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Therapeutic HPV vaccines

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High-risk human papillomavirus (HPV) infection is known to be a necessary factor for cervical and anogenital malignancies. Cervical cancers account for over a quarter of a million deaths annually. Despite the availability of prophylactic vaccines, HPV infections remain extremely common worldwide. Furthermore, these vaccines are ineffective at clearing pre-existing infections and associated preinvasive lesions. As cervical dysplasia can regress spontaneously, a therapeutic HPV vaccine that boosts host immunity could have a significant impact on the morbidity and mortality associated with HPV. Therapeutic vaccines differ from prophylactic vaccines in that they are aimed at generating cell-mediated immunity rather than neutralising antibodies. This review will cover various therapeutic vaccine strategies in development for the treatment of HPV-associated lesions and cancers.

Why is there a need for a therapeutic vaccine

Human papillomavirus (HPV) was discovered to be the causative agent of cervical cancer in the 1970s by the Zur Hausen group [1]. The majority of HPV infections are transient and subclinical because of rapid immune clearance. However, persistent high-risk HPV (hrHPV) infection may lead to the development of precancerous lesions. HPV-16 and 18 have been identified as the two most prevalent hrHPV types and are accountable for approximately 62.6% and 15.7%, respectively, of invasive cervical

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cancers [2]. Additionally, these two hrHPV types are responsible for 80–86% of vulvar and vaginal cancers, 89–95% of oropharyngeal cancers, 93% of anal cancers and 63–80% of penile cancers [3].

In the trials that led to the approval of the prophylactic vaccines, Gardasil and Cervarix, these vaccines were found to provide nearly 100% protection against persistent cervical infections with HPV types 16 and 18 [4]. The vaccines are also highly efficacious in preventing precancerous cervical lesions (cervical intraepithelial neoplasia, CIN) caused by these genotypes [5,6]. Despite this, there is still a considerable population suffering from hrHPV infections and associated disease worldwide. There are several reasons for this:

1. Limited global vaccine uptake. By the end of 2016, nearly 70 countries had introduced a prophylactic HPV vaccine into their national immunisation schedule [7]. However, the countries with the highest morbidity owing to cervical cancer and anogenital warts were also the least likely to have introduced prophylactic vaccination. By mid-2016, only 8% of low- to middle-income countries had introduced the HPV vaccine, compared to 71% of high-income countries [8].
2. Limited cross-protection. The current bivalent and quadrivalent vaccines provide 100% protection against infection with HPV-16 and 18 [4]. However non-vaccine hrHPV types are responsible for about 30% of cervical cancers [9]. Two independent analyses of the phase III studies FUTURE I and FUTURE II assessed cross-protection afforded by the quadrivalent vaccine. Brown [10] and Wheeler [11] reported a reduction in the incidence of HPV-31/45 infection by 40.3% and 31.6%, respectively, and of 31/33/45/52/58 infection by 25.0% and 17.7%, respectively. They also reported similar reductions in the incidence of HPV-31/45 CIN1-3 by 43.6% and 22.2% and HPV-31/33/45/52/58 CIN1-3 by 29.2% and 18.8%, respectively, in the naïve population.
3. Prophylactic vaccines provide no therapeutic benefit and are only effective in HPV-naïve individuals as their mechanism of action is the induction of antibodies against the L1 capsid protein. L1 is only expressed in the granular epithelium just prior to viral shedding, and therefore, current prophylactic vaccines are unable to eliminate pre-existing infections and associated lesions. Clearance of infected cells is dependent on induction of a cell-mediated immune response [12,13].
4. Cost and requirement for a cold chain. This restricts wide-scale deployment in developing countries, where ~87% of cervical cancer deaths occur [14].
5. Because of the long time required for the development of precancerous lesions, it is estimated that an impact on cancer incidence may not become apparent for at least 20 years from the implementation of mass HPV vaccination.

Women with low-grade disease (CIN1), which is known to have high regression rates [15,16], are often observed and cervical cytology repeated annually until disease progression is detected [17,18]. For high-grade disease (CIN2-3) treatment options include excisional approaches using electric loops (loop electrosurgical excision procedure or large loop excision of the transformation zone (TZ)), and carbon dioxide lasers or scalpels (cold knife) to perform conisation, in which the entire TZ is removed. Although there is no difference in haemorrhage or CIN recurrence between the three conisation approaches [18], adverse obstetric outcome, such as preterm birth and second-trimester miscarriage, is increased with cold knife conisation [19–21]. Local destruction of the TZ by cryotherapy or laser ablation is more time-consuming than excisional procedures, and as these techniques do not yield tissue for histological assessment, they are only appropriate after diagnosis via biopsy and when no invasion is suspected. Hysterectomy is not part of standard therapy for CIN and only considered in certain circumstances. Treatment of high-grade cervical lesions in LMIC countries has been estimated to cost $12.6 million annually [22]. The infrastructure required to deliver and monitor the efficacy of ablative treatment, which is a major challenge for LMICs carrying the vast burden of HPV-associated disease, makes non-surgical therapies for preinvasive hrHPV lesions highly desirable.

