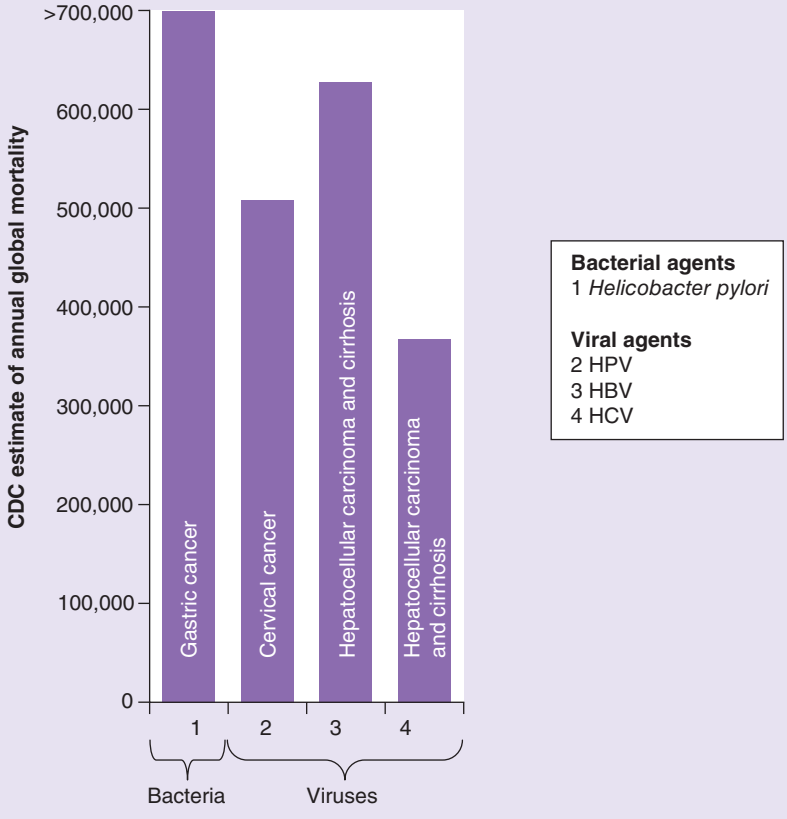

Figure 1. Cancers attributable to infectious agents and their associated global mortality.
Data taken from [109].

and 25%, respectively (Figure 2A & B). However, closely related types form 'species' and most of the high-risk types are phylogenetically related to either the HPV species 7 (HPV-18, -39, -45, -59 and -68) or species 9 (HPV-16, -31, -33, -35, -52 and -58) based on the sequences of their L1 capsid protein [7–9].

Within the context of the divergent genomes, HPV-16 and HPV-18 types have been shown to account for approximately 70% of invasive cervical cancers [5]. HPV-16 has been strongly associated with both anogenital and oropharyngeal cancers as well, and the rate of new oropharyngeal diagnosis is increasing to such a degree that it has been estimated that this form of HPV-induced cancer will surpass cervical cancers caused by HPV by the year 2020 in the USA [10]. The fact that two types alone account for the vast majority of aggressive infections suggests that intervention directed specifically at these types has the potential to significantly reduce the burden of disease. To that end, two prophylactic vaccines are currently available that have been shown to be highly effective at preventing infection of cervical tissue by the HPV-16 and -18 types [11–14], which in turn may lead to a lower incidence of disease. However, unfortunately, data released by the CDC reveals that only 32% of teenagers who qualify for immunization are getting all three of the recommended doses of the bi- or quadra-valent vaccines in the USA [104], suggesting that the majority of teens remain susceptible to infection with the high-risk HPV-16 and -18 types, and thus susceptible to high-grade disease. Study of these same vaccines in patients who are already infected with either of these types suggests that immunization affords no therapeutic effect on pre-existing cervical infection or the presence of cervical lesions [15,105]. Therefore, a gap in immune-based coverage of established cervical disease currently exists in patients who were infected prior to approval of the prophylactic vaccines, as well as those who were approved for vaccination but declined to initiate or finish the three-dose regimen prior to exposure and infection. The development of an immune-based interventional therapy is therefore of paramount importance for this group of patients, as it would afford them the possibility of a noninvasive, nonsurgical option for treatment of HPV infection.

Etiology of HPV-associated disease & recruitment of the host immune system

The progression of cervical HPV infection from clinical presentations of atypical squamous cells of unknown significance to cervical intraepithelial neoplasia (CIN) to overt cervical cancer represents the establishment and loss of control of a chronic viral infection within the cervical tissue [16–21]. While precancerous lesions leading to HPV-induced oropharyngeal and anogenital cancers are far harder to detect prior to the onset of a cancerous state, it is also possible that a similar progression of cellular changes occurs in these tissues as occurs in the cervix. Interestingly, while approximately 25% of the oropharyngeal cancers have an HPV-associated etiology [22–25], with as many as 90% of those associated with HPV-16, some reports suggest that patients with an HPV-positive carcinoma of the upper respiratory tract have a more favorable prognosis compared with the HPV-negative head and neck cancers, which are associated with other risk factors such as excessive tobacco and alcohol use. This may have to do with a different genetic profile in HPV-associated cancers and the mechanism of E6 and E7-mediated carcinogenesis [26,27]. The aforementioned prophylactic vaccines work based on the concept of antibody responses to L1 capsid proteins of the virus [12–14,28]; control or elimination of established infection is not mediated by the immune responses driven by these vaccines. Historically, the control or elimination of a chronic viral infection is a task that has fallen to the T-cell arm of the immune response [29–35] and thus it is believed that an effective immunotherapy for established HPV infection will also require this type of response [36–39]. Moreover, as HPV establishes not only chronic infection,

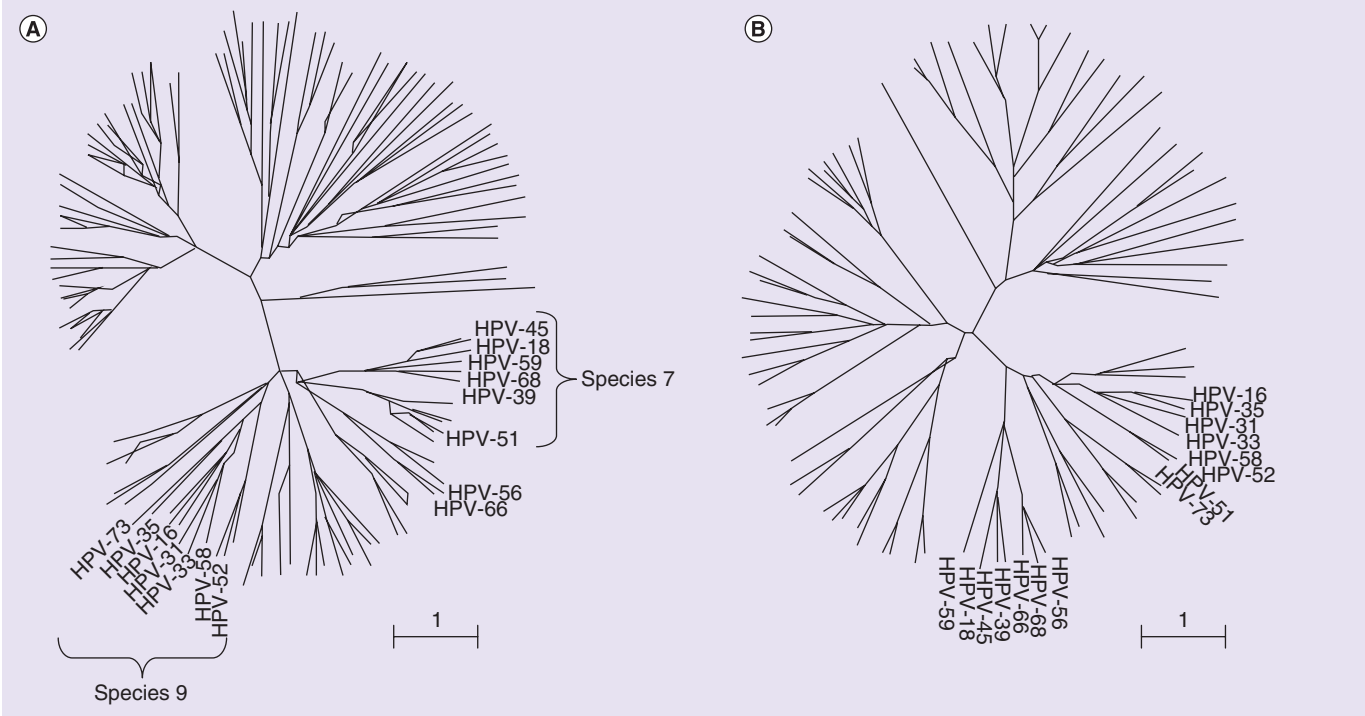

Figure 2. Divergence of human papillomavirus sequences and tumorigenic potential based on the viral protein.
(A) Phylogenetic tree of L1 proteins of different HPV types. A total of 145 L1 protein sequences, each representing a HPV type, were included in the phylogenetic analysis. Types comprising species 7 and 9 are indicated as reported by [7–9]. **(B)** Phylogenetic tree of E6 proteins of different HPV types. A total of 86 E6 protein sequences, each representing a HPV type, were included in the phylogenetic analysis. In both **(A)** and **(B)**, the placements of the 15 most common HPV types that are associated with cervical cancer are indicated. The bar represents genetic distance corresponding to 1% divergence between sequences.
Data taken from [Inovio, Unpublished Data].

