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Anti-Tumour Treatment

The promise of combining cancer vaccine and checkpoint blockade for treating HPV-related cancer

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ABSTRACT

Human papillomavirus (HPV)-associated intraepithelial neoplasia or cancers are ideal candidates for cancer immunotherapy since HPV oncoproteins, such as E6 and E7 proteins of high-risk HPVs, could be utilized as foreign antigens. In HPV-associated cancers as well as nonviral cancers, the cancer cells may evade host immunity through the expression of immune checkpoint molecules, downregulation of human leukocyte antigen, and activation of immune regulatory cells. Because of these immune suppressive mechanisms, HPV therapeutic vaccines have shown little efficacy against HPV-associated cancers, although they have shown efficacy in treating HPV-associated intraepithelial neoplasias. Recently, checkpoint blockade emerged as a promising new treatment for solid cancers; however, these therapies have shown only modest efficacy against HPV-associated cancers. Here we reviewed literature analyzing a combinatory therapy using an immune checkpoint inhibitor and an HPV therapeutic vaccine for treating HPV-associated cancers to compensate for shortfalls of each monotherapy. Complimentary modes of T cell activation would be deployed; as vaccines would directly stimulate the T cells, while checkpoint inhibitors would do so by releasing inhibition. Some promising studies using animal models and early human clinical trials raised a possibility that such combinations may be efficacious in regressing HPV-associated cancers. Epitope spreading (the phenomenon in which non-targeted antigens become new targets of immune response) may play a critical role mechanistically. Currently ongoing studies will shed light as to whether such combination therapy would indeed be a promising new treatment paradigm. Current and future studies must also determine the adverse effect profile of such a combination treatment.

Introduction

Although human papillomavirus (HPV) strains had previously been described in the literature, the earliest published evidence of the link between HPV and cervical cancer dates to 1983 by Harald zur Hausen and colleagues [1]. Since then, over 200 HPV genotypes have been described [2], and the 2008 Nobel Prize was awarded to zur Hausen for his pioneering work in the field. HPV is a common virus that is efficiently transmitted by sexual exposure and skin-to-skin contact [2]. Certain genotypes of HPV (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) are categorized as high-risk (HR) due to their causative association with not only cervical disease (cervical intraepithelial neoplasia [CIN] and cervical cancer), but also penile, anal, vulvar, vaginal, oropharyngeal, and laryngeal precancers or cancers [3,4]. The number of yearly new cancer cases worldwide attributed to HPV infection is approximately 640,000 [5].

Virus-associated cancers, such as those caused by HPV and Epstein–Barr virus, are ideal candidates for immunotherapy because of the consistent presence of foreign antigens in comparison to nonviral cancers [6]. HPV proteins, particularly the E6 and E7 proteins, are not only thought to contribute to carcinogenicity [2,7], but are also rational cancer antigen targets for immunotherapy due to their foreignness [8]. Hence, E6 and E7 are perhaps the most common antigen targets for numerous HPV therapeutic vaccines that are at various stages of clinical development for HPV-related precancers, such as CIN and vulvar intraepithelial neoplasia (VIN) [9]. However, such therapeutic vaccines are currently not approved. Current treatment options for CIN, a representative HPV-related precancer, include excisional surgeries such as conization, which is associated with elevated risk of preterm delivery, premature rupture of membranes, and low birth weight [10]. HPV-related cancers are typically treated with multimodal therapy including surgery, chemotherapy, and radiation [11].

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Immune checkpoint inhibitors have recently emerged as promising new therapeutics for numerous cancers. The 2018 Nobel Prize was jointly awarded to James P. Allison, who showed anti-tumor effects of cytotoxic T lymphocyte-associated protein 4 (CTLA-4) blockade [12], and Tasuku Honjo, who discovered anti-tumor effects of programmed cell death 1 (PD-1) blockade [13]. Since the US Food and Drug Administration (FDA) approval of ipilimumab (an anti-CTLA-4 antibody) in 2011 and approval of pembrolizumab and nivolumab (both anti-PD-1 antibodies) for solid tumor treatment in 2014 [14], indications for checkpoint blockade continue to expand [15]. However, efficacy of checkpoint blockade has been variable across cancer types [15]. Good response rates of immune checkpoint blockade have been shown in a limited number of cancers, including melanoma with high mutational load [16], cancers with mismatch repair deficiency [17], and polyomavirus-induced Merkel cell carcinoma [18]. In contrast, the therapeutic response of checkpoint inhibitors in numerous other cancers has not been as promising, with reported response rates of approximately 15–40% [15].

Similarly, checkpoint blockade against HPV-related cancer has demonstrated low response rates in clinical trials. Overall response rates (ORR) of ipilimumab or pembrolizumab in cervical cancer, and pembrolizumab in HPV-associated head and neck squamous cell carcinoma have been reported at 3%, 14%, and 24%, respectively [19–21]. Nevertheless, pembrolizumab was recently approved for the treatment of advanced, treatment-resistant cervical cancer that expresses programmed death-ligand 1 (PD-L1) [21].