Thus, there is an urgent need to develop therapeutic vaccines against hrHPV, which could have an immediate impact on women who are already infected by preventing the progression of low-grade disease, inducing regression of existing lesions, and/or preventing recurrence following treatment. hrHPV infection is an attractive target for immunotherapy because the preinvasive stages are well defined and progression to cancer typically takes 10–30 years, providing ample opportunity for

therapeutic intervention. Furthermore, spontaneous regression of high-grade (CIN2/3) lesions occurs in 30% of cases [23], indicating that host immune responses can reverse the course of disease.

The requirement for a cell-mediated immune response

While humoral immune responses have important preventive utility, several lines of evidence suggest that cell-mediated immune responses play a crucial role in regression of precancerous lesions and clearance of infection. First, persistent hrHPV infections and preinvasive lesions are significantly more frequent in immunosuppressed states such as untreated HIV infection [24] or receipt of anti-rejection therapy post-transplant [25].

Spontaneous regression of high-grade CIN has been reported to correlate with cell-mediated immune responses. Regressing lesions are typically infiltrated by $CD8^+$T cells specific for the oncoproteins, E6 and E7. In addition, $CD4^+$T cell helper responses to E2, a key regulator of transcription, have been detected in individuals following regression of CIN [26–30]. Increased CD4:CD8 ratios in the stroma have also been described. In contrast, reduced numbers of $CD4^+$T cells are present in persistent and/or progressing CIN lesions [31].

Regulatory T cells (Tregs) have been found in persistent HPV infections. The frequency of Tregs increases with the size of genital warts [32]. Clinical studies have suggested that Tregs may facilitate both viral persistence [33,34] and disease progression [35].

Patients with anal intraepithelial neoplasia and vulval intraepithelial neoplasia (VIN) treated with imiquimod, a Toll-like receptor (TLR)-7 agonist that induces innate immune responses, developed HPV-16-specific $CD4^+$T cell responses that correlated with remission of lesions [36].

Cross-sectional human studies provide only a snapshot of what is a dynamic process; however, animal studies have been informative in revealing the immunological events of the entire cycle from infection to regression. The canine oral papillomavirus model confirms observations in human studies that the predominant infiltrate associated with regression of lesions comprises $CD4^+$T and $CD8^+$T cells, specific for the early proteins E2 and E6, respectively [37].

In summary, induction of potent cellular immune responses to E6 and E7, and possibly other viral antigens, is likely to be crucial to the efficacy of a therapeutic HPV vaccine.

How to induce a cell-mediated immune response by vaccination

The generation of virus-specific cytotoxic T cells (CTLs), which target and kill infected cells, and T helper cells, which secrete cytokines and orchestrate the maturation of CTLs and B cells, is dependent on priming by professional antigen-presenting cells (APCs) such as dendritic cells (DCs). Thus, therapeutic vaccines require rational design to achieve concentrated antigen delivery to DCs and DC activation. When a pathogen, or component thereof, penetrates epithelial and mucosal barriers, the first line of defence is the innate immune response. Pathogen-associated molecular patterns (PAMPs) in viral capsids or bacterial membranes are recognised by pathogen recognition receptors (PRRs), such as TLRs on the cell surface. Activation of PRRs leads to the production of pro-inflammatory cytokines and chemokines and an influx of non-specific inflammatory cells such as macrophages, DCs and natural killer (NK) cells to the site of infection. The main cytokines involved in an antiviral response are the interferons (IFN-α, IFN-β and IFN-γ) and the tumour necrosis family (TNF-α and TNF-β); these have direct antiviral effects and immunomodulatory effects. Professional APCs (DCs, monocytes, macrophages and B cells) carrying foreign antigen migrate from the site of infection to the draining lymph nodes, where they present this antigen to naïve T cells in the form of peptides complexed to major histocompatibility complex (MHC) class I or II molecules, initiating the primary adaptive immune response. The defining feature of professional APCs is the expression of co-stimulatory molecules (CD80 and CD86), which are essential for activation of naïve T cells. The MHC class I pathway processes proteins from the cytosol. Many viruses are not trophic for DCs and are therefore not present in the cytosol of professional APCs; however, exogenous antigens can access MHC class I molecules through a process called cross-presentation. Viral antigens derived from phagocytosed/endocytosed apoptotic or

necrotic virus-infected cells can leave the vacuole in which they have been engulfed and gain entry to the cytosol, where they are subsequently degraded by the proteasome, and presented by MHC class I to naive $CD8^+$T cells. These cells subsequently proliferate and differentiate into effector cells that can directly eliminate virus-infected cells. Upon clearance of the pathogen, a small fraction of $CD8^+$T cells persist as effector memory cells that patrol the periphery and rapidly upregulate effector molecules upon encounter with cognate antigen. Other $CD8^+$T cells differentiate into central memory cells, which reside in lymphatic tissue where they are maintained for years.