but in the case of HPV-16 or -18 may also induce a cancerous disease state, it is worth noting that many immunotherapies being developed for the treatment of solid tumors also heavily rely on T-cell responses [40–44]. Taken together, such concepts reinforce the notion that a strong T-cell response may be important not only for control or elimination of infection in precancerous states of HPV-16 and -18 infection, but also possibly for control or elimination of HPV-induced cervical cancer. Such notions are not purely speculative; a number of published clinical reports of control and regression of HPV-related disease highlight the associations and correlations of HPV-specific T-cell responses in peripheral blood as well as in tissue into which lymphocytes have successfully penetrated [45–48]. For instance, in a study of a cohort of patients with biopsy-confirmed CIN, Kadish *et al.* found a statistically significant correlation ($p < 0.05$) between cellular immune responses to the E6 and/or E7 antigens and HPV negativity in follow-up clinic visits [49]. Moreover, in a year-long study of over 100 patients with diagnosed cervical disease, this same group found that cell-mediated immune responses to E6 or E7 peptides correlated with a 60.0 and 68.3% spontaneous regression rate, respectively [50].

While HPV-related oropharyngeal and anogenital disease is on the rise, to date the greatest number of studies of HPV-driven pathology has occurred in patients presenting with infection of cervical tissue. Of particular interest in terms of informing the construction of an effective immunotherapy for HPV-16 or -18 is the detection of cellular immune responses in patients who show control and regression of HPV-associated changes to cervical tissue [46–50]. A number of retrospective clinical research studies have been completed, with both immune phenotyping from peripheral blood as well as immunohistochemical analysis of archived pathological samples being performed. One study in particular by Peng *et al.* identified a specific HLA class II-restricted epitope within the HPV E7 viral protein in patients who had regressed from high-grade intraepithelial lesions to a state of cleared CIN lesions and reduced HPV viral load [47]. Immune profiling of peripheral blood mononuclear cells (PBMCs) that activated in the presence of peptides spanning this epitope *in vitro* revealed that the response was restricted to the $CD4^+$ T-cell subset, and that these $CD4^+$ T cells showed a Th1-biased cytokine production profile in which IFN-γ and TNF-α were produced in abundance (up to 12.3% and 40.41%, respectively), whereas no robust increase in production of the Th2-associated cytokines IL-4 and IL-10 were noted [47]. Moreover, analysis of pathological tissue samples has revealed that patients who have undergone spontaneous regression exhibit a significantly increased number of infiltrating $CD8^+$ T cells within cervical tissue ($p < 0.007$ for regressors vs nonregressors), whereas those that have persistent disease do not follow this profile [46]. Conversely, Trimble *et al.* note that the presence of HPV-16-associated CIN actively inhibits the incursion of $CD8^+$ T cells into

the infected tissue, a finding that underscores possible mechanisms of viral persistence as well as the importance of the presence and activity of $CD8^+$ T cells within infected tissue for control and elimination of infection [51]. Prospective studies have largely supported the data generated by retrospective studies, especially in regards to the antigens to which patients are responding and the types of responses being generated. Specifically, T-cell responses are frequently noted to the E6 or E7 antigens within regressed patients [47–50,52,53] and cytokine profiling of the T-cell responses from these patients skews towards a Th1 bias [47,48,50,52,53]. One study of patients showing regression of HPV-driven pathology postintervention without disease recurrence found that T-cell responses were dominated by production of IFN-γ to both the E6 and E7 viral antigens. Analysis of patients who had undergone regression but subsequently had been diagnosed with recurrent disease did not show this type of profile [53]. Moreover, the recurrence-free group showed a high T-cell proliferative index in response to HPV antigen, while the group experiencing disease recurrence, as well as a control group that was in the process of active HPV infection, did not exhibit any robust proliferative function [53]. These data are in agreement with data released from Dillon *et al.* in which proliferative capacities of T cells isolated from patients with persistent HPV infection were approximately twofold lower than those isolated from patients who exhibit spontaneous resolution of infection [45]. Taken together, the outcomes of studies such as these paint a picture in which a cellular immune response, particularly a Th1-biased response, seems to correlate strongly with spontaneous regression of HPV-associated disease within cervical tissue. It would therefore stand to reason that these data serve as a template for the creation of immunotherapies for active HPV infection. The logical first step in the creation of such a therapy would be to aim for the induction of HPV-specific $CD8^+$ T cells as well as $CD4^+$ T cells whose functional profile skews towards a Th1-biased immune response in support of CD8 activity.

Genomic diversity & antigen targets for *de novo* HPV immunotherapy

As can be seen from Figure 2A & B, genome diversity has played an important role in the development of anti-HPV preventive and therapeutic vaccines. The L1 protein sequences between two species share approximately 62–65% identities, while the E6 protein sequences between two species share approximately 44–58% identities. As expected, the L1 and E6 protein sequences within each species 7 or 9 are phylogenetically closer and the identities range from 76 to 91% for the L1 proteins and from 58 to 85% for the E6 proteins. The high degree of divergence across genotypes suggests that effective interventions would likely need to target specific genotypes. Indeed, while studies have shown that the HPV vaccines could induce cross-protection against phylogenetically related HPV types [11,54], the commercially available prophylactic vaccines (Gardasil® [Merck & Co.] -16, -18, -6 and -11; Cervarix® [GlaxoSmithKline] -16 and -18) and the therapeutic vaccines in clinical development have all focused on a strategy of developing type-matched vaccines. It remains to be seen whether 'species-matched' vaccines covering multiple related HPV genotypes are feasible as a means to better handle genomic diversity. But for now, much of the attention has focused on targeting HPV-16 and -18, because they account for the bulk of invasive cervical cancer.

A number of immune-based therapies have been tested for the treatment of HPV-16 and HPV-18 infection (Table 1) [45,55–61,106,107]. While the L1 protein, which encodes for the viral coat, is the main target of prophylactic vaccination [12,14], this viral antigen is less desirable as a target for an immune response once infection has been established. This is due in part to the fact that it is expressed predominantly in terminally differentiated keratinocytes as opposed to all layers of epithelium [62], and due to the fact that advanced-stage infections of cervical tissue show a reduced level of L1 transcript and protein [62–64]. In contrast to L1, proteins expressed early in the HPV replication cycle, such as E1, E2, E6 and E7, have all been identified as strong targets for the generation of an immunotherapy due largely to the fact that their expression is retained through multiple stages of infection and, in the case of E6 and E7, may even increase as the severity of pathology increases [65,66]. While E1 acts as an ATPase and helicase, E2 is a sequence-specific DNA binding protein that has significant roles in DNA replication, transcription and involvement in regulation of migration of viral genomes to daughter cells during mitosis of infected cells [18,62,64]. Thus due to their critical role in viral DNA replication, expression of E1 and E2 proteins are retained throughout multiple stages of infection, making them an attractive target for immune responses aimed at eliminating cells that are persistently infected, regardless of the stage of pathogenesis.

