Immunotherapy for Precancerous Lesions of the Uterine Cervix

7

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Uterine cervical cancer is the fourth most common neoplasia in women and the seventh overall. In 2012, there were 528,000 new cases and 266,000 deaths from cervical cancer worldwide, accounting for 7.5% of all female cancer deaths. It is the most frequent gynecological cancer in developing countries [1, 2]. The frequency of cervical cancer after treatment for dysplasia is less than 1% and mortality is less than 0.5% [3]. The increasing incidence of the disease in developing countries is related to the high number of multiple partners, early age at first intercourse, infrequent use of condoms, multiple pregnancies with chlamydia association, and immunosuppression with HIV [4]. It was noted that HIV-infected women have a higher risk and persistence of multiple HPV infections which are associated with increased risk of progression to precancerous cervical lesions compared to HIV-noninfected women [5]. About 10–15% of women have oncogenic HPV types (HPV high risk, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, and 82, and HPV low risk, 6, 11, 40, 42, 43, 44, 54, 61, 72, and 81) [6]. In the United States of America (USA), HPV-16 and HPV-18 types are detected in 70% of high-grade squamous intraepithelial lesions (HGSIL) and in invasive cervical cancer in women [7]. It has been shown that oral contraceptives are associated with increased risk of the disease (administration for >5-year-double risk, >10-year-quadruple risk). In addition, other risk factors such as sexual activity, frequency of gynecological examinations, and medication-free interval time are observed [7, 8]. Interestingly, smoking is thought to have unclear relation to the disease [9]. There are several mechanisms by which cancers can avoid immune defenses. Cancers can directly inhibit immune reactivity by secreting soluble immune inhibitory mediators such as PGE2, TGF-β, and IL-10

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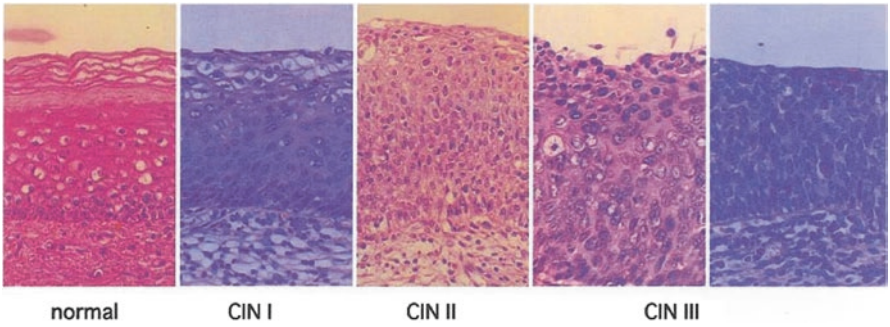

Fig. 7.1 Histopathologic features of cervical intraepithelial neoplasia (CIN) stages (From Fukumoto and Irahara [236], with permission)

[10–12]. They also express checkpoint inhibitory ligands such as PD-L1 that block immune reaction [13]. In addition, inhibition by cancers is mediated by their induction of host immune inhibitory cell populations. These include macrophages, Treg cells, Th2-skewed T-cells, myeloid-derived suppressor cells (MDSC), and $CD34^+$ progenitor cells [14–18]. Within the tumor environment, there are also immune inhibitory endothelial cells and fibroblasts [19, 20]. Histopathologic features of cervical intraepithelial neoplasia (CIN) stages are shown in Fig. 7.1 [21].

Immunological Aspects of Precancerous Lesions of the Uterine Cervix

Noted efforts have been exerted on cancer prevention such as improved diet, smoking cessation, and reduced sun exposure. Less emphasis has been placed on immunological approaches to prevent cancer development or progression prior to when cancers subvert immune defenses. However, an advancement toward this effort is the availability of HPV vaccines, which aim to prevent cervical cancer and can become effective in preventing other HPV-associated malignancies such as squamous cell carcinomas (SCC) of the head and neck [22, 23]. Non-HPV-associated malignancies might be preventable in individuals that are at high risk for development of cancer. In general, premalignant lesions are tissues that can progress to become malignant. Examples of these precancerous tissues include polyps in the colon, actinic keratosis of the skin, dysplasia of the cervix, metaplasia of the lung, and leukoplakias of the mouth. Premalignant lesions of the oral cavity, including leukoplakias and erythroplakias, are routinely screened during dental examinations [24]. Colonoscopies are performed routinely to detect colon polyps which, in turn, reduce colon cancer [25, 26]. Dysplasia of the cervix is routinely screened for by Pap smears [27]. The standard treatment for these premalignant tissues often includes their excision; however such treatment does not remove premalignant cells that have not yet been detected and does not prevent development of secondary lesions. A study compared the immunological microenvironment of intraepidermal

