REVIEW

Current therapeutic vaccination and immunotherapy strategies for HPV-related diseases

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ABSTRACT

Carcinomas of the anogenital tract, in particular cervical cancer, remains one of the most common cancers in women, and represent the most frequent gynecological malignancies and the fourth leading cause of cancer death in women worldwide. Human papillomavirus (HPV)-induced lesions are immunologically distinct in that they express viral antigens, which are necessary to maintain the cancerous phenotype. The causal relationship between HPV infection and anogenital cancer has prompted substantial interest in the development of therapeutic vaccines against high-risk HPV types targeting the viral oncoproteins E6 and E7. This review will focus on the most recent clinical trials for immunotherapies for mucosal HPV-induced lesions as well as emerging therapeutic strategies that have been tested in pre-clinical models for HPV-induced diseases. Progress in peptide- and protein-based vaccines, DNA-based vaccines, viral/bacterial vector-based vaccines, immune checkpoint inhibition, immune response modifiers, and adoptive cell therapy for HPV will be discussed.

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Introduction

Human papillomavirus (HPV) is currently the number one sexually transmitted virus.¹ The lifetime risk of acquiring one or more anogenital HPV-types is >80%, and it is estimated that two-thirds of women worldwide acquire an HPV infection within 2 years after becoming sexually active.² The World Health Organization estimates that there are approximately 14 million new HPV infections each year.³ While it has been estimated that 90% of HPV infections are cleared naturally by the immune system, infections by cancer-causing high-risk HPVs (hrHPVs) can persist, remaining largely asymptomatic.^{4–6} Persistent infection with a hrHPV genotype is associated with an increased risk of developing anogenital cancers. Specifically, hrHPV DNA can be detected in over 99% of cervical cancers with the most common hrHPV types 16 and 18 accounting for 50% and 20% of cervical cancers, respectively.^{7,8} In addition to HPV16 and HPV18, there are currently 13 other identified hrHPVs including types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82.⁹ In 2012, 85% of the 528,000 newly diagnosed cervical cancer cases and 87% of the 266,000 cervical cancer deaths occurred in less developed regions, highlighting socio-economic disparities that exist for HPV-associated diseases.^{10,11} Even in the US, where screening programs have reduced the overall rate of cervical cancer, inequality exists in the incidence of cervical cancer between white, black, Hispanic, and economically disadvantaged women.^{12,13} Beyond cervical cancer, hrHPV infections have also been linked to approximately 70% of head and neck squamous cell carcinomas (HNSCC) with HPV16 being detected in over 90% of these cases.^{14,15}

Moreover, it is estimated that the number of HPV-positive HNSCC will surpass the annual number of cervical cancers diagnosed within the US by the year 2020.¹⁶

HPVs are non-enveloped, double-stranded DNA viruses of the Papillomaviridae family that consists of over 170 identified members.⁹ The 8 kb genome encodes for 6 'early' proteins (E1, E2, E4, E5, E6 and E7) and 2 'late' proteins (L1 and L2).¹⁷ The early proteins interact with cellular gene products and facilitate viral DNA replication, while the late proteins provide the structural components of the viral capsid and are involved in the packaging of DNA into progeny virions.¹⁸ Though HPV DNA usually replicates in episomal form, it has the potential to integrate into host DNA. This event often coincides with the loss of several early (*E2*, *E4* and *E5*) and late (*L1* and *L2*) genes due to integration, leaving *E6* and *E7* as the primary protein products expressed within the infected cell.¹⁹ Moreover, *E2* expression acts as a transcriptional repressor of *E6* and *E7*, and thus the loss of *E2* results in upregulation of the *E6* and *E7* genes, and hence increases downstream protein production. *E6* and *E7* interact with p53 and retinoblastoma (Rb) proteins respectively, which are important cell cycle regulatory proteins. These interactions result in the disruption of cell cycle regulation and genomic integrity, and are the primary contributors in the transformation of HPV-infected cells into cancer.²⁰