Indeed, previous studies in canines employing immunization with DNA vaccines encoding codon-optimized E1 or E2 antigens resulted in either complete protection from the growth of papillomas after viral challenge [67,68], or a complete regression of papillomas after postchallenge immunization [67,68], reinforcing the concept that these antigens may serve as strong targets for the induction of a protective therapeutic immune response. Additionally, studies performed in rabbits employing DNA vaccination have shown that immunization with a combination of the E1 and E2 antigens results in a significantly decreased number of papilloma formations after challenge, as well as a significantly increased frequency of papilloma regression when immunization occurs after challenge [67,68]. As E1 and E2 are not expressed on the surface of infected cells or virions, one can easily speculate that protective immune responses generated in these animal studies are cellular responses in which the immune system recognized infected cells in the form of antigen presentation through MHC class I or class II. The promising data generated from these animal studies, in addition to retrospective clinical data correlating T-cell proliferative responses to E2 with spontaneous regression of lesions in infected patients [67,68], aided in the establishment of clinical trials aimed at the induction of cellular immune responses to the E2 antigen. In one series of studies, a modified vaccinia ankara vector encoding the E2 antigen was given to patients who had established HPV-induced cervical lesions, ranging from CIN1 to CIN3 (Phase I/II trial) to high grade (Phase II trial). Patients treated with this therapy

showed a variety of positive responses, ranging from complete elimination of cervical lesions, to regression from CIN3 to CIN1, to significant reductions in HPV viral load [58,69]. While antibody responses to E2 were measured within these patients, review of trial data suggests that cellular immune responses, and in particular cytotoxic T lymphocyte (CTL) responses were of paramount importance in regards to positive post-treatment outcome [58,69], reinforcing the notion that Th1-biased cellular immune responses are important for control of HPV infection not only in animal models but also in clinical settings. Unfortunately, vector-specific antibodies did develop in immunized patients enrolled in the trial, blunting the ability to continue to boost poorer responders and indeed, some patients were noted to have recurrence of lesions after study termination [58,69]. Moreover, the therapy required direct injection into uterine tissues in order to be effective, which is considered to be an unpleasant, invasive procedure.

In contrast to L1 and E2, the E6 and E7 proteins are constitutively expressed in all levels of infected epithelium at high levels and integrate into the host genomes as the host cells transform into cancer, making them ideal targets for immunotherapies aimed at the control and/or elimination of chronic infection or transformed precancerous/cancerous tissue [62–64]. As they impart oncogenic potential to HPV viruses [62–64,70], these proteins are of particular interest in the creation of an immunotherapy for high-risk HPV types such as HPV-16 and HPV-18. While the E6 protein interferes with p53, disrupting normal cell-cycle checkpoint functions [62–64,70], E7 interacts with and disrupts the natural tumor-suppressor functions of the Rb protein [62–64,70] further aiding in cellular transformation. Similarly to the E1 and E2 antigens, a number of animal studies of immunization using E6 and E7 as targets for cellular immune responses have been performed, with many promising results, including a study by Yan *et al.* resulting in 100% prophylactic protection from the establishment of tumors after challenge, as well as significant retardation ($p < 0.01$) of the growth of established, aggressive tumors [62–64,70,71]. Similar tumor challenge results were generated by Sharma *et al.* using chimeric virus-like particles expressing E7 and L1 [72]. Such studies have been aided by the development of aggressive HPV-based tumor cell lines, such as the TC-1 cell line, which has been invaluable for animal studies of HPV-16 E6 and E7-driven tumors [62–64,70]. In addition, as noted above, clinical studies of spontaneous regression of HPV-associated disease in patient populations further supports the notion of strong cellular responses to these antigens contributing to the elimination of HPV-driven pathology, as multiple groups have noted a correlation between this arm of the immune response specifically directed towards the E6 and E7 antigens and the resolution of infection. Natural progression of these analyses has lead to the initiation of a wide number of clinical trials aimed at the recapitulation of these types of immune responses through immunization.

E6- and/or E7-based immunotherapies in the clinic

A number of trials have targeted the E7 protein alone as the key antigen [36,62–64,70,73–76], and the outcomes of these trials have varied in regards to immunogenicity and clinical impact. For example, HPV-16 E7 peptide-based vaccinations of patients with refractive cervical cancer showed little benefit with regards to disease progression with 15 of the 19 enrolled patients showing progressive disease, while two showed stable disease and two showed regression only after the administration of chemotherapy [75]. However, of note, specific immune responses driven by administration of the peptide in this case could not be detected. Conversely, patients in trials receiving E7 lipopeptide, E7 fusion protein or E7 DNA-based immunotherapies saw antigen-specific immune responses following immunization in the form of IFN-γ production from isolated PBMCs [74]. One trial conducted with the E7 fusion protein (E7 linked to *Haemophilus influenzae* protein D) was a Phase I study for which efficacy was not an end point [1,77] but stringent immune analysis from patient PBMCs was able to identify vaccine-induced IFN-γ production from both the $CD4^+$ and $CD8^+$ T-cell compartments. Additional trials linked the E7 antigen to HSP65 from *Mycobacterium bovis* and immunized patients who had been diagnosed as CIN3 positive [77–79]. Results from these trials suggest that immune responses were mounted to the vaccine in terms of inflammatory responses within cervical tissues after vaccination, which were found to correlate strongly with CIN regression [77–79]. However, the percentage of women displaying resolution of CIN3 to below CIN1 was 22.5%, which is too similar to reported rates of spontaneous regression in an unvaccinated cohort [78] to be able to ascribe this phenomenon solely on vaccination. By contrast, 55% of enrollees in this study mounted a partial response in the form of reduction in lesion size of 50% or greater, which was seen as an encouraging result. Follow-up studies of this same platform were, indeed, able to detect Th1-biased cellular immune responses in the form of IFN-γ production in an ELISpot assay, and found that the majority of the patients exhibiting complete regression of cervical disease showed concominant increases in ELISpot magnitude. Unfortunately, as with the previous study, a complete response was not noted in a high enough percentage of patients to be able to ascribe resolution solely through immunization (35% complete response [79]). A lipopeptide-based trial revealed that responses generated did not lead to control or regression of disease [74] and the first DNA-based therapies have shown limited promise. A dose-escalation trial of plasmid DNA encoding a transgene that produced E7 linked to HSP70 showed limited efficacy at the highest dose, with low induction of responses in the IFN-γ ELISpot assay and a resolution rate of 33% [76]. An additional trial using plasmid DNA encoding a 13-amino acid sequence with high homology to E7 was able to identify a subset of patients that, upon therapy completion, showed no histological or cytological evidence of disease and no detectable level of HPV DNA by PCR reaction [80]. It should additionally be noted that the trial in which the DNA-based therapy was tested restricted enrollees to an HLA-A2 haplotype, which constitutes a drawback in this therapy. While helpful in the context of early immunotherapy development, a truly effective therapy would need to be functional irrespective of patient HLA haplotypes. The differences in outcomes of these many trials targeted specifically at E7 may speak to the method of

Table 1. Selected human papillomavirus immunotherapeutic clinical trials.