It would be prudent to ask whether the efficacy of HPV antigen-based immunotherapies, such as therapeutic vaccines and adoptive cell therapy, could possibly be augmented by checkpoint blockade. Considering the promise of therapeutic vaccination and checkpoint inhibition in the therapy of HPV-related precancers and cancers, respectively, we here review literature suggesting that a combinatory therapeutic approach may compensate for shortfalls of each separate monotherapy.

Promising HPV E6 and E7 antigen for immunotherapy

E6 and E7 antigens are considered ideal tumor antigens for clearance of HPV-related precancer and cancer. While up to 79% of women suffer from at least one HPV infection between 20 and 79 years of age [22], approximately two thirds of cervical HPV infections become undetectable by 12 months [23]. Therefore, not all HPV infections progress to invasive cancer; indeed, most HPV infections are cleared by host immunity [24]. The HPV genome contains several open reading frames (*L1*, *L2*, *E1*, *E2, E4*, *E5*, *E6*, and *E7*) [25], and the HPV E6 and E7 proteins are essential drivers for oncogenic transformation. Both E6 and E7 interact with products of tumor suppressor genes: E6 has been shown to bind and promote degradation of cell-encoded p53 and E7 interacts with the retinoblastoma susceptibility gene product, Rb [26,27]. Expression of E6 and E7 proteins has been shown to be necessary and sufficient for HPV 16 transformation of human cells [28,29].

Mechanisms by which HPV infection and/or CIN are eliminated by the immune system have been analyzed by examining T cell responses to HPV proteins, including those to E6 and E7 proteins [30–34]. In particular, the $CD8^+$ T cell responses to HPV 16 E6, detected using interferon gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assay, were most dominant among patients with various grades of CIN [31]. Our group followed HPV 16-postive women without cervical disease, and showed that cytotoxic T cell responses to E6 were significantly associated with clearance of HPV 16 infection [32]. When $CD4^+$ and $CD8^+$ T cell responses were examined in women being followed for abnormal Papanicolaou (Pap) smear results, responses to E6 were significantly associated with regression of lesions for both $CD4^+$ and $CD8^+$ T cells [33,34]. On the other hand, responses to E7 were only significantly associated with $CD4^+$ responses [34]. Therefore, E6 may be a better suited than E7 protein to be used as an antigen in HPV therapeutic vaccines.

The utility of E6 and E7 proteins as suitable antigens to treat HPV-related precancers has been demonstrated [35–39], and multiple candidate HPV therapeutic vaccines are in development. One version of peptide-based HPV therapeutic vaccine consisted of the HPV 16 E6 and E7 proteins with long peptides and an incomplete Freund's adjuvant (Montanide ISA 51) [37]. When women with HPV 16-positive high-grade VIN were treated, ORR was 79% (15 of 19 patients) [complete response (CR) in 47% (9 of 19 patients) and partial response (PR) in 32% (6 of 19 patients)] while 3 non-responders and 1 newly developed invasive cancer were observed at 12 months [37]. Patients who had CR at 3 months had significantly higher INF-γ-producing $CD4^+$ T cells and stronger proliferative responses when compared to patients who had no clinical response in their post hoc subgroups analyses [37]. The most common adverse event (AE) was swelling observed in all patients, and no AEs exceeding grade 2 were reported [37]. Similarly, another version of peptide-based vaccine consisting of HPV 16 E6 peptides and *Candida* skin test antigen as a vaccine adjuvant demonstrated 45% (14 of 31 patients) regression of cervical high-grade squamous intraepithelial lesions (HSILs), a significant increase in circulating T-helper type 1 (Th1) cells, and a significant decrease in HPV 16 viral load [35,36]. No vaccine-related AEs beyond grade 2 were observed [35,36]. Encouraging responses have also been reported from clinical trials testing DNA-based HPV therapeutic vaccines [38,40]. One such DNA-based vaccine, VGX-3100, which contains HPV 16 and 18 E6 and E7 genes, has shown efficacy in women with cervical HSILs in a randomized, double-blind, placebo-controlled phase IIb clinical trial. In per-protocol analysis, 49.5% (53 of 107 patients) of VGX-3100-treated patients showed histopathological regression to low grade squamous intraepithelial lesion (LSIL) or normal pathology at 36 weeks, compared to 30.6% (11 of 36 patients) in the placebo group ($p = 0.034$). Intriguingly, histopathological regression and clearance of HPV 16 were observed significantly more frequently in vaccinated patients compared to the placebo group for those with multiple HPV type infections as well with HPV 16 single infection. Erythema was significantly more common in the vaccinated group compared to the placebo group ($p = 0.007$) [38]. Given these results, a phase III trial of VGX-3100 for women with CIN is anticipated to start in 2019 [ClinicalTrials.gov registry number (NCT) 03185013 and NCT03721978]. Similarly, another DNA-based vaccine (GX-188E), which contains the same antigens arranged in a different manner, has been developed [40]. The investigators engineered a construct in which the HPV proteins are fused with the extracellular domain of FMS-like tyrosine kinase 3 ligand (Flt3L) and the signal sequence of tissue plasminogen activator in order to promote antigen presentation and trafficking [40]. They showed electroporation-enhanced immunization with GX-188E resulted in histological regression from CIN3 to normal and clearance of HPV in 78% (7 of 9 patients) patients in phase I study [40]. Interestingly, IFN-γ secreting cells against HPV 16/18 E6/E7 peptides in peripheral blood mononuclear cells (PBMCs) were detected in all patients in ELISPOT assay. Notably, responses to E6 in the assay were more prominently observed, compared to those to E7 [40]. Chills, injection site pain, swelling and hypoaesthesia were reported as vaccine-related grade 1 AEs [40]. E6 and E7 antigens are also suited to create not only HPV therapeutic vaccines, but also engineered T cell receptor T cells (TCR-T). Using retroviral transduction, Draper et al. constructed TCR-T cells to express the TCR against E6 [39]. These TCR-T cells recognized and killed HPV 16-positive cervical (CaSki) and head and neck cancer cell lines (SCC90 and SCC152) *in vitro*.