The MHC class II pathway generates peptides by degradation of exogenous antigens internalised by phagocytosis/endocytosis for presentation to $CD4^+$T cells. In the presence of cytokines such as interleukin 12 and intercellular adhesion molecule 1, naïve $CD4^+$T cells differentiate into T helper 1 (Th1) cells. Th1 cells release cytokines that provide signals necessary for both the priming of naïve $CD8^+$T cells and maintenance of their effector functions. Cytokines including OX-40 ligand, IL-10 and transforming growth factor β induce Th2 cell polarisation, and these cells promote B-cell differentiation, proliferation and antibody production in response to extracellular pathogens.

Target antigen

The HPV genome encodes seven early proteins (E1, 2, 4, 5, 6, 7, 8) and two late proteins (L1 and L2). L1 and L2 encode the viral capsid proteins that are necessary for the initial infection of the basal layer of epithelium. Following virion internalisation, the E1 and E2 proteins initiate and maintain viral replication. Because they are essential for HPV replication within host cells and are expressed early in the virus life cycle, they are potential targets for vaccines that aim to treat early stages of disease, such as CIN1. Additionally, responses to E2 are associated with regression of CIN and virus clearance [27,28].

E6 and E7 drive oncogenesis by targeting host cell-cycle control. E7 binds to and inactivates the retinoblastoma gene product, pRb, leading to uncontrolled cellular proliferation. The tumour suppressor, p53, would normally induce apoptosis in this situation; however, E6 binds to p53, resulting in its ubiquitination and subsequent degradation, thereby inhibiting apoptosis. The concerted actions of E6 and E7 result in malignant transformation of HPV-infected cells and uncontrolled tumour growth. E6 and E7 are constitutively expressed in both premalignant and invasive lesions but are absent on healthy cells, making them ideal targets for immunotherapeutic approaches for HPV-induced malignancies. They have therefore been included in most therapeutic vaccine candidates developed to date. Additionally, because E6 and E7 are foreign proteins there is minimal risk of induction of immune tolerance.

E5 cooperates with E6 and E7 oncogenes to promote hyper-proliferation of infected cells and is therefore regarded as being crucial for malignant progression. Despite this, few studies have investigated its potential as a target antigen.

Vector of choice

Nucleic acid-based vaccines

DNA

DNA vaccination involves the delivery of plasmid DNA encoding a protein of interest into the host's tissues, with subsequent transfection of cells, leading to expression of the transgene and production of proteins that can access the cellular processing machinery. A DNA vaccine typically consists of a bacterial plasmid containing a strong viral promoter, the gene of interest and a polyadenylation/transcriptional termination sequence. After injection, the DNA may be taken up by myocytes or DCs. The bacterial DNA backbone can induce an innate immune response, through recognition of CpG motifs that are a ligand for TLR9 on DCs, B cells and NK cells [38]. TLR9 ligation triggers a cascade of pro-inflammatory responses with subsequent cytokine production leading to the recruitment and activation of other immune cells.

DNA vaccines are believed to induce adaptive cellular immune responses by any or all of the following mechanisms.

1. Transfection of somatic cells and subsequent presentation of encoded antigens to $CD8^+$T cells via the MHC class I pathway. This is more common when the vaccine is delivered by intramuscular injection, which tends to result in transfection of myocytes. However, because myocytes are not professional APCs, they are incapable of activating strong specific immune responses.
2. Direct transfection of professional APCs leading to MHC class I-associated antigen presentation to naïve $CD8^+$T cells. This is more common when the DNA vaccine is delivered intradermally as it can access Langerhans cells residing in the skin.
3. Phagocytosis of transfected somatic cells by professional APCs, leading to processing and presentation of exogenous antigens via MHC class II molecules to $CD4^+$T cells helper cells or cross-presentation to $CD8^+$T cells.

The advantages of DNA vaccines are (1) simplicity and low cost of manufacture; (2) thermostability; (3) possibility of repeating dosing because of the absence of anti-vector immune responses; (4) potential to elicit both cellular and humoral immunity; (5) excellent safety and tolerability in humans. The primary safety concern for DNA vaccines is the potential to integrate into host cellular DNA; however, little evidence of this has been found [39,40].