Antigens	Regimen	Patient population	Phase	Immune response/clinical efficacy	Ref.
Protein/peptide platform					
L2/E6/E7	Weeks 0, 4 and 8	Normal/healthy	I	Eight out of 23 patients show IgG, 25 out of 32 show proliferation, eight patients show IFN-γ ELISpot postivity/NA	[55]
L2/E2	Weeks 0, 1 and 4, or weeks 0, 4 and 8	Genital wart (HPV-6)	I	Ten patients show IgM and/or IgA, 32 patients show IgG, 32 patients show proliferation/ NA	[56]
E7 (fused with *Haemophilus influenzae* protein D)	Weeks 0, 2 and 4	CIN (HPV-16)	I/II	$CD4^+$ and $CD8^+$ IFN-γ production by ICS, IFN-γ production by ELISA, IgG/NR	[69]
E6/E7	Weeks 0, 3, 6 and 9	VIN (HPV-16)	II	$CD4^+$ activity in 94% of patients, $CD8^+$ activity in 78% of patients, IFN-γ ELISpot positivity in 83% of patients, reduced Tregs in responders/15 out of 19 patients with objective clinical response on 12-month follow-up; nine with complete response and six with partial response	[52,92]
E7	Weeks 0, 3, 6 and 9	Cervical cancer (HPV-16)	I/II	NR/four out of 18 with stable disease in 2-week follow-up. None with stable disease in 2–22-month follow-up	[67]
Viral vector platform					
E6 and E7 (vaccinia)	Week 0	Cervical cancer (HPV-16 and 18)	I/II	IgG in three out of eight, CTL response in one patient/NR	[57]
E6 and E7 (vaccinia)	Weeks 0 and 2	Cervical cancer (HPV-16 and -18)	I	IgG in eight out of 29 patients, CTL response in four out of 29 patients/NA	[78]
E2 (vaccinia)	Weeks 0, 1, 2, 3, 4 and 5	CIN (HPV-16 and -18)	II	NR/19 out of 32 patients with complete regression of at least one lesion present at enrollment, 12 out of 32 patients HPV DNA negative after treatment	[58]
E6/E7 (MVA also encoding IL-2)	Three doses	CIN (HPV-16)	II	NR/nine out of 18 women regressed from to CIN1 or below in a 6-month follow-up	[106]
E6/E7 (MVA also encoding IL-2)	Three doses	CIN (HPV-16)	IIb	NR/11 out of 55 patients with histological resolution of CIN; 20 out of 52 patients with viral clearance	[107]
DNA platform					
E7	Weeks 0, 3 and 6	CIN (HPV-16)	I	11 out of 15 patients show IFN-γ by ELISA; five out of 15 patients show regression	[77]
E7	Weeks 0, 4 and 8	CIN (HPV-16)	I	Five out of 15 patients exhibited IFN-γ ELISpot responses/NA	[73]
E6/E7	Weeks 0, 4 and 12	CIN (postintervention HPV-16 and -18)	I	14 out of 18 patients show IFN-γ ELISpot, 18 out of 18 show serum Ab; CTL in ten out of 11 subjects by flow cytometry/NA	[88]

CIN: Cervical intraepithelial neoplasia; CTL: Cytotoxic T lymphocyte; ICS: Intracellular cytokine staining; MVA: Modified vaccinia ankara; NA: Not applicable; NR: Not reported; VIN: Vulvar intraepithelial neoplasia.

Table 1. Selected human papillomavirus immunotherapeutic clinical trials (cont.).

Antigens	Regimen	Patient population	Phase	Immune response/clinical efficacy	Ref.
Dendritic cell platform					
Pulsed with E7	1–4 doses	Cervical cancer (HPV-16 and -18)	I	Four out of 11 patients with proliferation, three out of 11 patients with ELISpot responses	[59]
Pulsed with E7	First 5 injections at 10–14-day intervals, injections 6–14 at 30–60-day intervals	Cervical cancer (HPV-18)	Pilot	$CD4^+$ and $CD8^+$ infiltration at injection sites/CT scans reveal no tumor progression during therapy. Fine needle biopsy shows no viable tumor cells. Recurrence at 23 months	[60]
Pulsed with E7	Days 0, 5, 10, 15 and 20	Cervical cancer (HPV-16 and -18)	I	Ten out of ten patients showing serum Ab; ten out of ten show $CD4^+$ ELISpot; eight out of ten show $CD8^+$ ELISpot/NA	[61]

CIN: Cervical intraepithelial neoplasia; CTL: Cytotoxic T lymphocyte; ICS: Intracellular cytokine staining; MVA: Modified vaccinia ankara; NA: Not applicable; NR: Not reported; VIN: Vulvar intraepithelial neoplasia.

immunization or perhaps to the patient population in which the trial was performed, as the peptide-based therapies were evaluated in patients who had already progressed to cancer, while the protein and DNA-based E7 therapy was performed in patients who had been diagnosed as being in the precancerous or CIN3 state. This may also speak to a possible need for intervention prior to the onset of cancer in the context of cervical infection with HPV.

By way of engendering broader immune responses, several trials have expanded the therapeutic targets to both the E6 and E7 antigens together, as opposed to E7 alone, and most have fared better than trials that focused on E7 exclusively. For example, immunization with long peptides spanning the entire sequence of E6 and E7 in cervical cancer patients who had undergone resection revealed clear immunization-driven IFN-γ production in an ELISpot assay after completion of the protocol [81]. When this same platform was tested in immunization of cervical cancer patients with active disease, both $CD4^+$ and $CD8^+$ T-cell IFN-γ responses were detected to both antigens [82]. Additionally, significant increases in proliferative capacity were also noted in responding T cells [82], reminiscent of the type of response noted in spontaneous regressors. However, a possible drawback of this immunization was noted in the resected population in which it was determined that prolonged antigenic exposure lead to an outgrowth of Tregs ($CD4^+$/$FoxP3^+$/$CD25^+$ as defined by the authors), which could blunt immune responses [81]. This theory was further tested when this platform was used for immunotherapy of vulvar intraepithelial neoplasia in which half of the patients showed regression, and this regression was found to correlate not only with the production of IFN-γ, but also with the frequency of Tregs present [52,82]. Recombinant viral vectors have also been tested for their ability to drive cellular immune responses to both E6 and E7, and studies using vaccinia viral vectors have yielded intriguing results. In an experimental clinical trial, 29 patients with stage I or II cervical cancer were vaccinated twice via scarification with a vaccinia vector encoding HPV-16 and HPV-18 E6 and E7 antigens [83]. Results from this study showed that immune responses were noted in eight out of 29 patients, with half of those patients showing readily detectable CTL responses in the form of target cell lysis by isolated PBMCs (range of 10.4–27.1% lysis by Cr51 release assay) [83]. While clinical outcomes were not measured due to surgical intervention in all patients, the induction of CTL responses is both noteworthy and important in the context of the development of an effective therapy.

Expert commentary & five-year view

Cumulatively, the retrospective and prospective clinical studies as well as the data gleaned from clinical trials begin to uncover a path forward for the development of an effective therapy for HPV infection. Of note are the consistent reports of spontaneous regressors exhibiting Th1-biased cellular immune responses to HPV antigens. This observation coupled with the notion of antigenic selection of the E6 and E7 proteins due to their persistent expression throughout infection, has meant that all the therapeutic clinical trials to date seek to drive the induction of the same general immune profile; therapy-induced Th1 immune responses such as

IFN-γ to HPV-16 and HPV-18 E6 and E7. While the basis for the construction of therapies with this notion in mind is on sound footing, one does wonder if strictly looking at Th1-biased cytokine profiles is a sufficient immune correlate for the induction of an immune response that can clear an established HPV infection. The current clinical trials have focused on cellular responses in general, and yet very few have looked at the most logical functional responses that would likely be associated with viral clearance and resolution of pathology – that of cytolytic T cells. Indeed, measurement of CTL activity has been central to the understanding of control in other examples of chronic infection as well as responses driven by immunization, notably HIV long-term nonprogressors and patients enrolled in the STEP trial, respectively [84,85]. CTL activity has historically been measured by Cr51 release assays or more recently using nonradioactive enzyme release, ELISpot-based systems, flow cytometry measuring markers of CTL phenotypes (granzyme, perforin, CD107a) or assays that directly measure the transfer of lytic enzymes such as granzyme B from CTLs to targets [84–87]. With the exception of the trial listed above [83], none of the previous clinical trials of HPV immunotherapies have sought to specifically interrogate $CD8^+$ T cells for their ability to home to cervical tissues (if immunization is occurring in the periphery), or to load granzyme B or perforin (the main components of lytic granules) or kill target cells in an antigen-specific fashion. Another issue to consider in the immune analysis is that of measuring CTL activity in the cervical tissue in addition to the periphery, since clinical data shows that dysplastic tissue from patients with progressive infection can specifically exclude $CD8^+$ T cells [51] and that pathological analysis of infected tissue has noted that $CD8^+$ T-cell presence trends with spontaneous resolution of infection [46,48]. While being desirable, specifically analyzing $CD8^+$ T cells from cervical tissue has proved difficult in routine practice because of inability to access samples using noninvasive techniques. Nevertheless, characterization of immune responses from the periphery remains an important tool for analysis, especially within the context of clinical data showing detectable immune responses in the periphery of spontaneous regressors. However, a necessary precondition to a comprehensive analysis of T-cell subsets with increasingly sophisticated multicolor flow-based approaches is the availability of PBMCs and in particular the induction of a strong antigen-specific T-cell response to the immunotherapeutic. A common theme emerging from a review of the HPV clinical literature is that in contrast to the natural immune responses seen in subjects who have spontaneously regressed lesions, vaccine approaches have generally led to weak cellular immune responses from peripheral lymphocytes even in cases where some clinical efficacy was noted [74,76]. In these studies, the use of cultured ELISpot assays with prolonged stimulation (5–7 days) has often been required to detect antigen-specific responses in contrast to the more typical overnight stimulation protocols more commonly in use for other antigens. Several factors may be contributing to the historically weak vaccine-induced cellular responses noted with HPV antigens using the platforms tested to date, making it difficult to separate whether the issues are related to the host immune system or the candidate vaccine/platform. E6 and E7 are relatively small antigens (~100–110 amino acids). As such, the development of more potent vaccine platforms and candidate immunotherapies is critical to help further characterize relevant immune responses, particularly the T-cell subsets, and ultimately lead to substantially improved clinical efficacy. Towards this goal, the authors recently reported data from a Phase I dose-escalation trial of a combined HPV-16 and HPV-18 E6 and E7 DNA vaccine delivered by electroporation in patients previously treated for CIN2/3 (Table 1). In total, 78% of patients mounted a cellular immune response as measured by IFN-γ ELISpot and analysis using flow cytometry determined that both the $CD4^+$ and $CD8^+$ T-cell compartments played a role in IFN-γ synthesis. Additional analysis of immune responses revealed statistically significant increases in the ability of patient $CD8^+$ T cells to activate in the presence of HPV-16 and HPV-18 E6 and E7 antigens (via expression of the activation markers HLA-DR and CD38), as well as a concomitant increase in the synthesis of both granzyme B and perforin within these cells. Moreover, these lytic enzymes were able to be effectively employed in CTL activities, as a functional killing assay revealed that 91% of analyzed patients exhibited clear, HPV-specific killing activity in the form of cytolytic degranulation [88].

