuelan Equine Encephalitis virus (41, 128). Self-replication of the RNA replicon allows a sustained level of antigen expression, enhancing

immunogenicity and making them superior to DNA vaccines in this manner. The replicon vectors are modified to exclude viral structural genes, preventing production of viral particles and ensuring safe administration. This also allows repeat administration without the generation of neutralizing antibodies.

Unfortunately RNA is less stable than DNA. Attempts to overcome this have used more stable `suicidal DNA', which is translated into RNA replicons within the transfected cell. Transfected cells eventually undergo apoptosis, alleviating concerns of possible genomic integration and cellular transformation, an anxiety associated with DNA vaccines. Despite this advantage, the apoptosis leads to poor immunogenicity in DCs directly transfected with RNA replicons. Apoptosis can be delayed by suicidal DNA encoding the E7 antigen linked to anti-apoptotic proteins, such as BCL-xL, which in mice produces significantly higher E7-specific CD8+ T cell immune responses than wild type E7 alone, due to prolonged survival of DCs (129). Another strategy to overcome the problem of apoptosis is to exploit the flavivirus Kunjin (KUN) vector to deliver replicons. The advantage of KUN is that it does not induce apoptosis in transfected cells, prolonging antigen presentation by DCs. DNA-launched KUN replicons encoding HPV-16 E7 have been shown to generate E7-specific T cell responses and protect mice against a challenge of E7-expressing tumor (130).

RNA replicon-based vaccines can be enhanced through employment of intercellular spreading and intracellular targeting strategies as utilised in DNA based vaccines (126, 131, 132). Despite the relative success of RNA replicon vaccines in preclinical models, there has not yet been progression to clinical trails.

5. Combinational Approach

Prime-Boost Regimen

The variety of therapeutic vaccines available creates opportunities to enhance overall potency through prime-boost regimens. For example, an initial priming HPV-16 E6/E7 DNA vaccine can be followed by a boost with recombinant vaccinia (133), adenovirus (134) or with HPV-16 E6/E7 expressing tumor cell-based vaccine (135) eliciting greater HPV-specific CD8+ T cell responses than the vaccines delivered alone. Several prime-boost studies in mice have shown significantly increased E7-specific CTL responses, for example through priming with a Sindbis virus RNA replicon containing HPV-16 E7 linked with hsp70 (E7/hsp70) and boosting with a recombinant vaccinia virus encoding E7/hsp70 (136).

Prime-boost regimens have been evaluated in therapeutic clinical trials. TA-CIN (HPV-16 L2/E6/E7 fusion protein vaccine) has been boosted with a recombinant vaccinia virus encoding HPV-16/18 E6/E7 fusion protein (TA-HPV) in patients with anogenital intraepithelial neoplasia. Increases in HPV-16 antigen-specific T cell mediated immune responses were shown in 5 out of 29 patients (137, 138). However, this is not a significant advantage over TA-HPV alone as no additional efficacy is observed (137). A second study using TA-HPV followed by TA-CIN in 10 women with HPV-16+ high grade vulvar intraepithelial neoplasia reduced lesion size in 3 patients and created HPV-16 antigen-specific T cell responses in 9 of the 10 vaccinated patients. Unfortunately, there is no correlation between immunological and clinical responses (139). A clinical trial using a DNA vaccine encoding a signalling peptide (Sig), the mutated E7 antigen (E7(detox)) and hsp70 (i.e. pNGVL4a/Sig/E7(detox)/Hsp70), boosted with TA-HPV is currently in progress at Johns Hopkins University in women with CIN II/III lesions (http://clinicaltrials.gov/ct2/show/NCT00788164?term=pNHVL4a-Sig%2FE7%28detox%29%2FHSP70%rank=2).