Prospective well-controlled phase 3 trials of prophylactic HPV vaccines have demonstrated effective prevention of high-grade cervical lesions associated with HPV16 and HPV18.^{21,22} More recently, the nonavalent Gardasil vaccine, 9vHPV, has

been FDA approved to cover 7 high-risk genotypes associated with cervical, vulvar, vaginal, and anal cancers (16, 18, 31, 33, 45, 52, and 58), and 2 low-risk genotypes that cause genital warts (6 and 11).^{23,24} Despite the effectiveness of these vaccines, widespread general adoption and administration of HPV vaccinations is poor in most countries.²⁵ The percentage of girls aged 13-17 receiving at least one dose has improved from 25.1% to 60.0% from 2007 to 2014 in the US, however the percentage receiving the recommended 3 doses is still only 39.7% as of 2014, and this figure remains low at 21.6% in boys aged 13-17 for whom the vaccine has been routinely recommended since 2011.²⁶

Diagnostic tests that detect HPV infections have only been carried out when women present with abnormal cytology on a Papanicolaou (Pap) smear or when women have been in the age range of 30-65.²⁷ However, in April 2014 the FDA approved the use of the Cobas HPV DNA test (Roche) as a primary screening method for detecting HPV in females 25 years of age and older.²⁸ This approval was largely due to results from the Addressing THE Need for Advanced HPV Diagnostics (ATHENA) trial of over 47,000 women in the US, where it was found that 1 in 10 women aged 30 and older who tested positive for HPV16 or 18 had cervical precancer despite exhibiting normal cytology on Pap smears.^{29,30} Importantly, as HPV DNA testing becomes widely implemented in conjunction with cytological review, there will be a larger number of women who test positive for infection with hrHPV genotypes, potentially with no abnormal cytology or with low-grade cervical intraepithelial neoplastic (CIN) lesions. Furthermore, there are currently no screening recommendations or standardized testing procedures for detecting HPV infection in males, a problem that limits assessing rates of detectable HPV infection to half of the population.

Given that the lifetime risk of HPV infection is high, and that populations severely underrepresented in vaccine coverage will likely continue to develop HPV-related cancers, it is clear that there is a need to develop new therapeutic strategies for HPV. When properly engaged, the human immune system has the ability to eradicate virus-infected cells that express non-self viral antigens. Immunotherapies are a promising strategy against HPV-induced cancers. Therefore, this review will begin with a brief comparison of prophylactic vaccinations and immunotherapeutic approaches, and then will summarize current information on HPV therapeutic vaccinations and other immunotherapeutic strategies for HPV-related diseases, followed by a summary of challenges in HPV immunotherapies.

Comparison of prophylactic versus immunotherapeutic strategies for HPV-driven cancers

The primary goal of prophylactic vaccination is to induce a humoral response such that high-titers of HPV-neutralizing antibodies are produced that are capable of preventing initial infections, making HPV antigen-specific B cells the target cell type for these vaccines. HPV vaccines are composed of HPV L1 containing virus-like particles (VLP), mainly of high-risk genotypes, and adjuvants to enhance immune responses.³¹ When overexpressed in cells, the L1 major

capsid protein is capable of spontaneous formation of the icosahedral capsid of the HPV virion,³² mimicking the shape of naturally occurring virions and able to illicit high-titers of HPV-neutralizing antibodies in vaccinated individuals.³³ Routine vaccination of girls and young women aged 13-26 against HPV was recommended by The Advisory Committee on Immunization Practices in 2006, followed by boys aged 11-26 in 2011.³⁴ To enhance the immune responses Gardasil has been formulated with the adjuvant amorphous aluminum hydroxyphosphate sulfate,^{35,36} whereas Cervarix is formulated in the AS04 adjuvant (3-O-desacyl-40-monophosphoryl lipid A and aluminum hydroxide).³⁷ While HPV prophylactic vaccines may induce some degree of cell-mediated immune responses, they are considered irrelevant to cancer prevention given that infected basal epithelial cells that would form tumor cells do not to express significant levels of L1 protein,³⁸ which is only expressed much later in the viral life cycle when infected cells have advanced into the upper epithelial stratum and are dying naturally.³⁹ Due to this, it is no surprise that prophylactic vaccination platforms have failed to report any therapeutic activity in individuals presenting with HPV-positive cancers.