Taken together, the available data suggest that the induction of a Th1-biased cellular immune response is important, but that this response likely must include the induction of fully functional $CD8^+$ T cells that can infiltrate into tissues and kill infected cells. If this is indeed the case, the specific interrogation of $CD8^+$ T cells for these phenotypes and functions is of paramount importance as a number of groups have noted that IFN-γ production alone does not specifically identify $CD8^+$ T cells capable of cytolytic activity. In fact, one study found that the two functions could be mutually exclusive [89]. Moreover, the presence of such cells would need to occur in the context of persistent antigenic stimulation, as extravazation of $CD8^+$ T cells into infected tissue will result in repeated, heavy antigenic exposure as well as possibly subject these cells to the presence of localized Tregs, which will function to suppress cytolytic activity. The ability to recapitulate these types of scenarios *in vitro* may prove to be of significant use in the development of an effective therapy.

It is the opinion of the authors that the most effective and tolerable immunotherapy for HPV infection will likely include peripheral immunization that ultimately induces the production of $CD8^+$ T cells with the capacity to home to and extravasate into infected tissues and mediate cell killing via cytolytic degranulation. Such $CD8^+$ T cells will need to be able to remain active even after heavy antigenic stimulation as well as possibly in the presence of a suppressive cytokine milieu generated by Tregs. Thus immunotherapy platforms with a track record of inducing strong $CD8^+$ T-cell responses in patients may be the best candidates for the development of an effective therapy via the induction of this type of immune response. While immunization directly into the cervical tissue may seem attractive in the context of generating an immune response where infection is occurring, the invasiveness of the procedure coupled with the fact that actively infected dysplastic tissue seeks to suppress robust immune responses make this method of immunization generally less desirable than peripheral immunization. Moreover, the retrospective and prospective clinical data as well

as clinical trial data also suggest that intervention in precancerous states such as CIN1, CIN 2 or CIN3 may yield better results than immunization of women with progressive cervical cancer. This may be because the immunotherapies tested to date may not have been potent enough to circumvent the tumor microenvironment and/or dysregulation of the immune system or perhaps, more likely, that a combination approach utilizing a therapeutic vaccine together with immunomodulating molecules (anti-PD1, CTLA4 [90]) is critical to combat late-stage disease (Figure 3). Within the context of a broadly Th1-directed immune response, issues such as breadth of T-cell epitopes recognized, magnitudes and types of cytokine responses produced as well as induction or direction of antigen-specific CTL activity within the target tissue will be critical to addressing chronic infections and cancer.

The authors note that while much of the attention has focused on the better management of CIN2 or CIN3 disease by developing immunotherapeutic approaches as an alternative to invasive surgery, a strong case can be made for immunotherapies targeting CIN1 disease as well. The standard of care for CIN1 differs from CIN2 and CIN3. The rates of spontaneous clearance of CIN1 lesions is substantially higher, upwards of 50% [27], and diagnosis of CIN1 generally results in a repeat Pap and/or colposcopy and a 'wait-and-watch' approach. An estimated 20 million HPV-infected people are living in the USA. The rate of new HPV infections is approximately 6.2 million each year and the prevalence rate for CIN1 dysplasias in the USA alone is at approximately 1.4 million cases [91,108]. The personal cost to the subject of living with a lesion and the healthcare cost to society of screening and managing a large cohort of 'wait and watch' subjects is enormous. Indeed, effective anti-HPV immunotherapies could well be positioned to treat all HPV-related disease, in particular CIN1, and more ideally be used as therapeutic vaccines for HPV infection (i.e., as adult vaccines for HPV-positive individuals for whom the L1-based preventive vaccines are ineffective).

Over the next 5 years, we see advances in our understanding of immunology and cancer immunobiology through better characterization of the functional immune responses increasingly driving therapeutic vaccine development. In these regards, the recent