carcinomas, and SCC showed an increased level of T-cells, and mainly $CD8^+$ T-cells, within the lesions compared to the levels of these cells in cancer tissue [28]. In another study, the investigators showed that premalignant oral leukoplakias are infiltrated by $CD3^+$ T-cells, with those containing lower numbers of $CD3^+$ cells having a higher incidence of progression to cancer [29]. It has also been shown that leukoplakias with dysplasia and oral SCC have a higher dendritic Langerhans cell and T-cell content than leukoplakias without dysplasia [30]. The conclusions of these studies suggest that the higher level of immune cell infiltration is indicative of ongoing immune reactivity against premalignant lesions and against cancers. Other studies showed that premalignant oral lesion tissues of patients and of a mouse model of premalignant oral lesions that progress to cancer contained increased levels of Th1 and inflammatory cytokines compared to levels within oral cancers [31]. Studies of the immune phenotypes have shown Barrett's esophageal tissues contain an elevated pro-tumorigenic Th2 immune phenotype, but this shifts to a less activated T-cell phenotype once the cancer is developed that consists of a mixed Th1 and Th2 cytokine profile [32]. Also, infiltration by M2 macrophages and Treg cells was hypothesized to contribute to esophageal cancer development in a rat model of chronic duodenal content reflux esophagitis [33]. Similarly, studies with *Helicobacter pylori*-infected patients having precancerous gastric lesions and *H. pylori*-infected mice concluded that increased myeloid cell infiltration and increased IFN-γ expression may be contributing to progression of lesions toward cancer [34]. Additionally, genetic expression profiles of colon polyp tissues and unaffected colon mucosa of patients having colon polyps showed an overlap of changes in gene expression compared to gene expression profiles of healthy individuals [35]. It was noted that patients with ulcerative colitis had a similar frequency of developing polyps as did healthy controls, although the histological types of polyps differed with an increase in inflammatory (pseudo)polyps [36]. Studies indicating immune involvement in progression of premalignant states toward cancer using the TRAMP mouse model showed the development of hyperplasia, prostatic intraepithelial neoplasia, and carcinoma. The presence of T-cells was shown to facilitate the process of progression [37]. Another study with a murine model of prostatic hyperplasia suggested immune involvement in stimulating prostatic epithelial proliferation, and the inflammatory reaction was mediated by macrophage-derived IL-1 [38]. It was suggested that macrophage recruitment promotes the formation and progression of pancreatic premalignant lesions [39]. Inflammation along the gastrointestinal tract appears to have a closer connection to progression of premalignant states to cancer than what has been described for other sites. Such inflammation-associated disorders with increased risk of cancer include Barrett's esophagus, Crohn's disease, and ulcerative colitis [40, 41]. Levels of inflammatory indicators such a C-reactive protein and IL-6 were shown to be increased in the peripheral blood of subjects with Barrett's esophagus, and these increases were associated with a higher risk of premalignant progressing to esophageal adenocarcinoma [42]. Subjects with premalignant oral lesions have increased levels of inflammatory mediators, TNF-α and IL-6 in their saliva, although salivary levels of these cytokines were shown to be higher in subjects with oral squamous cell carcinoma [43]. It was noted increased