In contrast to prophylactics, immunotherapies for HPV-driven cancers focus on the generation of cellular immune responses against antigens associated with cellular transformation, where antigen specific cytotoxic CD8⁺ T cells, also known as cytotoxic T-lymphocytes (CTLs), are the primary cell type involved. Given that the HPV16 E6 and E7 proteins are both drivers of unchecked cell proliferation in the most common oncogenic HPV types and are constitutively expressed,^{40,41} they are the most frequently targeted antigens in HPV therapy development.⁴¹ While HPV16 E2 and E5 have recently been investigated as therapeutic targets,⁴² this review will focus on therapies against E6 and E7 of HPV16 unless otherwise noted. HPV immunotherapies can be broken down into therapeutic vaccines, cell based approaches, and immunomodulators, each of which will be discussed in more detail below.

Therapeutic vaccines

While prophylactic vaccines aim to prevent HPV infections, the benefits of therapeutic vaccine strategies against HPV-driven cancers lie in their potential to generate robust CTL responses against the viral proteins E6 and E7 to clear infected cells. CTL responses against HPV are generated by the presentation of foreign viral antigens on the surface of antigen presenting cells (APC) through MHC class I molecules in the presence of costimulatory molecules to antigen-specific T cells expressing the corresponding T cell receptor (TCR). For example, E6 and E7 contain immunogenic epitopes that are processed and presented in association with the HLA*0201 allele.^{43,44} Functionally, therapeutic vaccines need to deliver adequate amounts of the viral antigens along with other immune stimulatory components to generate the appropriate immune cells in order to achieve the desired therapeutic outcome, though stimulation against the viral antigens themselves can take on many forms depending on the vaccine platform as discussed below.

Protein and peptide vaccines

The use of whole HPV proteins, like E6 and E7, or HPV fusion proteins as the antigenic source in early HPV therapeutic vaccines has been widely employed.⁴⁵⁻⁴⁸ An advantage of these vaccines is that they do not require the identification of particular HLA-specific epitopes, and the use of whole proteins theoretically covers all available epitopes. However, non-immunogenic peptides may be dominant during antigen processing and presentation, limiting responses to immunogenic peptide sequences. Despite this, the trend of utilizing whole protein is due to the desire to allow presentation of more CTL epitopes and T helper epitopes.⁴⁹ Many protein-based vaccines have induced HPV-specific CTLs and have induced tumor regression in mice, including those utilizing an E7-*Bordetella pertussis* CyaA fusion,⁵⁰ and an E7-HBcAg-Hsp65 fusion.⁵¹ Nevertheless, only a handful of protein-based vaccines have reached clinical trials such as a *Mycobacterium bovis* Hsp65 fused to HPV16 E7 (for review see ref. ⁵²).

HPV peptide vaccines utilize only a portion of E6 or E7,⁵³⁻⁵⁶ and have several advantages over other platforms including ease of production and safety.⁵² These vaccines have included a wide range of both short and long E6 and E7 peptides. Short, defined, HLA class I-restricted HPV peptides have thus been used to elicit the desired CTL response.⁵⁷⁻⁵⁹ However, where it is an advantage of whole proteins, a limitation of short peptide-based vaccines includes the necessity to define epitopes for specific HLA alleles. More specifically, at least one epitope for each HLA class I allele are required for wide clinical application of peptide vaccines as patients who have different HLA genetic backgrounds inherently recognize different HPV epitopes.⁵⁴ To overcome this limitation, a synthetic overlapping long peptide approach has been used since they contain all potential CD4+ and CD8+ epitopes. For example, a mix of long synthetic peptides of E6 and E7 in Montanide ISA-51 (incomplete Freund's adjuvant) were used to vaccinate vulvar intraepithelial neoplasia (VIN) patients, and durable complete regression was observed in nearly half of those vaccinated.⁶⁰ Recent improvements in the peptide vaccine platform have focused on enhanced peptide-fusion proteins; for example, data from an HPV16 E7 fusion-peptide (TheVax Genetic Vaccine Company) coupled with a GP100 adjuvant (Hawaii Biotech) demonstrated a strong HPV16 E7 CTL response and protected against a tumor challenge in mice. The fusion protein itself contains a full length HPV16 E7 peptide sequence, flanked by an exotoxin from *P. aeruginosa* and an endoplasmic reticulum retention signal.⁶¹ However, even with these promising studies in animal models it still remains that while HPV protein/peptide-based vaccine trials in humans are able to demonstrate HPV-specific CTL response in some patients, often these CTL responses do not correlate with clinical outcome, and tumor regression in most cases has been minimal.⁴⁹