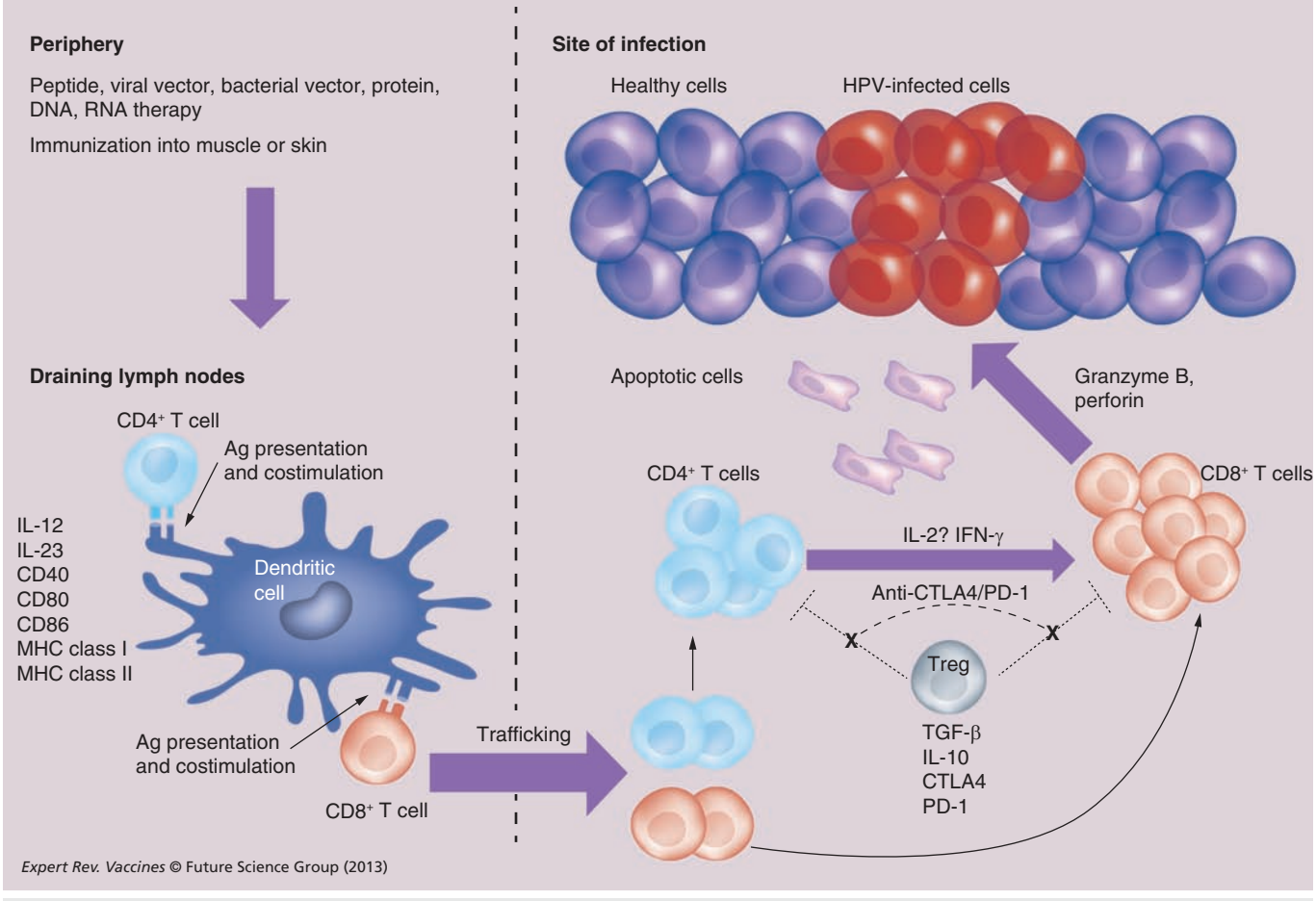

Figure 3. Proposed mechanism for an effective immunotherapy for human papillomavirus infection. An ideal scenario for the treatment of HPV-induced disease would be peripheral immunization that drives the induction of T cells that can traffic to site of infection and eliminate infected cells. $CD4^+$ T cells that traffic to the site of infection would provide support for $CD8^+$ T cells in the form of cytokine secretion, including IFN-γ and possibly IL-2. $CD8^+$ T cells would need to have significant cytolytic function, principally in the form of granzyme B and perforin release. Both cell types would need to be able to function under heavy antigenic load and in the presence of Tregs that would be producing suppressive cytokines such as TGF-β and IL-10 as well as mediating contact inhibition via CTLA4 and PD-1 ligation. Compounds could further be administered to blunt Treg function and aid CD8 activity.

data from more potent vaccine platforms demonstrating clinical efficacy and/or CTL activity lead to optimism that vaccine-induced immune responses may translate into clinical management of precancerous lesions and/or cancer. It will require additional clinical trials with longer-term monitoring to see if immunotherapy will be adjunctive to surgery or has the potential to delay or replace surgery at least in the precancerous setting. In the meantime, we are left with the sobering conclusion that just as with other malignancies, when it comes to cancers with a viral etiology, early detection is the key to control, elimination of infection and possible prevention of cancer.

Financial & competing interests disclosure

The authors thank ongoing grant and contract support from the NIH, PATH-MVI and DOD on vaccine development programs. MP Morrow, J Yan and NY Sardesai are employees of Inovio Pharmaceuticals, Inc. and receive salary and benefits and own stock/stock options in the company. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Therapeutic vaccines and immunotherapy approaches are an exciting addition to chemotherapy, radiation or surgery to address precancerous disease and cancer. In this article the authors discuss the progress in the development of therapeutic vaccines based on viral antigens to target HPV and HPV- associated diseases including cancer. Briefly, the authors note that:
  - Epidemiologic and clinical data support the correlation between induction of T-cell responses and clearance of HPV-associated lesions.
  - E6 and E7 (HPV regulatory proteins) have been widely targeted as viral antigens covering a wide variety of vaccine platforms and data from clinical trials support their use as targets for HPV immunotherapy.
  - Induction of Th1-biased immune responses have been recognized as being crucial for immunotherapy.
- The authors conclude that improvements in our understanding of tumor immunology and development of more potent Th1-directed vaccine platforms make it feasible to foresee an HPV therapeutic vaccine in the coming years.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 de Martel C, Ferlay J, Franceschi S *et al.* Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol.* 13(6), 607–615 (2012).

2 WHO – Department of Immunization, Vaccines and Biologicals. *Human Papillomavirus and HPV Vaccines: Technical Information for Policymakers and Health Professionals.* WHO Press, Geneva, Switzerland (2007).

3 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Cancer incidence and mortality worldwide. *IARC CancerBase* 10 (2008).

4 CDC. Sexual transmitted disease treatment guidelines – human papillomavirus (HPV) infection. *MMWR* 59(RR12), 1–110 (2010).

5 Romanowski B. Long-term protection against cervical infection with the human papillomavirus: review of currently available vaccines. *Hum. Vaccines* 7(2), 161–169 (2011).

6 Smith JS, Lindsay L, Hoots B *et al.* Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int. J. Cancer* 121(3), 621–632 (2007).

7 Chan PJ, Seraj IM, Kalugdan TH, King A. Blastocysts exhibit preferential uptake of DNA fragments from the E6–E7 conserved region of the human papillomavirus. *Gynecol. Oncol.* 58(2), 194–197 (1995).

8 Muñoz N, Bosch FX, de Sanjosé S *et al.*; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N. Engl. J. Med.* 348(6), 518–527 (2003).

9 Trottier H, Franco EL. The epidemiology of genital human papillomavirus infection. *Vaccine* 24(Suppl. 1), S1–S15 (2006).

10 Chaturvedi AK, Engels EA, Pfeiffer RM *et al.* Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J. Clin. Oncol.* 29(32), 4294–4301 (2011).

11 Harper DM, Franco EL, Wheeler CM *et al.* Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. *Lancet* 367(9518), 1247–1255 (2006).

12 Villa LL, Costa RL, Petta CA *et al.* Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre Phase II efficacy trial. *Lancet Oncol.* 5(6), 271–278 (2005).

13 Villa LL, Ault KA, Giuliano AR *et al.* Immunologic responses following administration of a vaccine targeting human papillomavirus types 6, 11, 16, and 18. *Vaccine* 7(24), 5571–5583 (2006).

14 Harper DM, Franco EL, Wheeler C *et al.* Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *Lancet* 364(9447), 1757–1765 (2004).

15 Hildesheim A, Herrero R, Wacholder S *et al.* Effect of human papillomavirus 16/18 L1 viruslike particle vaccine among young women with preexisting infection. *JAMA* 298(7), 743–753 (2007).

16 Goldstone SE, Vuocolo S. A prophylactic quadrivalent vaccine for the prevention of infection and disease related to HPV-6, -11, -16 and -18. *Expert Rev. Vaccines* 11(4), 395–406 (2012).

17 Miller DL, Puricelli MD, Stack MS. Virology and molecular pathogenesis of HPV (human papillomavirus)-associated oropharyngeal squamous cell carcinoma. *Biochem. J.* 443(2), 339–353 (2012).

18 Ibeanu OA. Molecular pathogenesis of cervical cancer. *Cancer Biol. Ther.* 11(3), 295–306 (2011).

19 Gissmann L, Wolnik L, Ikenberg H, Koldovsky U, Schnürch HG, zur Hausen H. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas

and in some cervical cancers. *Proc. Natl Acad. Sci. USA* 80(2), 560–563 (1983).

20 Dürst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc. Natl Acad. Sci. USA* 80(12), 3812–3815 (1983).

•• **The authors first report the discovery of the relationship of HPV-16 to cervical cancer. The identification of HPV-18 in cancer specimens followed shortly thereafter.**

21 Ghittoni R, Accardi R, Hasan U, Gheit T, Sylla B, Tommasino M. The biological properties of E6 and E7 oncoproteins from human papillomaviruses. *Virus Genes* 40(1), 1–13 (2010).

22 Gillison ML, Koch WM, Capone RB *et al.* Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J. Natl Cancer Inst.* 92(9), 709–720 (2000).

23 Syrjänen S. Human papillomavirus (HPV) in head and neck cancer. *J. Clin. Virol.* 32(Suppl. 1), S59–S66 (2005).

24 Applebaum KM, Furniss CS, Zeka A *et al.* Lack of association of alcohol and tobacco with HPV16-associated head and neck cancer. *J. Natl Cancer Inst.* 99(23), 1801–1810 (2007).

25 Münger K, Scheffner M, Huibregtse JM, Howley PM. Interactions of HPV E6 and E7 oncoproteins with tumour suppressor gene products. *Cancer Surv.* 12, 197–217 (1992).

26 Darnell GA, Schroder WA, Antalis TM *et al.* Human papillomavirus E7 requires the protease calpain to degrade the retinoblastoma protein. *J. Biol. Chem.* 282(52), 37492–37500 (2007).

27 Ostor AG. Natural history of cervical intraepithelial neoplasia: a critical review. *Int. J. Gynecol. Pathol.* 12, 186–192 (1993).