HPV Therapeutic Vaccines with other Therapies

Combinational approaches employ HPV therapeutic vaccines in addition to other therapeutic modalities such as chemotherapy, radiotherapy or biotherapeutic agents have been described. For example, agipenin, a chemotherapeutic agent that induces apoptotic tumor cell death in vitro in a dose-dependent manner has been tested in conjunction with HPV DNA vaccines. Mice bearing E7-expressing tumors treated with apigenin combined with HPV E7 DNA vaccines show enhanced E7-specific CD8+ responses and potent antitumor effects as apigenin increases tumor cell susceptibility to the CD8+ cells (140). Low-dose radiotherapy has also been combined with therapeutic HPV DNA vaccines (CRT/E7(detox)) to control E7-expressing tumors in TC-1 tumor-bearing mice (141)

Tumor Microenvironment

Effective immunotherapy for HPV-associated lesions must consider modulation of the tumor microenvironment, which may be hindering the success of therapeutic vaccines. For example, T regulatory cells release immunosuppressive cytokines, such as IL-10 (142) and TGF-β (143) in the microenvironment, which can paralyze T cell function, preventing clearance of HPV-associated lesions. Depletion of T regulatory cells from the tumor microenvironment significantly enhances the potency of therapeutic HPV DNA vaccines (144). The tumor also induces a state of immunosuppression through B7 homolog-1 (B7-H1) (145), signal transducer and activator of transcription 3 (STAT-3) (review:(146), the enzyme indoleamine 2,3-dioxygenase (IDO) (147), galectin-1 (148) and MHC class I polypeptide-related sequence A and B (MICA/MICB) (149). Each of these are potential targets of immune modulation which may enhance therapeutic effects of HPV vaccines in the future (for review, see (150)).

Summary

While the approval of Gardasil and Cervarix preventive HPV vaccines represents a breakthrough in the development of HPV immunotherapy, the much-needed therapeutic vaccines require further development before full-scale implementation. The high prevalence of HPV malignancies and HPV-associated lesions worldwide represents a pressing need for effective therapeutic HPV vaccines. Further study into the tumor microenvironment and molecular mechanisms impeding immune attack against HPV will lead to novel targets for therapeutic intervention in the future. Discovery of such targets, development of new adjuvants, and improved understanding of tumor biology will allow HPV vaccines to be used in combinational therapies in a synergistic manner in the future.