Unfortunately, clinical studies of DNA vaccines have been disappointing and have failed to reproduce the high levels of immunogenicity observed in small animal models. Cellular uptake of naked DNA is very inefficient *in vivo* and the bulk of the injected DNA remains extracellular [41]. The immunogenicity of DNA vaccines can be improved by optimisation of codon usage to improve expression *in vivo* [42] and the addition of a leader sequence to target antigens to the endoplasmic reticulum. The ability to incorporate additional genes into the vector creates opportunities to modulate cellular routing and the subsequent immune response (e.g. IL-2 [43]). In addition, electroporation (EP), which involves applying brief electric pulses to the skin, can greatly enhance vaccine uptake and subsequent antigen delivery by inducing transient permeabilisation of the cell membrane. EP also induces damage to the application site and therefore acts as a form of adjuvant that leads to inflammation and cytokine release with subsequent recruitment of APCs to the area [44]. Immunogenicity of DNA vaccines can also be improved by using them in a heterologous prime-boost vaccination strategy. In the combination of DNA prime viral vector boost, the lack of viral antigens in the DNA vector helps focus the immune response on the key antigen and the additional innate antiviral responses that develop following the viral vector boost result in high-magnitude antigen-specific responses [45]. Such a regimen may generate immune responses that are higher in magnitude and better in quality compared with homologous prime boost and avoid anti-vector immunity [46].

In 2015, proof of concept for a therapeutic DNA-vectored HPV vaccine was demonstrated in phase IIb study by Trimble et al. [47]. VGX-3100 consists of two DNA plasmids encoding optimised synthetic consensus E6 and E7 genes of HPV-16 and 18. One hundred and sixty-seven women with histologically confirmed HPV-16/18-positive CIN2/3 were randomised 3:1 to receive VGX-3100 or placebo, given intramuscularly at 0, 4 and 12 weeks, followed by EP. Histopathological regression occurred in 49.5% of vaccinees compared with 30.6% of placebo recipients (percentage point difference 19.0, $p = 0.034$). In a post hoc analysis, the magnitude of T-cell responses to E6 was associated with clinical outcome [47]. This trial addressed two important issues; first, it demonstrated that a therapeutic vaccine could induce adaptive immune responses in patients with existing disease. Second, administration of a vaccine systemically can elicit adaptive immune responses that have a therapeutic effect distally in a mucosal target lesion. A phase III trial (REVEAL) is currently underway to confirm the efficacy, safety and tolerability of VGX-3100 followed by EP in women with histologically confirmed HPV-16/18-positive CIN2/3. It aims to recruit 198 patients with an expected completion date of 2020 (NCT03185013). A phase II study of VGX-3100 followed by EP alone or in combination with imiquimod is also underway in women with HPV-16/18-positive high-grade lesions of the vulva (NCT03180684).

RNA

Once administered and internalised by host cells, mRNA transcripts are translated directly in the cytoplasm. Therefore, in contrast to DNA vaccines, RNA vaccines only have to cross the plasma membrane but not the nuclear membrane, which may improve probability of successful transfection. Translation of RNA occurs with the resulting antigens presented by MHC class I molecules. RNA vaccines can also activate the innate immune system by acting as agonists for TLR7 and TLR8 [48]. RNA vaccines are notoriously unstable and as of yet have not been used in any clinical settings for HPV-associated diseases. Encapsulating mRNA in nanoparticles may protect the mRNA from nuclease degradation resulting in enhanced cell uptake and delivery efficiency [49].

Viral vectors

Because of their natural propensity to transduce their own genetic information into host cells for replication, viruses are attractive modalities for the development of therapeutic HPV vaccines. Non-essential viral genes are replaced with foreign genes coding for immunogenic proteins from pathogens of interest. Recombinant viral vectors can then transduce their usual target cell, with subsequent intracellular expression of the encoded antigen. Antigen production is efficient and leads to presentation of both vector and transgene-derived peptides in the context of MHC class I molecules at the cell surface. In addition, viral nucleic acids contain PAMPs that stimulate the innate immune responses. The main drawback of viral vector vaccines is vector-specific immunity: pre-existing antibodies can block uptake of the vector into the target cell, while de novo responses can be amplified with each subsequent vaccine boost and outcompete responses to the transgene. The most extensively tested viral vectors are adenoviruses (Ad), adeno-associated viruses, alphaviruses and vaccinia virus (such as Modified Vaccinia Ankara, MVA).

Adenoviruses

Adenoviruses display extensive tropism for many different cell types, which is one of the reasons adenoviruses were pioneered for gene therapy and subsequently developed as vaccine vectors. The adenovirus genome has five early regions, E1A, E1B, E2, E3 and E4. E1A proteins transactivate other viral genes and are critical for the initiation of virus replication; deletion of this region and replacement with the target gene results in replication-defective virions that are safe for human use. The majority of studies in animals and in humans utilise E1-deleted adenovirus vectors. Additional deletions in the E2, E3 and E4 coding sequences increase the capacity for transgene insertion. Replication-deficient E1/E3-deleted vectors can accommodate approximately 6.5 kb of foreign DNA. After entering the nucleus, adenoviruses persist episomally, rather than integrating into the host genome, minimising the risk of insertional oncogenesis. Adenovirus vectors can be administered by various systemic and mucosal routes and are able to transduce both quiescent and actively dividing cells.