Bacterial-based and viral vectors

Attenuated bacteria (e.g. *Salmonella*, *Shigella*, *Yersinia*, *Saccharomyces cerevisiae*, *Escherichia coli*, and *Listeria monocytogenes*) can serve as vectors to deliver proteins of interest or plasmids encoding genes in vaccines.^{62,63} The rationale for the use of

microbes as delivery vehicles is based on the ability of these agents to interact with multiple pattern-recognition receptors, such as Toll-like receptors (TLRs), and stimulate robust responses in APCs to induce CTLs against target antigens.⁶⁴ TLR signaling events play a central role in the stimulation and activation of both innate and adaptive immunity.⁶⁵ Additionally, viral vectors including adenovirus, vaccinia virus, and a modified vaccinia Ankara (MVA) have been evaluated and tested as platforms for HPV vaccination. Given that adenovirus and vaccinia virus infect dendritic cells (DCs), upregulation of co-stimulatory molecules along with increased cytokine and chemokine production accompanying the infection can contribute to effective CTL induction. For example, an adenoviral vaccine against HPV16 E2 is able to reduce papilloma-forming sites in rabbits.⁶⁶ Original clinical trials utilizing the vaccinia platform to generate a CTLs against HPV16 and 18 E6 and E7 showed no clinical response for patients with late stage cervical cancer,⁶⁷ however an MVA vaccine inducing expression of HPV16 E6 and E7 along with the immunostimulant IL-2 (TG4001 from Transgene) showed a clinical response in 7 out of 10 patients, specifically cytologic clearance of CIN2/3 lesions and elimination of HPV16 mRNA and DNA in previously infected tissue.⁶⁸ Another study utilized a calreticulin (CRT)-E7 fusion protein delivered by a replication-deficient adenovirus vector (Ad-CRT-E7), which had a therapeutic effect against established tumors in mice, and a later tumor re-challenge showed that the generated CTLs had long-term immunological memory against E7-expressing cells.⁶⁹

The use of replication-deficient forms of viruses such as alphavirus replicon particles, HPV pseudovirions, and adeno-associated viruses have been used to generate potent immunogenic responses with minimal toxicity.⁷⁰ Alphaviruses such as Semliki Forest virus (SFV) or Venezuelan equine encephalitis virus (VEE) as vaccine platforms have several advantages. Alphaviruses that are engineered to express the RNA of the E6 and E7 oncogenes do so only in the cytosol, eliminating the potential for integration of oncogenes into host cell DNA. Furthermore, VEE replicons have a DC tropism, and there is no preexisting immunity to these viruses in a vast majority of the population.⁷¹ SFV- and VEE-based vectors expressing an E6-E7 fusion protein were shown to induce HPV-specific CTLs, prevent tumor establishment of an HPV16-E6/E7 transformed cell line, and eliminate tumors in mice.^{72,73} Another study showed that the VEE platform with mutated-fused E6 and E7 proteins was able to generate 100% protection from tumor challenge in vaccinated mice and eliminate 90% of established tumors in HLA-A*0201 transgenic mice,⁷⁴ which is important given that HLA-A*02 is the most prevalent MHC I allele family in humans.⁷⁵ As for both bacterial and viral based HPV vaccines, immune recognition of components inhibits repeat vaccination with the same delivery system, therefore a heterologous prime-boost strategy may be best for these platforms to maximize the immune response to target HPV antigens.

DNA vaccines

DNA vaccines are yet another potentially powerful and economical vaccine platform. The ability of DNA plasmids to generate full-length peptide sequences and allow processing and

presentation of multiple epitopes on both MHC I and II, in addition to their efficient production and ease of storage, could lead to vaccines that provide exceptional immune-stimulating function and that are cost effective.⁷⁶ Typically, DNA vaccines code for antigens of interest (i.e. E6 and/or E7) that have been cloned into a bacterial vector, which is then optimized for expression in mammalian cells. Importantly, all completed trials have shown that DNA vaccines are safe and well tolerated. However, DNA vaccines designed to induce immune responses against full-length E6 and E7 hold the risk of causing cellular transformation. This hazard can be partially circumvented by mutating the P53 and Rb binding sites in E6 and E7 or more completely through shuffling the gene sequence in a way that allows for expression of putative HLA epitopes but disables the resultant protein.^{77,78} Although DNA vaccines in animal models showed induction of E6 and E7-specific CTLs and the elimination of established tumors in mice,^{69,79} clinical trials using DNA vaccines in humans showed no significant responses over spontaneous clearance of CIN lesions.^{80,81}