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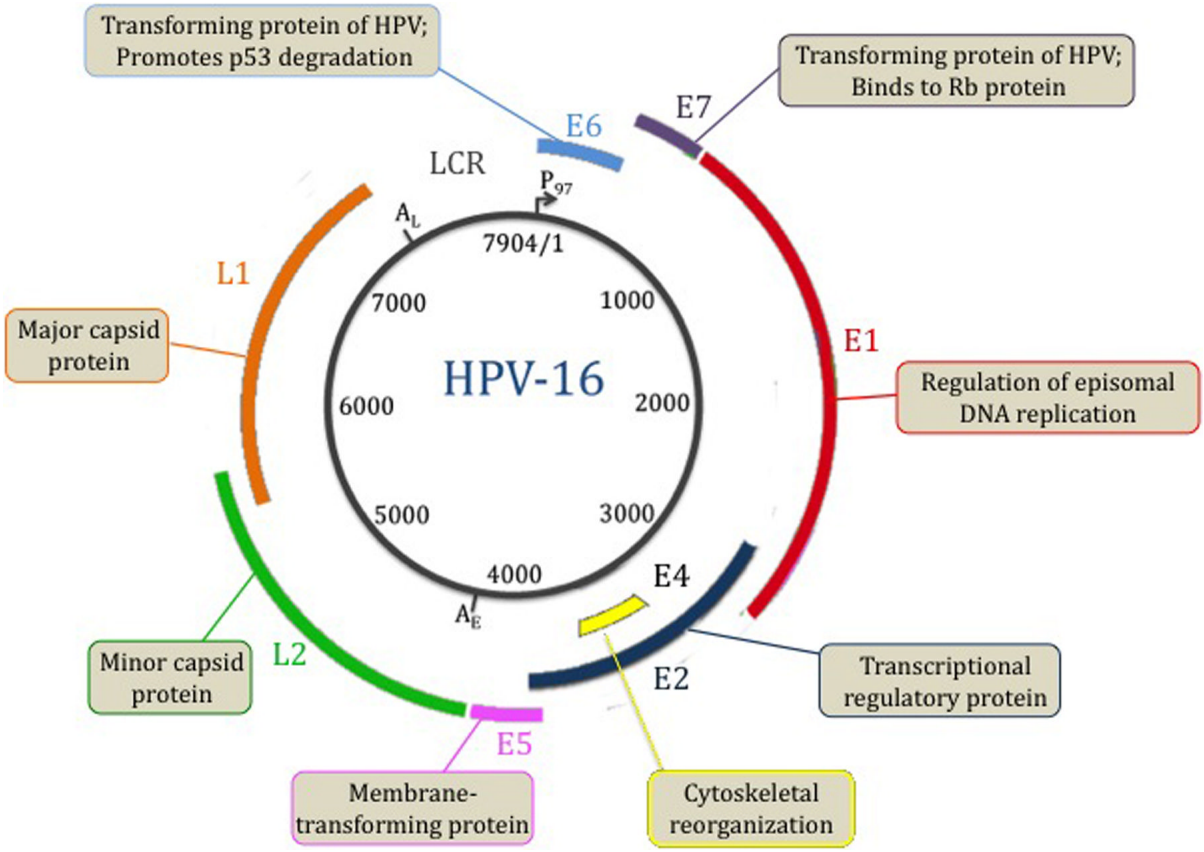

Figure 1.

HPV-16 genome and protein function. HPV-16 has a 7904 base pair, double-stranded circular DNA genome. The transcriptional promoter is designated P_{97}. A_E and A_L are the early and late polyadenylation sites, respectively. The viral long control region (LCR) contains transcriptional and replication regulatory elements. The HPV-16 genome contains six early genes (E1, E2, E4, E5, E6, E7) and two late genes (L1, L2). The late genes comprise the viral capsid while the early genes are involved in viral replication. E1 regulates episomal viral DNA replication. E2 is a transcriptional regulator of E6 and E7. E4 is involved in cytoskeletal reorganization. E5 is involved in cellular transformation. E6 and E7 are responsible for the induction of malignant transformation by binding to p53 and retinoblastoma (Rb) protein, respectively.

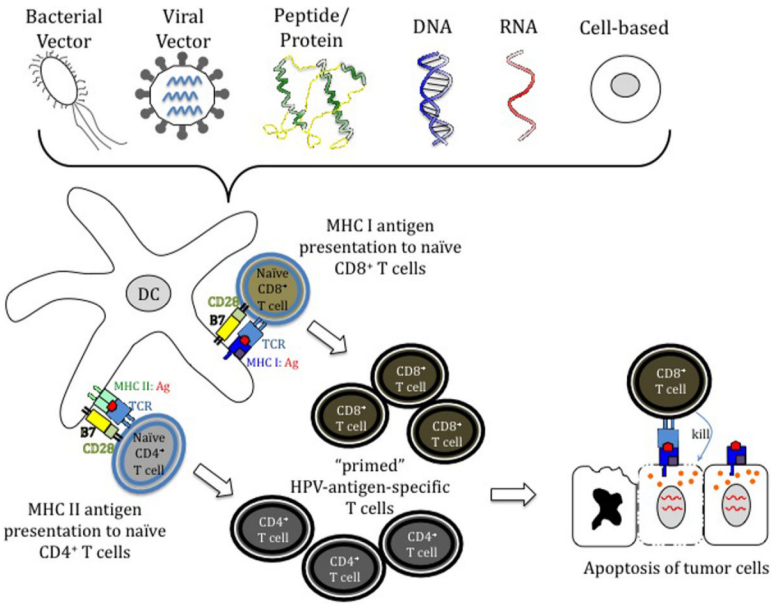

Figure 2.