As adenoviruses are ubiquitous in humans, pre-existing immunity is common: up to 60% of adults living in Europe and the USA have high titres of neutralising antibodies to adenovirus type 5 (Ad5), the most commonly used human serotype for gene therapy and vaccine trials. Prevalence increases to over 90% in individuals living in sub-Saharan Africa; therefore, a number of alternatives are being evaluated, including rare human serotypes such as Ad26 and Ad35 and non-human adenoviruses from ovine, porcine, bovine and chimpanzee sources. Adenoviruses from chimpanzees (ChAd) were advanced to the clinic because of their low/no seroprevalence in the human population and comparable immunological potency to human Ad5 (reviewed in Ref. [50]).

Khan et al. [51] investigated the use of rare human adenoviruses, Ad26 and Ad35, encoding E2, E6 and E7 as fusion proteins for the treatment of HPV-16/18-related disease. E6 and E7 genes were reordered to abrogate their oncogenic activity. In preclinical studies, robust T-cell immunogenicity and protection against tumour challenge was observed upon immunisation of mice. This fusion protein has also been expressed in MVA for use in a prime-boost strategy. A phase I trial is planned for 2017.

While most therapeutic vaccine candidates to date have targeted HPV-16 and 18, Ragonnaud et al. have explored a novel approach to increase the coverage of circulating oncogenic papillomavirus [52]. They designed a papillomavirus ancestor antigen, which corresponded to the root of a phylogenetic tree of all oncogenic human and macaque papillomavirus E1 and E2 sequences (CDSE1E2 antigen), as

well as two other sequences that were ancestral to the clades containing HPV-16/31/35 and HPV-18/45, respectively. A molecular T-cell adjuvant, the MHC class II invariant chain (Ii) was fused to the HPV transgene to increase antigen presentation in transduced DCs. Fusion of Ii to various antigens was shown to increase $CD4^+$T and $CD8^+$T cell responses [53]. Replication-deficient ChAd3 and ChAd63 vectors encoding CDSE1E2 were shown to induce potent and long-lasting CDSE1E2-specific T-cell responses in outbred mice. However, $CD8^+$T cell cross-reactivity against HPV-16 E1 and E2 was disappointingly limited. A heterologous (ChAd3/ChAd63) prime-boost regimen was subsequently tested in female macaques naturally infected with *Macaca fascicularis* papillomaviruses (MfPVs). All immunised animals developed IFN-ɣ producing $CD8^+$T cell responses to CDSE1E2, yet also demonstrated poor cross-reactivity against circulating papillomaviruses (HPV-16, HPV-18 MfPV3) and no reduction in HPV virus load [52,54].

Poxviruses

Poxviruses have a large, stable genome, which can support stable expression of large amounts of transgenic DNA whilst retaining transcriptional and translational capacity [55]. Recombinant genes are expressed for ~7 days before the infected cell is cleared by the immune system [55]. Their entire life cycle takes place in the cytoplasm of somatic human cells, which minimises the risk of insertional mutagenesis. Furthermore, poxviruses infect cells though a passive membrane fusion, thus conferring a broad tropism for mammalian cells, including monocytes and immature myeloid-derived DCs [56]. The extensive use of replication-competent vaccinia and latterly MVA as smallpox vaccines provided evidence for the safety of poxviruses in humans. MVA was highly attenuated by ~570 serial passages of the Ankara strain in primary chick embryo fibroblasts, which resulted in several gene deletions and loss of host immune evasion proteins. Pre-existing immunity is only present in vaccinated individuals, and is therefore diminishing with time. MVA and other attenuated vaccinia strains such as NYVAC are easy to manufacture at a low cost and have been safely administered by the intradermal, intranasal, intravaginal and intrarectal routes. MVA and NYVAC are particularly immunogenic when used in heterologous prime-boost strategies.

An MVA vaccine candidate containing the E2 gene from bovine papillomavirus has been evaluated in several phase I/II clinical trials in patients with established HPV-induced CIN lesions [57–59]. In a phase I/II trial, 36 women with CIN1–CIN3 lesions received MVA-E2 injected directly into the uterus weekly over a 6-week period. Ninety-four percent of patients showed complete elimination of precancerous lesions after treatment [57]. The remaining two patients showed regression from CIN3 to CIN1. Following this, in a phase II clinical trial, 56% of women with HPV-16/18-positive high-grade lesions had a complete regression following vaccination [58].