Recent improvements in HPV DNA vaccine delivery including the use of intramuscular (*i.m.*) injection coupled with electroporation (EP) have shown promising results.^{82,83} Briefly, EP consists of applying a series of short electrical pulses at the site of the DNA vaccine injection. These pulses increase plasmid uptake and jumpstart the immune response to the vaccine. In a phase I study, it was shown that the DNA vaccine VGX-3100 combined with EP was able to generate CTLs that could target cells expressing E6 or E7.⁸² Findings from a phase 2 trial that examined patients with HPV16 or 18 positive CIN2-3 showed histological regression in 53 out of the 107 patients enrolled (49.5%) compared to the 11 out of 36 (30%) in the placebo group. It is important to note that this is the first randomized control trial of a therapeutic vaccine that has shown significant regression in CIN2+ patients.⁸⁴ Additionally, treatment with GX-188E, a pGX27 vector with shuffled overlapping N- and C-termini of HPV16 and 18 E6 and E7 flanked by the secretory signal sequence of tissue plasminogen activator and the extracellular domain of Fms-related tyrosine kinase 3 ligand, resulted in enhanced poly-functional CTL responses as shown by an increase in cytolytic activity, proliferative capacity, and secretion of effector molecules when exposed to E6 and E7 peptides. Notably, 7 out of 9 patients exhibited complete regression of their lesions and had viral clearance within 36 weeks of follow up.⁸³ Most recently a plasmid encoding HPV-16 E7 antigen linked with calreticulin (CRT), CRT/E7, was co-administered with DNA encoding for bovine papillomavirus (BPV) L1 or L2 resulting in enhanced CD4⁺ T cell help for improved HPV CTL responses in mice.⁸⁵

Cell based therapies

Cell-based cancer immunotherapies include DC vaccines and adoptive cell transfer (ACT). These therapies entail the isolation of target cells (e.g., DCs or T cells) from the patient, manipulation of the cells *ex vivo* (e.g. activation, genetic manipulation, or expansion), and delivery of the cells back into the patient to elicit effector functions.

DC vaccines

DCs are potent APCs, and with proper stimulation they efficiently present antigens with significant levels of costimulation to induce strong CTL responses. As such, DCs have been investigated for usage as cell-based vaccines, in which autologous DCs are either loaded with peptide/protein antigens or transduced to express antigens and then returned to the patient (for review see ref. ⁸⁶). A small number of HPV-targeting DC vaccines have already been investigated in cervical cancer.⁸⁷⁻⁸⁹ For example, autologous DCs pulsed with recombinant HPV16 or HPV18 E7 protein were utilized in a pilot study conducted in stage IV cervical cancer patients. Although, HPV-specific CTLs were measured in some patients, no clinical responses were observed.⁸⁷ A similar study with DC pulsed with HPV16 and HPV18 E7 delivered with daily low-doses IL-2 for several days post vaccination in late-stage cervical cancer patients detected E7-specific CTLs in all patients, though again no clinical responses were observed.⁸⁸ Another strategy utilized has been DCs transduced with a CD40-targeted adenoviral vector with a mutated HPV16 E7, which resulted in protection against E7-expressing tumors in mice.⁹⁰ Most recently, a recombinant adenovirus expressing codon-optimized HPV16 E6 and E7 fusion protein (Ad-ofE6E7) was used to transduce DCs, which resulted in robust CTL induction and protective immunity against tumor challenge in mice.⁹¹ Limitations of DC vaccines include the requirement of sufficient autologous DCs from each patient, low efficiency of transduction, the inability of terminally differentiated DC to expand *ex vivo*, and limited DC lifespan.