Therapeutic HPV vaccines. A number of therapeutic vaccines have been developed targeting HPV E6 and/or E7 antigen(s), including live vector-based vaccines, peptide/protein-based vaccines, nucleic acid-based vaccines and cell-based vaccines. These vaccines likely control HPV infection through cell-mediated immunity. Dendritic cells (DCs) prime naïve T cells through MHC:Antigen (Ag) complex with the help of costimulatory molecules (B7 on the DC and CD28 on the T cell). Antigens are processed and presented to CD4+ T cells via MHC class II pathway and presented to CD8+ T cells via MHC class I pathway. The primed effector T cells are subsequently HPV-antigen-specific T cells. Activated CD8+ T cells kill tumor cells by inducing apoptosis in the target cells. Induction of CD4+ T cell help can augment the CD8+ T cell immune response, supplementing tumor killing.

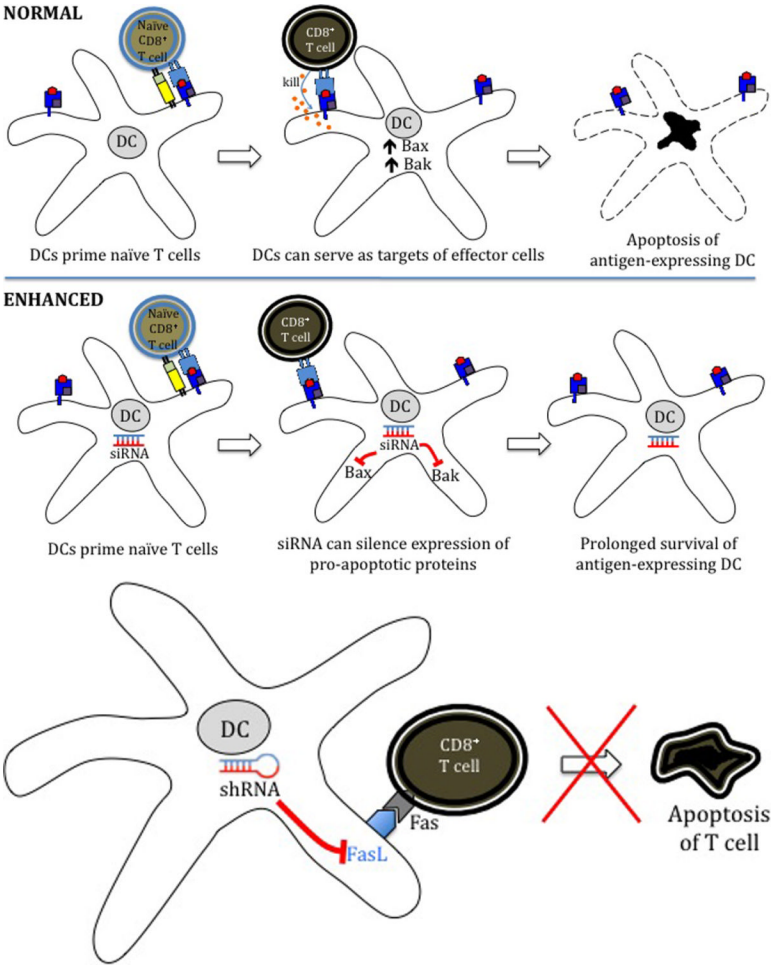

Figure 3.