On the other hand, Rosenberg showed that therapeutic vaccine for cancer cure only small numbers of patients presenting data of numerous vaccines for various cancers (breast cancer, colorectal cancer, melanoma, ovarian cancer, and renal cell cancer) [41]. Likewise, the use of E6 and E7 proteins in treating cancer patients have been disappointing. In phase I/II of HPV16 E7 peptides and a helper peptide emulsified in

Montanide ISA 51 to HPV 16-positive recurrence or residual cervical cancer patients, Driel et al. reported that 74% (14 of 19 patients) were deceased within 22 months [42]. Likewise, on the report of HPV16 E6 and E7 overlapping long peptides in Montanide ISA 51 adjuvant for advanced or recurrent HPV 16-related cancer composed of cervical, vaginal, and anal cancer in phase II study, van Poelgeest et al. reported that 95% of patients (19 of 20 patients) died of progressive disease (PD) and tumor regression was not observed, although 85% tested patients (11 of 13 patients) had detectable vaccine-induced T cell responses by IFN-γ ELISPOT [43]. In these peptide-based vaccines trials for HPV-related cancers, no AEs exceeding grade 2 were observed [42,43].

Another approach taken to develop an HPV therapeutic vaccine uses live-attenuated *Listeria monocytogenes* (*Lm*) as an "antigen vector" for dendritic cells (DCs), and the investigators focused on treating patients with previously treated metastatic, refractory, or recurrent cervical cancer. *Lm*-based HPV therapeutic vaccine is proposed to efficiently present HPV antigens through both major histocompatibility complex (MHC) class I and II to $CD8^+$ and $CD4^+$ T cells [44]. In a dose-escalation phase I clinical trial, Maciag et al. evaluated the safety of genetically modified *Lm* that secretes fusion protein of *Lm*-listeriolysin O and HPV 16 E7 (*Lm*-LLO-E7) [45]. Patients were admitted to a hospital for 5 days following each infusion of *Lm*-LLO-E7 for observation. Five patients each received the nominal doses of 1×10^9, 3.3×10^9, and 1×10^{10} colony forming units (CFU) (n = 15 total), and they experienced pyrexia (100%), vomiting (60%), chills (53.3%), headache (53.3%), anemia (53.3%), nausea (46.7%), tachycardia (46.7%), and myalgia (26.7%) that were evaluated to be drug-related [45]. As *Lm* is known to have liver tropism, drug-related elevations in liver enzymes (gamma-glutamyl transferase, lactate dehydrogenase, and alkaline phosphatase) that were observed in 40% of patients (6 of 15) was not surprising. Hypotension which met the criteria as a dose-limiting toxicity was observed in 3 patients in the 1×10^{10} CFU cohort, so the administration of a higher dose, 3.3×10^{10} CFU, was cancelled [45]. Huh et al. evaluated the efficacy of *Lm*-LLO-E7 (ADXS11-001) in a single arm phase II clinical trial performed by Gynecologic Oncology Group (GOG-0265) [46,47]. Overall survival (OS) at 12 months was 38% (19 of 50 patients). This result was encouraging since it was higher than the predicted model-based 12-month OS of 25% [46,47]. Phase III clinical trial of ADXS11-001 for locally advanced cervical cancer after concurrent chemoradiotherapy is not currently recruiting participants, but is still actively being conducted by the Gynecologic Oncology Group (NCT02853604) [48].

Doran et al. conducted a dose-escalation phase I/II clinical trial utilizing HPV 16 E6 TCR-T for metastatic or refractory/recurrent HPV 16-positive cancers for patients who received a prior first line systemic therapy and were human leukocyte antigen (HLA)-A*02:01-positive (n = 12) [49]. Transduced autologous T cells were administered with aldesleukin following a lymphodepleting conditioning. No dose-limiting toxicity (any grade 3 or higher AE except for chemotherapy-induced myelosuppression) was observed, and 2 of 9 patients in the high dose cohort (1.7×10^{11} T cells infused) experienced PR [49]. A clinical trial of HPV 16 E7 TCR-T targeting HPV16-associated anal, cervical, oropharyngeal, penile, and vaginal cancers is currently enrolling participants (NCT02858310) [50].