28 Firzlaff JM, Kiviat NB, Beckmann AM, Jenison SA, Galloway DA. Detection of human papillomavirus capsid antigens in various squamous epithelial lesions using antibodies directed against the L1 and L2 open reading frames. *Virology* 164(2), 467–477 (1988).

29 Petrovic D, Dempsey E, Doherty DG, Kelleher D, Long A. Hepatitis C virus – T-cell responses and viral escape mutations. *Eur. J. Immunol.* 42(1), 17–26 (2012).

30 Snyder CM. Buffered memory: a hypothesis for the maintenance of functional, virus-specific CD8(+) T cells during cytomegalovirus infection. *Immunol. Res.* 51(3), 195–204 (2011).

31 Haas A, Zimmermann K, Oxenius A. Antigen-dependent and -independent mechanisms of T and B cell hyperactivation during chronic HIV-1 infection. *J. Virol.* 85(23), 12102–12113 (2011).

32 Williams LD, Bansal A, Sabbaj S *et al.* Interleukin-21-producing HIV-1-specific CD8 T cells are preferentially seen in elite controllers. *J. Virol.* 85(5), 2316–2324 (2011).

33 Fröhlich A, Kisielow J, Schmitz I *et al.* IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 324(5934), 1576–1580 (2009).

34 Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. *Science* 324(5934), 1569–1572 (2009).

35 Bonilla WV, Fröhlich A, Senn K *et al.* The alarmin interleukin-33 drives protective antiviral CD8$^+$ T cell responses. *Science* 335(6071), 984–989 (2012).

36 Ma B, Xu Y, Hung CF, Wu TC. HPV and therapeutic vaccines: where are we in 2010? *Curr. Can. Ther. Rev.* 6, 81–103 (2010).

37 van der Burg SH, Melief MC. Therapeutic vaccination against human papilloma virus induced malignancies. *Curr. Opin. Immuol.* 23(2), 252–257 (2011).

38 Su JH, Wu A, Scotney E *et al.* Immunotherapy for cervical cancer: research status and clinical potential. *BioDrugs* 24(2), 109–129 (2010).

39 Lin K, Roosinovich E, Ma B, Hung CF, Wu TC. Therapeutic HPV DNA vaccines. *Immunol. Res.* 47(1), 86–112 (2010).

40 Abastado JP. The next challenge in cancer immunotherapy: controlling T-cell traffic to the tumor. *Cancer Res.* 72(9), 109–129 (2012).

41 Bernatchez C, Radvanyi LG, Hwu P. Advances in the treatment of metastatic melanoma: adoptive T-cell therapy. *Semin. Oncol.* 39(2), 215–226 (2012).

42 van Duikeren S, Fransen MF, Redeker A *et al.* Vaccine-induced effector-memory CD8$^+$ T cell responses predict therapeutic efficacy against tumors. *J. Immuol.* 189(7), 3397–3403 (2012).

43 Zhou Q, Schneider IC, Edes I *et al.* T cell receptor gene transfer exclusively to human CD8$^+$ cells enhances tumor cell killing. *Blood* 120(22), 4334–4342 (2012).

44 Lesterhuis WJ, Haanen JB, Punt CJ. Cancer immunotherapy – revisited. *Nat. Rev. Drug Disc.* 10, 591–600 (2011).

45 Dillon S, Sasagawa T, Crawford A *et al.* Resolution of cervical dysplasia is associated with T-cell proliferative responses to human papillomavirus type 16 E2. *J. Gen. Virol.* (88), 803–813 (2007).

46 Øvestad IT, Gudlaugsson E, Skaland I *et al.* Local immune response in the microenvironment of CIN2–3 with and without spontaneous regression. *Mod. Pathol.* 23(9), 1231–1240 (2010).

• **Reports analysis of patient samples taken prior to spontaneous regression of HPV infection. A survey of local immune responses within the tissue is performed, and the authors demonstrate a correlation between the infiltration of CD8$^+$ T cells into the tissue and the rate of spontaneous regression.**

47 Peng S, Trimble C, Wu L *et al.* HLA-DQB1*02-restricted HPV-16 E7 peptide-specific CD4$^+$ T-cell immune responses correlate with regression of HPV-16-associated high-grade squamous intraepithelial lesions. *Clin. Cancer Res.* 13(8), 2479–2487 (2007).

48 Jung AC, Guihard S, Krugell S *et al.* CD8-alpha T-cell infiltration in human papillomavirus-related oropharyngeal carcinoma correlates with improved patient prognosis. *Int. J. Cancer* 132(2), E26–E36 (2012).

49 Kadish AS, Ho GY, Burk RD *et al.* Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia. *J. Natl Cancer Inst.* 89(17), 1285–1293 (1997).

50 Kadish AS, Timmins P, Wang Y *et al.* Regression of cervical intraepithelial neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide. *Cancer Epidemiol. Biomarkers* 11(5), 483–488 (2002).

51 Trimble CL, Clark RA, Thoburn C *et al.* Human papillomavirus 16-associated cervical intraepithelial neoplasia in humans excludes CD8 T cells from dysplastic epithelium. *J. Immunol.* 185(11), 7107–7114 (2010).

• **Describes studies of samples of dysplastic tissue from HPV-infected patients and identify that a key attribute of this tissue in patients who have not regressed is its ability to exclude CD8$^+$ T cells from infiltration. These data imply an importance for a CD8$^+$ T-cell response in the context of clearing HPV infection.**

52 Welters MJ, Kenter GG, de Vos van Steenwijk *et al.* Success or failure of vaccination for HPV16-positive vulvar lesions correlates with kinetics and phenotype of induced T-cell responses. *Proc. Natl Acad. Sci. USA* 107(26), 11895–11899 (2010).

•• **The data presented in this study demonstrate the importance of the T-cell response in connection to clearing HPV infection in vulvar tissue after therapeutic immunization. Particular importance is placed on the presence and activity of Tregs, which may play a role in limiting the effectiveness of administered immunotherapies.**

53 Sarkar AK, Tortolero-Luna G, Follen M, Sastry KJ. Inverse correlation of cellular immune responses specific to synthetic peptides from the E6 and E7 oncoproteins of HPV-16 with recurrence of cervical intraepithelial neoplasia in a cross-sectional study. *Gynecol. Oncol.* 99(3), 251–261 (2005).

54 Paavonen J, Jenkins D, Bosch FX *et al.* Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a Phase III double-blind, randomised controlled trial. *Lancet* 369(9580), 2161–2170 (2007).

55 de Jong A, O'Neill T, Khan AY *et al.* Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T-cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. *Vaccine* 20(30), 3456–3464 (2002).

56 Thompson HS, Davies ML, Holding FP *et al.* Phase I safety and antigenicity of TA-GW: a recombinant HPV6 L2E7 vaccine for the treatment of genital warts. *Vaccine* 17(1), 40–49 (1999).

57 Borysiewicz LK, Fianders A, Nimako M *et al.* A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *Lancet* 347(9014), 1523–1527 (1996).

58 García-Hernández E, González-Sánchez J, Andrade-Manzano A *et al.* Regression of papilloma high-grade lesions (CIN 2 and CIN 3) is stimulated by therapeutic vaccination with P < E2 recombinant vaccine. *Cancer Gene Ther.* 13(6), 592–597 (2006).

59 Ferrara A, Nonn M, Schreckenberger C *et al.* Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. *J. Cancer Res. Clin. Oncol.* 129(9), 521–530 (2003).

60 Santin AD, Bellone S, Gokden M, Cannon MJ, Parham GP. Vaccination with HPV-18 E7-pulsed dendritic cells in a patient with metastatic cervical cancer. *N. Engl. J. Med.* 346(22), 1752–1753 (2002).

61 Santin AD, Bellone S, Palmieri M *et al.* HPV16/18 E7-pulsed dendritic cell vaccination in cervical cancer patients with recurrent disease refractory to standard treatment modalities. *Gynecol. Oncol.* 100(3), 469–478 (2006).

62 Lamains LA. The biology of human papillomaviruses: from warts to cancer. *Infect. Agents Dis.* 2(2), 74–86 (1993).

63 Burd EM. Human papillomavirus and cervical cancer. *Clin. Microbiol. Rev.* 16(1), 1–17 (2003).

64 Zur Hausen H. Papillomavirus infections – a major cause of human cancers. *Biochim. Biophys. Acta* 1288(2), 55–78 (1996).