Adoptive cell transfer

Adoptive cell transfer (ACT) involves the expansion of autologous antigen specific CTLs *ex vivo* prior to delivery back into a patient. ACT offers the following advantages: antigen-specific CTLs that can be expanded to large numbers *in vitro*; CTLs can be genetically engineered and/or activated *ex vivo* to acquire anti-antigenic functions; and the host can be manipulated before cell transfer to eliminate suppressor cells such as regulatory T cells (Treg). The transferred CTLs in ACT act as “living drugs” and can induce long-term protection.⁹² In a recent pilot study, complete regression was observed in 2 out of 9 metastatic cervical cancer patients after a single infusion of HPV16 E6 and E7 reactive CTLs.⁹³ Additionally, CTLs were transduced to express a TCR against an HPV E7 epitope, and these CTLs showed functional responses toward the target antigen *in vitro*.⁹⁴ Most recently, this approach was used to introduce another TCR against E6 into CTLs, which were then able to kill HPV+ cells from cervical and head and neck cancer cell lines,⁹⁵ and this is now being used in an ongoing clinical trial (NCT02280811).

Immunomodulators

It is important to note that successful therapeutic vaccines developed from the aforementioned platforms likely result in the generation of HPV-type specific responses to target proteins. While there have been a handful of studies that suggest

histological regression regardless of HPV-type after treatment, including one study that found significant regression of late-stage CIN over spontaneous clearance,⁹⁶⁻⁹⁸ there has yet to be an identified T-cell epitope that shows cross-reactivity across multiple HPV-types. Because of the limitations imposed by type-specificity in therapeutic vaccines, immunomodulators that can stimulate positive or negative immune system responses regardless of HPV-type are an attractive option for therapy. Here we will discuss a form of negative modulation known as checkpoint inhibition, as well as positive modulation with non-specific immunostimulants, and their use in HPV immunotherapies.

Checkpoint inhibitors

Though promising, multiple inhibitory mechanisms limit endogenous CTLs and modified T cells used in ACT therapies from effective tumor eradication. Beyond Tregs and myeloid-derived suppressor cells (MDSC) that populate the suppressive tumor microenvironment,⁹⁹ T cells have intrinsic regulatory mechanisms that abolish their effector functions. For example, activated CTLs upregulate inhibitory receptors including CTL-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1),^{100,101} of which ligand engagement transduces inhibitory signals to attenuate CTL activation.¹⁰² Briefly, CTLA-4 competes with CTL surface CD28 for CD86 binding on APC, and the interaction of CTLA-4 with CD86 results in CTL attenuation, decreased proliferation, and an increased threshold for activation.¹⁰³⁻¹⁰⁵ Moreover, HPV-positive tumor cells often present with upregulated levels of the PD-1 ligand, programmed death ligand-1 (PD-L1), on their surface,^{106,107} leading to an inverse correlation between PD-L1 expression and the number of CTLs within tumors.¹⁰⁸⁻¹¹⁰ As such, these receptors act as checkpoints that protect against unbridled CTL responses. This has led to new approaches in CTL-targeted immunotherapies focused on disrupting these receptor-ligand interactions, and compounds that accomplish this are known as checkpoint inhibitors.¹¹¹ Utilizing antibodies targeted against CTLA-4, PD-1, PDL-1, or more importantly a combination of these receptors results in a prevention of suppressive signaling events from taking place, resulting in enhanced CTL activity.^{112,113} Though agents against CTLA-4 and PD-1 were the first checkpoint inhibitors tested, combinations of PD-1 with LAG-3,¹¹⁴ TIM-3,^{115,116} TIGIT,¹¹⁷ or BTLA¹¹⁸ have been shown to bolster anti-tumor CTL function.

One benefit of checkpoint inhibitors compared to T cell therapies is the ease of generalizability to a spectrum of malignancies. Unlike ACT that relies on the presence of cancer-specific antigens and *ex vivo* manipulation, checkpoint inhibitors can be used as off-the-shelf treatments that can be applied across tumor types. There are currently several clinical trials examining the effects of checkpoint inhibitors in patients with advanced stage HPV-associated diseases. Ipilimumab (Yervoy by Bristol-Myers Squibb), an anti-CTLA-4 monoclonal antibody, is being tested by our lab in a phase I study of chemoradiation followed by ipilimumab for advanced cervical cancer patients (NCT01711515) as well as in a phase II study for HPV+ cervical cancer that is either metastatic or recurrent (NCT01693783). Dual checkpoint inhibition is being examined

with an anti-PD-L1 antibody combined with tremelimumab, another anti-CTLA-4 antibody, in 2 phase I trials for patients with solid tumors (NCT02261220) and patients with cervical cancer (NCT01975831). Furthermore, combination approaches that are examining a “brakes off, gas on” approach with the immunomodulator OX40 (MedImmune/AstraZeneca) and either anti-PD1 or anti-CTLA4 and are underway in phase I and II trials for patients with advanced stage solid tumors, including cervical cancers (NCT02205333).