While E6- and E7-targeted immunotherapy results, with a possible exception of those of the *Listeria*-based vaccine, have been disappointing when cancer patients were treated, the importance of targeting neoantigens and cancer germline antigens in HPV-related cancer has gained greater appreciation. Neoantigens and tumor antigens have been shown to be the effective targets of immunodominant response in patients who had complete regression of metastatic cervical cancer in a clinical trial by Stevanović et al. [51,52] utilizing an adaptive T cell transfer approach in which a large number of tumor-specific T cells individually harvested and expanded *in vitro* are re-infused in the same patient. This approach contrasts with a vaccination approach in which antigen-specific T cells are stimulated *in vivo*. In this clinical trial of infusion of T cells along with aldesleukin after preconditioning with cyclophosphamide and fludarabine for treating patients with metastatic cervical cancer (n = 9), 2 patients achieved CR and 1 patient achieved PR [51]. No cell infusion-related acute toxicities and no autoimmune AEs were reported. [51]. They evaluated the profile of antigen-specific tumor infiltrating lymphocytes (TILs) of 2 patients who achieved CR (HPV 16-positive squamous cell carcinoma and HPV 18-positive adenocarcinoma) with an IFN-γ ELISPOT assay using autologous DCs electroporated with neoantigens, cancer germline antigens, or HPV 16/18 E6/E7 antigens [52]. In the patient with HPV 16-positive squamous cell carcinoma, TILs responded not only to HPV 16 E6/E7 but also responded to 3 neoantigens [52]. In the patient with HPV 18-positive adenocarcinoma, TIL responses to cancer germline antigens (KK-LC-1) as well as HPV 18 E7 were identified [52,53]. Another group has reported that targetable neoantigens can be detected in most cervical tumors, commonly in known driver genes such as *KRAS*, *MAPK1*, *PIK3CA*, *ERBB2*, and *ERBB3* [54]. Therefore, targeting neoantigens would likely have an important role in developing effective immunotherapeutic measures even for HPV-related cancers.

Mechanisms of immune suppression in HPV-related cancers

Why would HPV antigen targeted vaccines be effective for patients with precancers, but not those with cancers? There are a number of immune suppressive mechanisms described in cancer patients that can explain why. The examples of these mechanisms are expression of immune checkpoint molecules, downregulation of HLA, activation of regulatory T cells (Tregs) and myeloid suppressor cells [55,56]. Yang et al. showed that the PD-1 expression on cervical lymphocytes increased significantly in HR-HPV-positive CIN 1 and CIN 2/3 in comparison to HR-HPV-negative cytologically normal cervical tissue, which suggests that the PD-1/PD-L1 axis plays a critical role in attenuating T cell responses [57]. Additionally, PD-1 expression on peripheral $CD8^+$ T cells of metastatic cervical cancer patients has been reported by Stevanović et al., and the amplification of PD-L1 and PD-L2 in cervical cancer has also been demonstrated using whole exome sequencing [52,58]. Furthermore, Kataoka et al. described cases of cervical and head and neck cancer in which HPV 16 integrated into the *PD-L1* locus resulting in an aberrant amplification of *PD-L1* gene and transcription of PD-L1 mRNA [59]. Therefore, it is possible that HPV itself may promote immune suppression.

The Cancer Genome Atlas Research Network reported somatic mutations in *HLA-A* (8% of 192 cases) and *HLA-B* (6% of 192 cases) genes exclusively in cervical squamous cell carcinoma using whole exome sequencing [58]. Interestingly, Ashrafi et al. demonstrated that surface HLA Class I in HPV 16 E5-expressing cells was decreased due to sequestration of HLA Class I in the Golgi apparatus [60]. Moreover, HPV alters methylation patterns in HPV-associated cancer, and Cicchini et al. showed decreased expression of HLA-A, B, C, E and G in HPV-positive keratinocytes in an E7-dependent manner [61]. Thus, HPV infection appears to play a role in inducing immune suppression by modulating the expression of HLA molecules.

Tregs may contribute to progression of HPV-related diseases. Molling et al. reported that Tregs in PBMCs were significantly increased in women who had persistent HPV 16 infection compared to patients who were negative for HPV or those who whom HPV 16 infection became undetectable [62]. Kojima et al. showed an inverse relationship between numbers of cervical Tregs in CIN lesions and their regression [63]. Scott et al. reported that expression of FoxP3, a transcription factor of Tregs, in the cervix was higher in HSILs than in LSILs [64]. Jordanova et al. reported that an overabundance of Tregs and a reduced $CD8^+$/Treg ratio were linked with poor prognosis in cervical cancer [65]. Therefore, Tregs appear to be a key biomarker worthy of monitoring during immunotherapy. On the reports of our clinical trial mentioned above (administration of HPV16 E6 synthetic peptides and *Candida* skin test as adjuvant for cervical HSILs), Greenfield et al. and

Coleman et al. stated that Tregs in PBMC at pre-vaccination were significantly lower in responders than in non-responders [35,36]. A study of the DNA-based VGX-3100 vaccine for CIN showed $CD8^+$/FoxP3 ratios were increased concomitantly with histologic CIN regression and viral clearance [66].