65 Jabbar SF, Park S, Schweizer J *et al.* Cervical cancers require the continuous expression of the human papillomavirus type 16 e7 oncoprotein even in the presence of the viral e6 oncoprotein. *Cancer Res.* 72(16), 4008–4016 (2012).

66 Duensing S, Lee LY, Duensing A *et al.* The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc. Natl Acad. Sci. USA* 97(18), 10002–10007 (2000).

67 Moore RA, Walcott S, White KL *et al.* Therapeutic immunisation with *COPV* early genes by epithelial DNA delivery. *Virology* 314(2), 630–635 (2003).

68 Moore RA, Santos EB, Nicholls PK *et al.* Intraepithelial DNA immunisation with a plasmid encoding a codon optimised *COPV* E1 gene sequence, but not the wild-type gene sequence completely protects against mucosal challenge with infectious COPV in beagles. *Virology* 304(2), 451–459 (2002).

69 Gutierrez C, Tinoco A, Navarro T *et al.* Therapeutic vaccination with P < E2 can eliminate precancerous lesions (CIN1, CIN2 and CIN3) associated with infection by oncogenic human papillomavirus. *Hum. Gene Ther.* 15(5), 421–431 (2004).

70 Yim EK, Park JS. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. *Cancer Res. Treat.* 37(6), 319–324 (2005).

71 Yan J, Harris K, Khan AS, Draghia-Akli R, Sewell D, Weiner DB. Cellular immunity induced by a novel HPV18 DNA vaccine encoding an E6/E7 fusion consensus protein in mice and rhesus macaques. *Vaccine* 26(40), 5210–5215 (2008).

72 Sharma C, Dey B, Wehiduzzaman M, Singh N. Human papillomavirus 16 L1-E7 chimeric virus like particles show prophylactic and therapeutic efficacy in murine model of cervical cancer. *Vaccine* 30 (36), 5417–5424 (2012).

73 Muderspach L, Wilczynski S, Roman L *et al.* A Phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. *Clin. Cancer Res.* 6(9), 3406–3416 (2000).

74 Steller MA, Gurski KJ, Murakami M *et al.* Cell-mediated immunological responses in cervical and vaginal cancer patients immunized with a lipidated epitope of human papillomavirus type 16 E7. *Clin. Cancer Res.* 4(9), 2103–2109 (1998).

75 van Driel WJ, Ressing ME, Kenter GG *et al.* Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a Phase I–II trial. *Eur. J. Cancer* 35(6), 946–952 (1999).

76 Trimble CL, Peng S, Kos F *et al.* A Phase I trial of a human papillomavirus DNA vaccine for HPV16$^+$ cervical intraepithelial neoplasia 2/3. *Clin. Cancer Res.* 15(1), 361–367 (2009).

77 Hallez S, Simon P, Maudoux F *et al.* Phase I/II trial of immunogenicity of a human papillomavirus (HPV) type 16 E7 protein-based vaccine in women with oncogenic HPV-positive cervical intraepithelial neoplasia. *Cancer Immunol. Immunother.* 53(7), 642–650 (2004).

78 Einstein MH, Kadish AS, Burk RD *et al.* Heat shock fusion protein-based immunotherapy for treatment of cervical intraepithelial neoplasia III. *Gynecol. Oncol.* 106(3), 453–460 (2007).

79 Van Doorslaer K, Reimers LL, Studentsov YY, Einstein MH, Burk RD. Serological response to an HPV16 E7 based therapeutic vaccine in women with high-grade cervical dysplasia. *Gynecol. Oncol.* 116(2), 208–212 (2010).

80 Sheets EE, Urban RG, Crum CP *et al.* Immunotherapy of human cervical high-grade cervical intraepithelial neoplasia with microparticle-delivered human papillomavirus 16 E7 plasmid DNA. *Am. J. Obstet. Gynecol.* 188(4), 916–926 (2003).

81 Welters MJ, Kenter GG, Piersma SJ *et al.* Induction of tumor-specific CD4$^+$ and

CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. *Clin. Cancer Res.* 14(1), 178–187 (2008).

82 Kenter GG, Welters MJ, Valentijn AR *et al.* Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. *Clin. Cancer Res.* 14(1), 169–177 (2008).

83 Kaufmann AM, Stern PL, Rankin EM *et al.* Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 *E6* and *E7* genes, in women with progressive cervical cancer. *Clin. Cancer Res.* 8(12), 3676–3685 (2002).

84 Migueles SA, Osborne CM, Royce C *et al.* Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* 29(6), 1009–1021 (2008).

• **Analyzes the influence and importance of a cellular immune response that includes a cytolytic component in the context of control of a chronic viral infection. This study demonstrates that a key difference between patients who controlled chronic HIV infection and patients who exhibited progression to a disease state was the relative ability of their immune systems to mount a cytolytic immune response as measured by the lytic granule loading of effectors and delivery of granzyme B to target cells.**

85 Migueles SA, Rood JE, Berkley AM *et al.* Trivalent adenovirus type 5 HIV recombinant vaccine primes for modest cytotoxic capacity that is greatest in humans with protective HLA class I alleles. *PLoS Pathog.* 7(2), e1002002 (2001).

86 Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytoxicity is mediated through perforin and Fas lytic pathways. *Nature* 370(6491), 650–652 (1994).

87 Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev. Vaccines* 9(6), 601–616 (2010).

88 Bagarazzi ML, Yan J, Morrow MP *et al.* Immunotherapy against HPV 16/18 generates potent Th1 and cytotoxic cellular immune responses. *Sci. Transl. Med.* 4(155), 155ra138 (2012).

•• **An immunotherapy for HPV infection is thought to require a robust cellular response that includes the ability to kill target cells infected with HPV. The Phase I clinical trial detailed in this paper describes a DNA vaccine against HPV-16 and -18 that was able to elicit strong Th1-biased cellular responses as well as lytic degranulation and killing in response to HPV antigen.**

89 Varadarajan N, Julg B, Yamanaka YJ *et al.* A high-throughput single-cell analysis of human CD8+ T cell functions reveals discordance for cytokine secretion and cytolysis. *J. Clin. Invest.* 121(11), 4322–4331 (2011).

90 Alexandrescu DT, Ichim TE, Riordan NH *et al.* Immunotherapy for melanoma: current status and perspectives. *J. Immunother.* 33(6), 570–590 (2010).

91 Schiffman M, Solomon D. Findings to date from the ASCUS-LSIL Triage Study (ALTS). *Arch. Pathol. Lab. Med.* 127, 946–949 (2003).

92 Kenter GG, Welters MJP, Valentjin R *et al.* Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N. Engl. J. Med.* 361, 1838–1847 (2009).

•• **Phase II clinical trial that describes immunization with a synthetic long peptide encoding a HPV antigen against HPV-16 infection of vulvar tissue. This is an important trial in the context of HPV immunotherapy as it describes strong cellular responses induced by vaccination as well as showing complete clinical responses in a subset of patients.**

Websites

101 WHO. Sexual and reproductive health. Cervical cancer, human papillomavirus (HPV) and HPV vaccines. www.who.int/reproductivehealth/publications/cancers/RHR_08_14/en/index.html

102 WHO. International Agency for Research on Cancer. GLOBOCAN 2008. http://globocan.iarc.fr

103 CDC. HPV 2010 STD Treatment Guidelines. www.cdc.gov/std/treatment/2010/hpv.htm

104 CDC press release. National survey shows HPV vaccine rates trail other teen vaccines. www.cdc.gov/media/releases/2011/p0825_hpv_vaccine.html

105 EMA. Annex I Summary of Product Characteristics for the HPV Vaccine 'Gardasil' 2008. Updated 2012. www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000703/WC500021142.pdf

106 Transgene announces positive Phase II results for its HPV therapeutic vaccine in precancerous cervical lesions. www.transgene.fr/images/stories/en/pdf/communique_presse/communiques_divers_2006/CP-US%20Eurogin%2025-04-06.pdf

107 Transgene reports randomized Phase 2b data with its therapeutic HPV vaccine TG4001 in women with CIN2/3 intraepithelial cervical neoplasia. www.transgene.fr/index.php?option=com_press_release&task=download&id=208&l=en

108 CDC. Sexually transmitted diseases. Genital HPV infection – fact sheet. www.cdc.gov/std/hpv/stdfact-hpv.htm

109 CDC. www.cdc.gov