Non-specific immunostimulants

Non-specific immunostimulants are agents that can stimulate the immune system through increasing the activity of certain immune cells, and that can act irrespective of antigen specificity. Toll-like receptors (TLRs) are a family of receptors expressed by APC and recognize pathogen associated molecular patterns (PAMPs), which are general patterns associated with different classes of pathogens. These PAMPs provide danger signals to APC to induce potent activation. For instance, TLR3 is found primarily in the endosomes of APC, including Langerhans cells (LC), the resident APC of the epithelial and mucosal layers where HPV infects, and recognizes viral dsRNA.^{119,120} Along these lines, it was shown that Poly-I:C, a dsRNA viral mimic TLR3 agonist, used as an adjuvant for an intravaginal HPV-vaccine increased mucosal E7-specific CTL responses in mice.¹²¹ A poly-lysine stabilized form of Poly-I:C, Poly-ICLC, was also shown to potently activate LC, which were subsequently able to induce robust E7-specific CTL responses *in vitro*.^{122,123} Similar results were seen via LC treatment with TLR8 or TLR7/8 agonists (3M-002 and resiquimod, respectively).¹²⁴ Importantly, these studies demonstrated that TLR agonists could activate LC that had naturally processed and presented E7 antigens, suggesting that such compounds could activate APC such as LC at the site of HPV infection where they take up antigens *in vivo*, and subsequently initiate HPV-specific CTL responses without the need for exogenous administration of HPV antigens. Alternative approaches have included using a mix of naturally produced cytokines from stimulated PBMC to activate HPV-exposed LC.¹²⁵

Immune modulating mechanisms caused by HPV-driven disease is a challenge for immunotherapies

HPV utilizes a variety of immune escape mechanisms to evade immune responses during initial and recurrent infections.¹²⁶ For example, HPV infection is associated with the suppression of MHC class I antigen presentation by infected cells via HPV16 E5, which functionally inhibits the transport of class I molecules through the golgi apparatus.¹²⁷ Additional evidence suggests that the interaction of α and β HPVs with LC (epithelial APC) may delay or prevent adaptive immune responses.^{128,129} Specifically, it has been shown that HPV16 entry via the annexin A2/S100A10 heterotetramer causes suppression of LC maturation resulting in failure of CTL induction.¹³⁰ These mechanisms may act as barriers for treatments of early stage disease (CIN I). In the case of progressive changes, while many of the aforementioned immunotherapies are able to overcome barriers associated with inducing

Table 1. – RECENT AND ONGOING CLINICAL TRIALS FOR IMMUNOTHERAPY OF HPV-INDUCED CANCERS

Platform	Treatment	Adjuvant	Route of Injection	Phase of Study	Patient Population	Immune Response	Clinical Response	References
Peptide	Four HPV16-E6 peptides	Yeast extract (Candin [®]) GM-CSF and Montanide ISA 51	IL SC	I	300 HSIL 90 recurrent, progressive or metastatic HNSCC	Ongoing	Ongoing	NCT01653249 NCT00257738
Peptide	MAG+B10:F24and HPV16 peptides					Ongoing	Ongoing	
Protein	peptidesMAGE-A3 and HPV16 peptides ProCervix	Imiquimod	ID	II	220 HPV16 ⁺ and/or 18 ⁺ women with normal cervical cytology or mild cervical cellular abnormalities	Ongoing	Ongoing	PC10VAC02 NCT01957878
Protein	HPV16 E7 + adeny/late cyclase (ProCervix)	Imiquimod	ID	I	47 HPV16 ⁺ and/or 18 ⁺ women with normal cervical cytology	Antigen-specific T cell responses	High viral clearance	PC10VAC01
DNA	Plasmid encoding HPV16 E7(detox) fused to calreticulin (CRT) (pNGVL-4a-CRT/E7(detox))	—	IM with electroporation and cyclophosphamide		21 HNSCC	Ongoing	ongoing	NCT01493154
DNA	Mixture of 2 plasmids encoding HPV16 and HPV18 E6 and E7 (VGX-3100)	—	IM with electroporation		167 CIN (II-III)	CTL Response See ref. 82	53/107 CR	84
Viral	Modified vaccinia virus Ankara encoding BPV E2(MVA-E2)	—	Intra-cervical		36 CIN(I-III)	HPV-specific CTL responses - all patients	34 CR	147
ACT	Autologous CTL generated and expanded from co-culture with partially resected tumor combined with low-dose IL2	—	IV	I	18 advanced stage cervical cancer	Ongoing	Ongoing	NCT01585428
Checkpoint Inhibitor	Ipilimumab (anti-CTLA-4) post chemo/radiation treatments	—	IV	I	28 advanced stage cervical cancer	Ongoing	Ongoing	NCT01711515