Suppressing myeloid cells may provide another mode of immunosuppression which could dampen responses to immunotherapy [56]. For a few decades, it has been known that the recruitment of myeloid suppressor cells at the tumor site negatively affected prognosis in patients with head and neck squamous cell carcinomas [67]. Furthermore, the presence of such myeloid suppressor cells in tumors of head and neck cancer patients has been shown to lead to the suppression lymphocytes by inhibiting interleukin 2 secretion [68]. Welters et al. have proposed a method of enhancing HPV therapeutic vaccine's effect by suppressing myeloid cells under conventional chemotherapy [69]. When 6 cycles of paclitaxel and carboplatin (TC) regimen were administered every 3 weeks to 6 patients with advanced recurrent or metastatic cervical cancer (first cohort), Welters et al. discovered that the nadir of myeloid cells was seen 1–2 weeks after the second cycle, while lymphoid cells increased in that same time period [69]. Then, HPV 16 peptide vaccine emulsified in Montanide ISA-51 was administered to a second cohort of 12 patients at 2 weeks after the second or third cycle of chemotherapy. The values of the lymphocyte stimulation test against HPV E6 and E7 peptide continued to be high even after chemotherapy [69]. Chemotherapy-related AEs in all patients and ulceration at the injection site in one patient were recorded [69]. Therefore, coordinating the timing of chemotherapy and therapeutic vaccination aiming to reverse the immune suppression due to myeloid cells may be a promising strategy.

The promise of combining E6 and E7 targeted therapy with checkpoint inhibitors

The indication for immunotherapy alone for cancer may be limited, as demonstrated by a number of studies in which HPV therapeutic vaccines were not able show clinical efficacy [42,43]. At the same time, in a phase I/II trial, ipilimumab monotherapy against 42 HPV-related metastatic or recurrent cervical cancers (29 squamous cell carcinoma and 13 adenocarcinoma) has not shown sufficient clinical response either, since only 2.9% (1 of 34 patients) showed PR and 29.4% (10 of 34 patients) had stable disease (SD) [19]. The median PFS and OS were 2.5 months and 8.5 months, respectively [19]. Likewise, clinical responses were marginal in a phase I/II trial (CheckMate 358) in which nivolumab monotherapy was utilized to treat recurrent or metastatic cervical, vaginal, and vulvar cancers (n = 24) with CR of 4.2% (1 patients), PR of 16.7% (4 patients), SD of 50.0% (12 patients), and PD of 29.2% (7 patients) at a median follow-up of 31 weeks [70]. Nevertheless, checkpoint inhibition is an important new treatment modality for PD-1/PD-L1-positive, advanced, and treatment-resistant cervical cancer as mentioned above. These results indicate that monotherapy treatment using checkpoint blockade or cancer vaccine for HPV-related cancer requires improvement. Therefore, there has been a shift to pair a stimulatory immune therapy with a checkpoint inhibitor.

The utility of this combination approach has been supported by some reports of preclinical and clinical studies (Tables 1 and 2). In a preclinical model using HPV E6/E7 expressing tumor cells, Rice et al. demonstrated robust anti-tumor activity of combination therapy comprised of checkpoint blockade (PD-1 inhibition) and cancer vaccine (adenovirus HPV 16 *E6/E7* construct). This combination treatment increased $CD8^+$ TILs and reduced PD-1 TILs after combination therapy [71]. In addition, the following combinations have been reported: combination of HPV 16 E7 long peptide with DCs and PD-L1 blockade in C57BL/6 mice, and combination of *Lm*-LLO fused-E6 vaccine (*Lm*-LLO-E6) and PD-L1 blockade in nude mice [72,73].

Interestingly, Dorta-Estremera et al. has combined three treatment modalities [HPV 16 E6/E7 peptides, CTLA-4 blockade, and 4-1BB

Table 1
Combination of stimulatory and inhibitory immune therapy for HPV-related preclinical cancer model.

Mice	Target antigens	Vaccine type	Checkpoint molecule	Cells	Summary
C57BL/6	HPV 16 E6/E7	DNA	PD-1	TC-1	Increase of $CD8^+$ TILs, decrease of Tregs/$CD8^+$ T cells, PD-L1 on tumor cells, and PD-1^+TILs were observed [71]
C57BL/6	HPV 16 E7	Peptide	PD-L1	TC-1	Tumor growth were suppressed by DCs targeting E7 and anti-PD-L1 treatment [72]
BALB/c nude	HPV 16 E6	DNA	PD-L1	TL-1, SiHa	Suppression of both of subcutaneous and metastatic HPV positive cancer were observed [73]
C57BL/6	HPV 16 E6/E7	Peptide	CTLA-4	mEER	Reduction of tumor growth and increase of $CD8^+$ T cells/MDSCs were observed by combination of HPV vaccine, 4-1BB agonist antibody, and CTLA-4 blockade [74]
C57BL/6	HPV 16 E6/E7	DNA	PD-L1	TC-1	Suppression of tumor growth were observed by antibodies of PD-L1 and HPV vaccine while no effect by antibodies of PD-L1 without vaccine [93]
C57BL/6	HPV 16 E7	DNA	PD-1/PD-L1	TC-1	HPV E7-specific $CD8^+$ T cells responses and were augmented by soluble PD-1 DNA [94]

HPV, human papillomavirus; PD-1, programmed cell death protein 1; TC-1, epithelial cell line of C57BL/6 transformed with HPV 16 E6/E7 and H-ras [95]; TILs, tumor infiltrating lymphocytes; Tregs, regulatory T cells; PD-L1, programmed death-ligand 1; DCs, dendritic cells; TL-1, HPV-infected lung cancer cell line [96]; CTLA-4, cytotoxic T lymphocyte-associated antigen; mEER, mouse tonsil epithelial cells expressing HPV 16 E6/E7 and H-ras [97]; MDSCs, myeloid-derived suppressor cells.