Strategies to improve DC-T cell interaction in enhancing therapeutic DNA vaccine potency. (A) Prolonging DC survival. Once DCs have primed naïve T cells, they can become the target of these effector T cells. The use of short interfering RNA (siRNA) targeting key pro-apoptotic proteins such as Bak and Bax can transiently silence expression of Bak and Bax, improving DNA-transfected DC resistance to apoptosis and improving DC-T cell interaction. (B) Prevention of activated CD8+ T cell apoptosis. The Fas ligand (FasL) found on the surface of DCs is a pro-apoptotic signalling protein that binds to the Fas receptor on T cells, causing them to undergo apoptosis. Creation of DNA encoding small hairpin RNA (shRNA) to block FasL can prevent apoptosis of activated T cells and improve DC-T cell interaction.

Table 1

Comparison of Cervarix and Gardasil preventive HPV vaccines

	Cervarix	Gardasil
Manufacturer	Merck & Co	GlaxoSmithKline
HPV types	16, 18	16, 18, 6, 11
Antigen (per dose)	20 μg HPV 16 L1 20 μg HPV 18 L1	40 μg HPV 16 L1 20 μg HPV 18 L1 20 μg HPV 6 L1 20 μg HPV 11 L1
Antigen source	Baculovirus	Yeast
Adjuvant	AS04 composed of: 500 μg aluminium hydroxide 50 μg MPL (3-O-desacyl-4'-monophosphoryl lipid A)	225 μg aluminum hydroxyphosphate sulfate
Recommended administration	0.5 mL dose at 0, 1, 6 months intramuscular dose	0.5 mL dose at 0, 2, 6 months intramuscular dose
Approx price (USD)	$100 per dose	$120 per dose
Approved for ages	10–25	9–26
Antibody titers 1 month after completed vaccination course compared to natural infection	HPV16: 107 times HPV 18: 82 times (151)	HPV 6: 11 times HPV 11: 7 times HPV 16: 105 times HPV 18: 19 times (152)
Geometric mean antibody titers at 7 months (age 18–26)	HPV 16: 31715 HPV 18: 13732	HPV 16: 8682 HPV 18: 1886

Table 2

Therapeutic HPV vaccine advantages and disadvantages and future strategies

	Advantages	Disadvantages	Future Prospects
Live Vector-based	Numerous vectors available. Highly immunogenic. Can be engineered to express cytokines and co-stimulatory molecules.	Pre-existing immunity. Possible dominance of immune response to viral vector rather than HPV antigen. Neutralizing antibodies restrict repeated administration. Risk of disease.	Enhancement of immunogenicity through adjuvant and fusion proteins. Circumvention of neutralizing antibodies to allow repeat dosage.
Peptide- based	Safe. Stable. Easy to produce. Can combine multiple epitopes in long chain peptides.	Epitopes must be determined. HLA-restriction. Low immunogenicity.	Enhancement of immunogenicity through. Epitope enhancement. Lipopeptide delivery.
Protein-based	Safe. Stable. No MHC restriction.	Low immunogenicity; limited CTL response.	Enhancement of immunogenicity through adjuvant and fusion proteins.
DC-based	Highly immunogenic. Multiple methods available to load antigen.	Expensive. Labor-intensive as individualized. Lack of agreed standards for preparation. DCs do not necessarily home to lymph nodes.	Increase survival of DCs. More efficient loading of antigen. Identification of the most effective delivery route.
Tumor-cell based	Likely to express tumor antigens.	Safety concerns. Labor-intensive as individualized. Weak antigen presentation by tumor cells.	Address safety issues. Immunogenicity enhanced by cytokines. Consistency in potency and purity established.
DNA-based	Safe. Stable. Easy to produce. Can administer multiple times. Several delivery methods possible. More sustained expression of antigen.	Low immunogenicity. No intercellular spreading. Risk of genomic integration.	Increase number and lifespan of antigen-expressing DCs. Enhanced DC antigen processing and presentation. Improve DC interaction with T cells.
RNA-based	Safe. Transient, non-infectious. Can administer multiple times. No risk of genomic integration.	Difficult to produce and store - unstable. Labor intensive to produce. No intercellular spreading.	Improved DNA-launched RNA replicons. Prevention of early apoptosis.