HPV-specific CTLs, they have all fallen short in effectiveness. Several underlying immunosuppressive mechanisms caused by HPV-driven disease are hypothesized to prevent CTLs from eliminating transformed cells or even homing into tumors. For example, the ability of CTLs to migrate into CIN lesions can be a predictor of disease regression, however it has been found that as patients develop higher grade CIN2/3 there are fewer CTL found within lesions due to multiple mechanisms including the dysregulation of addressin cell adhesion molecule-1.¹³¹ In addition to preventing CTL infiltration, as CIN progresses into high grade, the expression PD-1 and PDL-1 on cervical T-cells and DC is enhanced, respectively.¹³² These changes synergize with the increasingly immunosuppressive microenvironment created through the distorted equilibrium of type-1 T-helper (Th1) cells and Th2 cells.¹³³ Specifically, as disease advances from low grade CIN toward cancer it has been shown that Th1 cytokines (IL-2, IL-12, and TNF α) are reduced while Th2 cytokines are increased.^{132,134,135} As a result there is a parallel increase between CIN progression and the number of immunosuppressive cells found within the microenvironment of the CIN lesion. This cell population include Tregs^{136,137} and MDSC,^{138,139} both of which generate suppressive cytokines such as IL-10,^{140,141} IL-4, and TGF- β .¹⁴² and are involved in the inhibition of CTL responses.

Because these changes show that the microenvironment of lesions becomes more immunosuppressive as HPV-disease progresses it is reasonable to assume that patients with more advanced disease will be more difficult to treat with immunotherapy as they develop higher grades of CIN and eventually cancer. Work has shown that appearance of naturally occurring HPV-specific CTLs detected in peripheral blood capable of responding to the patient's HPV-type are not indicative of disease clearance.^{143,144} Paradoxically, some patients treated with a single dose of ACT comprised of a large number of ex vivo genetically engineered HPV-specific CTL have clinical responses involving regression of metastatic cancer lesions (2 complete and 1 partial response out of 9 patients).¹⁴⁵ These latter findings suggest that cervical cancer is amenable to T cell immunotherapy even in later stages of disease and CTL infiltration into tumors can occur, however it remains that single-pronged approaches as treatments in HPV-associated disease will most likely fail to overcome the multiple immune barriers generated within transforming/transformed tissue.

Summary and perspective

Promising clinical trials utilizing most of the aforementioned therapies that have recently been completed as of 2012 or are ongoing have been summarized in Table 1, for a comprehensive list see Kallhouf *et al.* 2014.¹⁴⁶ However, it still remains that initial attempts at generating effector T cell responses via therapeutic vaccinations have fallen short of expectations. Therefore, there is still a significant need to develop new treatments for HPV-associated diseases. Given our current understanding of HPV-cellular interactions, the interplay of the virus with the host immune system, and the hallmark changes that occur in the tumor microenvironment, the field may now be on the forefront of identifying clinically effective immunotherapies against HPV-transformed cells. One lesson learned from the previous

immunotherapy studies to date is that the *in vivo* induction or *ex vivo* generation of HPV-specific CTLs alone may not be sufficient for eliminating cancer cells in individuals with advanced-staged diseases. This is because HPV-driven cancers utilize multiple mechanisms to evade the immune system, some of which may still be unknown. Due to this, effective immunotherapeutic regimens in the future will likely require a multi-pronged approach. Therefore, in addition to the process of generating antigen-specific CTLs, the focus should shift to modulation of the tumor microenvironment, elimination of Tregs, inhibition of immune attenuation via checkpoint inhibitors, and stimulation of immune responses with immunostimulants, in a synergistic manner in order to most effectively battle HPV-associated diseases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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