Table 2
Combination of stimulatory and inhibitory immunotherapy for HPV-related cancer.

Phase	Stimulatory immunotherapy	Checkpoint molecule (drug name)	Cancer type	Status*	NCT (Location)
I	T cell targeting HPV 16/18 E6/E7 with mutant TGF-β receptor	PD-1 (nivolumab)	HR-HPV + cancer	Recruiting	02379520 (US)
I/Ib	Adenovirus vaccine and Maraba oncolytic virus expressing E6/E7	PD-L1 (atezolizumab)	HPV-related cancer	Active, not recruiting	03618953 (Canada/US)
I/II	DNA vaccine with HPV 16/18 E6/E7 and FLT3L	PD-1 (pembrolizumab)	HPV 16/18 + CC	Recruiting	03444376 (South Korea)
Ib/II	MVA vector expressing HPV 16 E6/E7/IL-2	PD-L1 (avelumab)	HPV 16 + cancer	Recruiting; Satisfactory on phase I was reported by sponsor in 2018	03260023 (France)
II	Synthetic long peptides from HPV16 E6/E7	PD-1 (cemiplimab)	HPV16 + OPC	Recruiting	03669718 (US and Europe)
II	Synthetic long peptides from HPV16 E6/E7	PD-1 (nivolumab)	HPV 16 + cancer	Active, not recruiting; ORR was 33% (8/24 patients) [77]	02426892 (US)
II	DNA plasmid encoding HPV 16/18 E6/E7 and IL-12	PD-L1 (durvalumab)	HPV 16/18 + cancer	Recruiting	03439085 (US)

* Status as of May 26, 2019; NCT, ClinicalTrials.gov registry number; HPV, human papillomavirus; TGF, transforming growth factor; PD-1, programmed cell death protein 1; HR, high risk; PD-L1, programmed death-ligand 1; FLT3L, Fms-like tyrosine kinase-3 ligand; CC, cervical cancer; MVA, modified vaccinia virus Ankara; IL, interleukin; OPC, oropharyngeal cancer; ORR, overall response rates.

(CD137) agonist] and demonstrated increased numbers of $CD8^+$ TILs and decreased numbers of Tregs in C57BL/6 mice injected with tumor cells expressing HPV 16 E6, HPV 16 E7, and H-Ras [74]. One component of this treatment cocktail, 4-1BB, is a tumor necrosis factor receptor family member which is transiently upregulated in T cell activation, and its activation has been shown to enhance T cell proliferation, survival, and cytotoxicity [75]. Safety and tolerability of combining utomilumab (a 4-1BB agonist) and pembrolizumab in patients with advanced solid tumors has been reported by Tolcher et al., with 26.1% patients (6 of 23 patients) having CR or PR [76]. The combination therapy-related AEs included grade 3 adrenal insufficiency, and grade 3 hypokalemia [76].

A phase II human clinical trial was conducted to determine whether the combination of PD-1 blockade and HPV therapeutic vaccine (HPV 16 synthetic long peptides in Montanide adjuvant) was used to treat incurable HPV 16-positive cancer [77]. The ORR of this trial, approximately 33% (2 CR and 6 PR of 24 total patients) [77], is approximately twice that which was reported with checkpoint inhibitors alone [20,78,79]. The combination treatment was discontinued due to dose-limiting toxicity in 2 patients: one patient had a grade 3 transaminase elevation, and the other patient had grade 4 lipase and amylase elevations [77]. Furthermore, the responsiveness to the combination therapy was associated with PD-L1 expression in tumors [77]. These findings suggest that HPV therapeutic vaccines may be more practical for treatment of HPV-related cancers when combined with checkpoint blockade. Massarelli and colleagues [77] have provided a proof of concept for efficacy in combining an HPV-targeted immunotherapy and a checkpoint inhibitor for treating HPV-related cancers. Additional ongoing clinical trials of similar combinations are summarized in Table 2.

Proposed mechanisms of immune activation by combined therapy of therapeutic vaccine and checkpoint blockade

HPV-targeting immunotherapies are designed to stimulate antigen-specific T cells directly whereas checkpoint inhibitors activate T cells indirectly by releasing inhibition by targeting checkpoint molecules. Therefore, the rationale for combining them would be to enhance anti-tumor immune response with a hope to exert an amplified anti-tumor effect.