Recently, this MVA E2 vaccine has been tested in a phase III trial of 1176 female subjects with HPV-driven oncogenic and non-oncogenic intraepithelial lesions and 180 male subjects with condylomata only. They were injected with MVA-E2 directly into their uterus, urethra, vulva or anus. Patients were monitored by colposcopy and cytology. Complete histological regression was observed in 89.3% of female patients and 100 of male patients, after MVA-E2 treatment. No description of E2-specific T cells was provided in any of the studies, and because no control vaccine was used in these studies, the true vaccine efficacy is uncertain [60].

Another MVA vectored vaccine, TG4001, developed by Transgene, comprises recombinant MVA encoding HPV-16 E6/E7 and human IL2. Safety and efficacy were evaluated in a phase IIa trial in 21 patients with HPV-16-related CIN2/3 lesions; 48% showed disease regression. Furthermore, of 10 patients showing regression to low-grade lesions, HPV-16 DNA clearance was observed in 8 and no recurrence of high-grade lesions was observed for 12 months after treatment [61]. Although these data were considered very encouraging, no immunological analysis was reported in this trial, and a correlation between vaccine-induced T cell and viral clearance has not been reported. A subsequent randomised placebo-controlled phase IIb trial (NCT01022346) in 206 women with CIN2/3 showed greater histological regression in vaccinees than placebos (25% vs 10%, $p = 0.0126$) as was viral clearance (37% vs 14%) 6 months after vaccination. As vaccine efficacy was well below that of excisional/ablative therapy, Transgene discontinued development of TG4001 in 2012.

Bacterial vectors

Live attenuated bacterial vectors have the capacity to induce both mucosal and systemic humoral and cell-mediated immunity. Upon administration, recombinant bacteria are likely to be phagocytosed by macrophages and other APCs [62]. Conserved molecular patterns such as lipopolysaccharides in gram-negative bacteria, lipoteichoic acid in gram-positive bacteria, peptidoglycans and flagellin are recognised by PRRs and can thus trigger innate immune responses [63,64]. Attenuation of bacteria is achieved by deleting essential genes involved in virulence regulatory systems or the aromatic amino acid biosynthesis pathway. These mutations render the bacteria unable to reproduce in the host whilst preserving their capacity to synthesise encoded antigens.

The most commonly used bacterial vector for vaccine development is Listeria monocytogenes (Lm), a gram-positive intracellular facultative anaerobe. Once inside the phagosome, Lm secretes the membrane-active virulence factors LLO and phospholipase C, which degrade the phagolysosomal membrane, enabling entry into the cytoplasm where expressed proteins gain access to the endogenous antigen-processing pathway [65,66]. Lm elicits durable $CD8^+$T cell responses. Once expressed in a phagosome, Lm-encoded proteins can also be presented directly to MHC class II molecules [67] and therefore induces both $CD4^+$ and $CD8^+$T cell responses.

Bacterial vectors are relatively simple and inexpensive to produce, suited to large-scale manufacture and stable without refrigeration (through lyophilisation). They have a large capacity for foreign DNA, can be eradicated with antibiotics if an adverse reaction occurs during large-scale trials and can be administered orally.

The first clinical trial of a live attenuated bacterial vectored therapeutic HPV vaccine evaluated Lm expressing a HPV-16 E7 antigen fused to a fragment of LLO, Lm-LLO-E7/ADXS11-001, in 15 patients with advanced carcinoma of the cervix who had failed prior chemotherapy, radiotherapy and/or surgery. The vaccine was shown to be safe, and a reduction in total tumour size was observed in 4 patients [68]. Three patients showed an increase in E7-specific T-cell responses post-vaccination. Phase I–II trials are underway in anorectal, head and neck and cervical cancers (NCT02399813, NCT02002182, NCT02291055, NCT01266460). Patients with persistent or recurrent metastatic cervical cancer enrolled in a phase II trial received ADXS11-001 intravenously, and overall 1-year survival was 38%, which compares favourably with the expected 12-month survival of 24%, established using a database of 500 patients with persistent or recurrent metastatic carcinoma of the cervix. A phase III trial (AIM2CERV) is investigating ADXS11-001 administered as adjuvant immunotherapy in patients with high-risk, locally advanced cervical cancer following chemoradiation (NCT02853604).

Peptide- and protein-based vaccines

Peptide vaccines

Peptide-based therapeutic vaccines have the advantages of stability, safety and feasibility of large-scale production [69]. They can be divided into specific epitope (short) peptides and synthetic long peptides (SLPs). Short peptides (<15 amino acids) do not require processing by professional APCs and can therefore bind exogenously to the MHC class I molecules of all nucleated cells [70]. However, presentation without optimal co-stimulation can result in a tolerising signal and short peptides have been shown to elicit immunological tolerance rather than protective immunity [71,72]. SLPs (>20 amino acids) are too long to stabilise MHC molecules, so they must be processed and presented by professional APCs [73,74]. SLPs typically harbour both $CD4^+$ and $CD8^+$T cell epitopes; however, peptide vaccines are poorly immunogenic and, therefore, require adjuvantation.