In the context of therapeutic vaccine treatments for cancer, epitope spreading (also referred as antigen spreading or determinant spreading) is the phenomenon through which non-vaccine-targeted antigens become additional targets of immune response [80–82]. Epitope spreading has been reported to occur in cancer vaccine recipients whose tumors have regressed [82]. For example, Butterfield et al. reported epitope spreading only in patients who showed CR to a single epitope immunization containing the melanoma antigen recognized by T cells (MART-1) peptide and not in non-responders [83]. Similarly, Corbière et al. detected a $CD8^+$ T cell clone specific for a neoantigen, specifically a mutated mitochondrial enzyme caseinolytic protease, among TILs from a patient with metastatic melanoma who responded to vaccination with the antigenic peptides melanoma antigen 1 (MAGE-1) and melanoma antigen 3 (MAGE-3) [84].

Similarly, epitope spreading may lead to clinical response in patients with HPV-related diseases after HPV therapeutic vaccination. Epitope spreading was demonstrated first, to our knowledge, in two patients who received HPV 16 E6-based HPV therapeutic vaccine in our clinical trial treating women with biopsy-proven cervical HSIL [35,36]. T cell reactions specific for non-vaccine targeted antigen (HPV 16 E7) were detected in two patients using IFN-γ ELISPOT assay [35,36]. One patient was a responder and no longer had HSIL after vaccination while the other one was a non-responder. As this was a phase I clinical trial focusing on evaluating safety with a follow-up time known to be too short for a full evaluation of efficacy, the role of epitope spreading will be further investigated in an ongoing phase II clinical trial. Similarly,

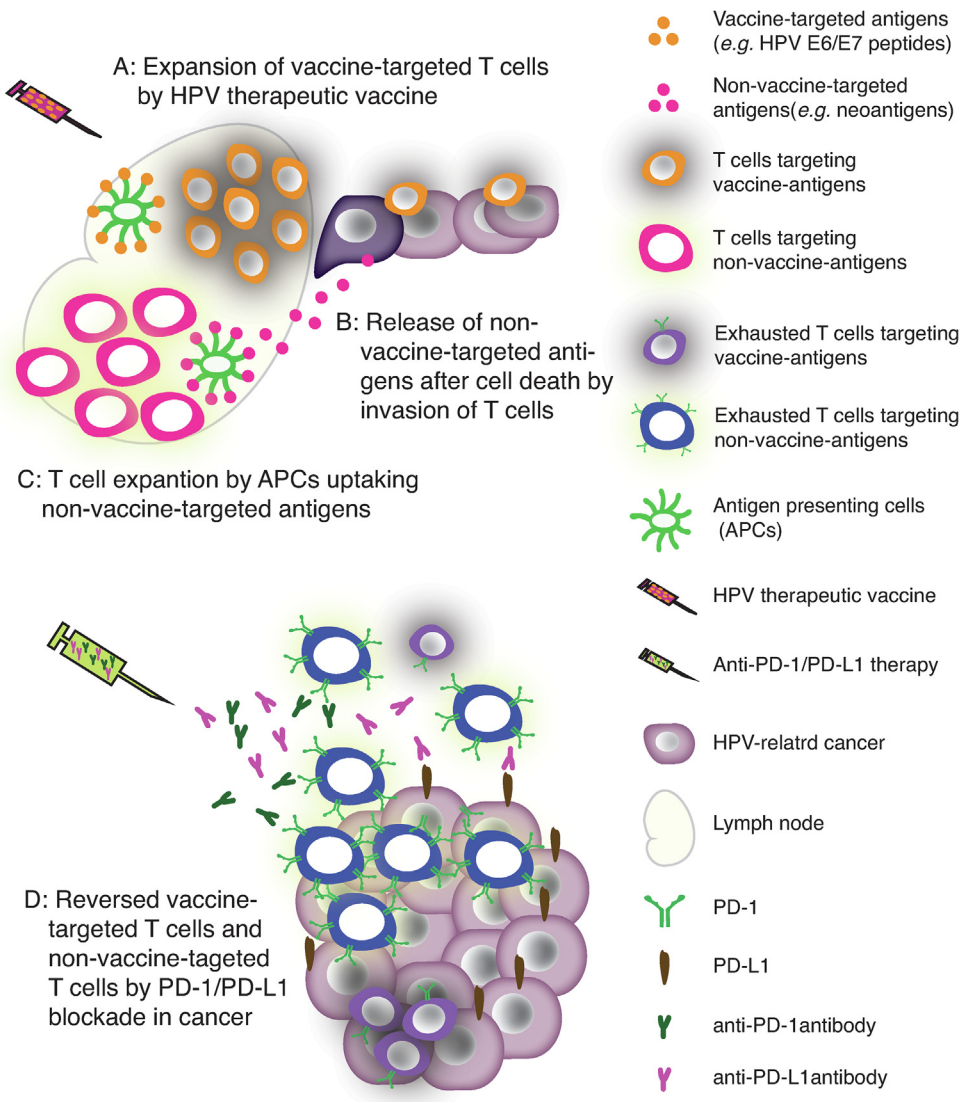

Fig. 1. Possible mechanisms of cancer vaccines and checkpoint blockade in therapy of HPV-related cancer. In lymph nodes, vaccine-targeted T cells are activated after priming of antigen presenting cells (APCs) such as dendritic cells (DCs) pulsed HPV antigens (A). After disruption in cancer cells by vaccine-targeted T cells, non-vaccine-targeted antigens (*e.g.* neoantigens) are released resulting in epitope spreading (B). DCs phagocyting non-vaccine-targeted antigens prime another T cell clone (C). Formation of oligoclonal T cell in HPV-related cancer by PD-1 or PD-L1 blockade. If PD-1 are expressed on vaccine-targeted T cells and non-vaccine-targeted T cells, these cells will be activated by PD-1 or PD-L1 blockade followed by expansion of T cell clone in tumor (D).