PepCan consists of four synthetic peptides covering the HPV-16 E6 protein with the *Candida* skin test reagent as a novel adjuvant. In a single-arm, dose-escalation phase I clinical trial, 24 women with biopsy-proven CIN2/3 were given four injections intradermally every 3 weeks. At a dose of 50 μg, a regression rate of 83% was observed; the overall rate was 52%. Vaccine-induced immune responses to E6 were detected in 65% of recipients. A phase II randomised and double-blinded trial of PepCan for treating cervical high-grade squamous intraepithelial lesions is ongoing (NCT02481414).

Another SLP vaccine, ISA101, consists of 13 SLPs spanning the entire HPV-16 E6 and E7 oncoproteins. Two phase I/II trials in women with HPV-16-positive high-grade VIN have shown clinical responses in >50% of patients at 12-month follow-up with 47% and 30% of patients having a complete response, respectively [75,76]. All responders were still free of disease at 24 months. Spontaneous clearance of HPV-16-induced high-grade lesions of the vulva is less than 1.5% [77]. Vaccine-induced T-cell responses were present in all patients, and post hoc analyses suggested that the magnitude of the HPV-16-specific immune response correlated with clinical efficacy of vaccination [76].

Protein-based vaccines

Protein-based vaccines include numerous $CD4^+$ and $CD8^+$T epitopes and avoid the limitation of MHC restriction, which is a disadvantage of short peptide vaccination. However, antigens are preferentially presented via MHC class II, which results in induction of antibodies rather than CTLs. Adjuvantation to enhance endogenous processing, or to target the antigen to DCs is therefore required.

GTL001 (Procervix) comprises HPV-16 and 18 E7, each fused to detoxified adenylate cyclase from *Bordetella pertussis* (CyaA) and adjuvanted by imiquimod. The N-terminus of CyaA binds to the adhesion molecule CD11b that is expressed on APCs [78]. A randomised, double-blind, placebo-controlled phase II of GTL001 enrolled 233 patients positive for HPV-16/18 with either normal or abnormal cervical cytology. All patients received at least one dose of vaccine or placebo along with topical imiquimod and were assessed for viral clearance at 2 years. The difference in viral clearance rates did not reach statistical significance and rates of progression to high-grade lesions were identical in the GTL001 and placebo groups [79] (NCT01957878). Further clinical development was therefore halted.

A second-generation vaccine has since been developed. GTL002 comprises modified E7 proteins from HPV-16, 18, 45, 31, 33 and 52. Preclinical immunogenicity of GTL002 has been evaluated in inbred and outbred mice and in Beagle dogs. E7-specific T-cell responses were induced against each of the six HPV genotypes and *in vivo* efficacy has been demonstrated using the widely used TC-1 tumour challenge model [80].

How to improve on current approaches

All the therapeutic vaccines tested in clinical trials to date have been safe and well tolerated. However, they have largely yielded disappointing clinical responses. Human HPV-induced cancers have been largely refractory to approaches shown to be successful in rodents, highlighting the limitations of currently used preclinical models. The efficacy of vaccine-induced T cells may be compromised by the numerous mechanisms of immunoevasion and immunosuppression exploited by tumours; therefore, treatment modalities that can down-modulate the regulatory mechanisms in the tumour microenvironment need consideration.

Breaking local immune regulation

Tregs, tumour-associated macrophages, fibroblasts, adipocytes and myeloid-derived suppressor cells present within the tumour microenvironment can inhibit tumour-specific CTLs and NK cells. Tregs suppress T-cell expansion and function [81]. The presence of large numbers of Tregs in relation to tumour-specific effector T cells has been associated with poor clinical prognosis in many tumours [82]. The ratio of $CD8^+$T:Tregs is an independent prognostic factor in cervical cancer [83]. To improve the success of therapeutic HPV vaccines, selective Treg depletion may be needed. Depletion of Tregs by an anti-CD25 antibody before DNA-E7 vaccination in tumour-bearing mice enhanced the number of vaccine-induced $CD8^+$T cells and resulted in enhanced survival [84].