Krupar et al. reported T cell epitope spreading to HPV 16 E6 by *Lm*-LLO-E7 vaccines (ADXS11-001) in patients with HPV-associated oropharyngeal cancer, as a vaccine-induced HPV 16 E6 specific IFN-γ response was detected [85].

Taken together, epitope spreading could be a mechanism by which the combination of cancer vaccines targeting HPV 16 E6 and/or E7 and checkpoint blockade may be effective (Fig. 1). After vaccination, the T cells in lymph node are primed by DCs pulsed with HPV therapeutic vaccine-targeted antigens (*e.g.*, E6 and E7 peptides) (Fig. 1A). After cancer cells are killed by the vaccine-targeted T cells, non-vaccine-targeted-antigens are released, thereby making them available to be primed and targets of T cells (Fig. 1B). DCs phagocytosing the non-vaccine-targeted antigens prime another T cell which can then eliminate cancer cells (Fig. 1C). Furthermore, checkpoint blockade, such as inhibition of PD-1/PD-L1, can help activation of T cells after epitope spreading in tumors (Fig. 1D), similar to the phenomenon described by Memarnejadian and colleagues [86].

Oligoclonal expansion of tumor-infiltrating T cells (presumably, expansion of tumor-specific T cells) has been shown in responders of checkpoint blockade such as anti-PD-1 and anti-PD-L1 therapies through high-throughput sequencing [87,88]. In melanoma patients with a positive response to nivolumab, expansion of T cell clones after treatment resulting in less T cell diversity was demonstrated by examining the distribution of unique CDR3 sequences of T cell receptor β (TCR-β). In addition, the diversity index of TCR-β repertoire, a measure of clonality of TILs, was significantly lower in responders compared to non-responders [87]. Similarly, a more restricted TCR-β chain usage significantly correlated with clinical response to pembrolizumab in patients with metastatic melanoma [88].

Expansion of T cell responses, including those elicited through epitope spreading, may contribute to side effects if the targets of newly generated T cell responses are self-antigens. Various manifestations of autoimmune disease are thought to be induced by high diversity of T cell clones resulting from cancer vaccines and checkpoint blockade. In the study of melanoma with nivolumab plus peptide vaccine, rare treatment-related adverse events (TRAEs) of grade 3 and 4 were reported, including bilateral optic neuritis (2%, 1 of 49 patients) and pneumonitis (4%, 2 of 49 patients) [89]. As mentioned earlier, Massarelli et al. reported rare grade 3 transaminitis and grade 4 lipase elevation in a study of HPV-positive cancer treated with combination of HPV therapeutic vaccine and nivolumab [77]. On the other hand, combining two checkpoint inhibitors has resulted in higher frequencies of severe autoimmune AEs. In a study of melanoma patients treated with combination ipilimumab and nivolumab (NCT02519322), 73% of patients (8 of 11 patients) showed grade 3 TRAEs (elevated liver enzymes, arthralgia, thyrotoxicosis, pneumonia, myositis, colitis, dehydration, and falls) [90]. In another study of melanoma patients treated with combination ipilimumab and nivolumab (NCT01844505), 59% of

patients (184 of 313 patients) showed grade 3 or 4 TRAEs (diarrhea, colitis, and increased liver enzyme) [91]. Therefore, combining a single immune checkpoint inhibitor with therapeutic vaccine may have a more favorable AE profile compared to dual immune checkpoint blocker treatments.

Conclusions

During the past decade, much progress has been seen in treating cancers by harnessing the power of the immune system through the use of checkpoint inhibitors. For HPV-related cancers, promising candidates for therapeutic vaccines for treating precancers are in development, but they have not shown efficacy against cancers. Available data in animal models and early stage clinical trials demonstrated encouraging data of combining a cancer vaccine and a checkpoint inhibitor for treating HPV-related cancers, and many more clinical trials are underway [92]. In time, the full potential of such combination therapies may be revealed along with mechanisms as to how the immune cells (DCs, T cells, and immune regulatory cells) and the antigens (viral antigens, cancer germline antigens, and neoantigens) are involved; the interaction of which may cause reversal of immune suppression, epitope spreading, and expansion of T cell clones.

Declaration of Competing Interest

M.N. is one of the inventors named in the patents and patent applications of HPV therapeutic vaccine (PepCan). Other authors have no conflicts of interests. This work was supported by the National Institutes of Health (R01CA143130, USA), the Arkansas Biosciences Institute (the major component of the Tobacco Settlement Proceeds Act of 2000, G1-52249-01, USA), and the Drs. Mae and Anderson Nettleship Endowed Chair of Oncologic Pathology (31005156, USA).

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