Checkpoint blockade

Immune checkpoint mechanisms such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) prevent excessive and uncontrolled immune responses. CTLA-4 competes with CD28 for binding to the CD80/86 ligands, thereby inhibiting T-cell proliferation,

blocking cytokine production and promoting T-cell anergy. PD-1 is transiently induced following immune activation. Chronic antigen exposure, such as in tumours, leads to persistently high levels of PD-1. Binding of PD-1 to its ligand, PD-L1, down-regulates T-cell receptor signalling, resulting in T-cell anergy and apoptosis and leading to immune suppression [85]. Many tumours express PD-L1, which may serve as an important mechanism for eradicating tumour-reactive T cells within the tumour microenvironment [86]. Similar mechanisms probably also limit the clonal expansion of T cells following vaccination, providing a rationale for combining therapeutic HPV vaccines with checkpoint inhibitors. A phase I/II trial is currently investigating ADXS11-001 and the fully humanised anti-PD-L1 antibody, MEDI4736, alone or in combination in previously treated locally advanced or metastatic cervical or HPV + head and neck cancer (NCT02291055).

Chemoimmunotherapy

Certain chemotherapeutics can enhance the anti-tumour effect of therapeutic vaccination. Although the precise mechanisms of this synergistic effect have not yet been completely defined, chemotherapy is believed to convert the tumour microenvironment into a site permissive for vaccination by enhancing antigen density and T-cell infiltration and by sensitising tumour cells to cytotoxic $CD8^+$T cell-mediated killing.

A phase I/II study is underway to assess the safety and tolerability of the SLP vaccine, ISA101, and the immune-modulating effects of ISA101 when combined with carboplatin and paclitaxel, with or without bevacizumab. The study aims to recruit 100 women with HPV-16-positive advanced or recurrent cervical cancer who have no curative treatment options. Study completion is expected in 2021 (NCT02128126).

Personalised antigens

An important consideration is whether cancer vaccines that use shared antigens not specific for individual tumours can succeed. The full repertoire of tumour antigens at the individual patient level can now be identified through immune profiling, owing to advances in sequencing technology. Neoantigens are newly formed mutated proteins that arise as a result of genomic instability of tumours. Both $CD4^+$ and $CD8^+$ neo-antigen-specific T cells have been identified in multiple human cancers and shown to be associated with a favourable clinical outcome [87]. These neo-antigens could be used in personalised immunotherapy. On obtaining a tumour biopsy, mutations expressed in tumour cells would be identified by whole-exome sequencing. Software algorithms then predict the binding affinity of identified neo-antigens to HLA in order to prioritise the most attractive targets. Prioritised neoantigens could then be delivered by either adoptive cell transfer or the vaccine platforms discussed earlier. Both approaches could be combined with other immune modulators to overcome immunosuppressive mechanisms in the tumour microenvironment that inhibit neoantigen-specific immune responses. Such an approach showed encouraging results in a patient with a cholangiocarcinoma [88].

Summary

While progress in the development of targeted therapeutics for HPV disease has been slow, encouraging results from a recent phase IIb trial showing vaccine-induced regression of high-grade CIN lesions are grounds for optimism. Further trials to improve on the clinical efficacy of DNA vaccines through adjuvantation are underway. Other promising strategies in development include viral and bacterial vectored vaccines, which offer the advantages of superior potency without the need for separate adjuvants, coupled with excellent safety. However, progress is still hampered by the lack of relevant animal models. Non-human primate species recapitulate some features of human disease but have not been studied in depth.

The historically poor efficacy of therapeutic vaccines in clinical trials could also be due to the immune-suppressive nature of the tumour microenvironment, which contributes to HPV pathogenesis. Further exploration of the intricate relationship between tumour cells, the tumour microenvironment and immune effectors may reveal pathways that are amenable to manipulation with immune

checkpoint inhibitors and other immunomodulators. As HPV-driven cancers express both viral and neo-antigens, the potential for synergy between these agents and therapeutic vaccines should be investigated.

Practice points

- HPV-16 and 18 have been identified as the two most prevalent high-risk HPV (hrHPV) types and are accountable for approximately 62% and 16% of invasive cervical cancers, respectively.
- Current prophylactic HPV vaccines protect against infection with high risk-type HPV-16 and 18 and provide limited cross-protection against infection with other hrHPVs. A nonavalent vaccine that provides coverage of an additional five high risk types has been licenced in Europe and the US, but its use is currently limited by cost.
- Prophylactic vaccines are ineffective at clearing pre-existing HPV infections and HPV-associated lesions.

Research agenda

- Several new platform technologies for antigen delivery have yielded encouraging results in other infectious diseases and are now being applied to the development of therapeutics for HPV disease.
- The principal targets for therapeutics have been E6 and E7; the potential benefits of targeting other viral proteins require further investigation.
- Therapeutic vaccines showing promise in animal models have yielded poor clinical results. The development of more relevant animal models is a priority.
- Tackling the global burden of HPV disease and reaching the most vulnerable populations will require scalable and affordable vaccine manufacturing processes and sustainable delivery in low-resource settings.

Conflicts of interest

The authors have no conflicts of interest.

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