levels of TNF-α in saliva of subjects with premalignant oral lesions and cancer were increased in the serum of these subjects [44]. Other studies showed increased splenic and regional lymph node pro-inflammatory activity with a Th1 and Th17 phenotype in a carcinogen-induced premalignant oral lesion animal model and in the blood of subjects with premalignant oral lesions [40, 45]. Studies to assess the mechanism by which premalignant oral lesion cells alter cytokine levels demonstrated that the stimulation of Th1 and Th17 cell-associated cytokines was through soluble mediators produced by premalignant lesion cells [41, 46]. The induction of some of the inflammatory mediators was blocked by inhibiting cyclooxygenase in premalignant lesion cells, hypothesizing that lesion cell-derived PGE2 could be contributing to some of the systemic inflammation [47]. The immune system is divided into two components: the innate immune system and the adaptive immune system. The latter is further subdivided into humoral immunity and cell-mediated immunity [44]. Innate and adaptive immune systems are intertwined, through several immune cells and cytokines that are involved in both the innate and adaptive immune responses. Innate immune response provides initial defense against pathogens by epithelial barriers, local inflammation and cytokines, complement system and phagocytic cells (neutrophils, monocytes, and macrophages), dendritic cells (DC), and natural killer (NK) cells [48]. NK-cells recognize tumor cells expressing histocompatibility complex (MHC) surface molecules and are responsible for killing these cancer cells by releasing perforin and granzyme that enter the cytoplasm and induce apoptosis [49]. Two functional types of receptors are expressed on the NK-cell surface: stimulatory receptors and inhibitory receptors. Natural killer group 2D (NKG2D) molecule is a known stimulatory receptor [50]. Binding of stress-related ligands on tumor cells with NKG2D stimulates NK-cells and results in secretion of interferon (IFN) gamma and perforin, release of inflammatory cytokines, and induction of apoptosis in cancer cells. Macrophages can phenotypically and functionally be categorized into M1-like, pro-inflammatory, tumor-suppressive macrophages (M1) and M2-like anti-inflammatory tumor-promoting (M2) macrophages [46]. M1 macrophages develop in response to bacterial products, acute inflammation, and IFN-a and recognize tumor cells expressing eat-me molecules at the cell surface. These signals include lipid phosphatidylserine (PS), oxidized PS, oxidized low-density lipoprotein, and calreticulin [51] which are translocated to the tumor cell surface during apoptosis [52]. Interaction between apoptotic tumor cells and these macrophages leads to immune tolerance in a tumor environment. M1 macrophages are also capable of extracellular killing of cancer cells by the release of cytokines, chemokines, and inflammatory mediators. In addition, M2 macrophage produces immunosuppressive cytokines and chemokines that result in alteration of the phenotype and function of local DCs and polarize T-cells to a x2 phenotype which decrease an antitumor immune response [53, 54]. Myeloid-derived suppressor cells (MDSC) hinder an antitumor immune response [55, 56] and are present in tumor microenvironment. Consequently, tumors attract myeloid cells and interfere with their differentiation. Dendritic cells (DCs) are highly specialized in antigen presentation to T-cells and act as bridges between the innate and the adaptive immune system. In cancer, tumor-infiltrating B-cells (TIL-Bs) play a key role

in the B-cell response. There is increasing evidence that the presence of TIL-Bs is associated with favorable clinical outcomes in cancer. In addition, B-cells can potentiate the antitumor response by producing chemokines and cytokines, as they serve as local APCs and organize lymphoid structures in the tumor that sustains the immune response [57]. Whereas B-cells recognize whole molecules and intact pathogens, T-cells possess T-cell receptors (TCR) that recognize small peptide antigens presented by MHC class I or II on the cell surface. Naïve T-cells need to recognize the antigen and receive a co-stimulatory signal to become activated, differentiated, and proliferated into effector cells. Co-stimulatory molecules provide signals which are involved in activating and regulating the development antigen-specific T-cells [58]. There are two major T-lymphocyte populations, CD8+ and CD4+ T-cells, which recognize distinct fragments of antigens and display distinct effector functions. CD8+ cytotoxic T-cells (CTLs) recognize small peptide antigens that are presented in MHC class I molecules on the cells. Ayer's recognition of the abnormally expressed antigen and CD8+ T-cells differentiate into cells that acquire cytolytic capacity, ending with a highly specific mature CTL that can kill the affected cell. CD4+ T-cells recognize antigens presented in MHC class II molecules. In addition to MHC class II expression by immune cells, such as APCs, MHC class II expression occurs in activated CD4+ T-cells and CD8+ T-cells and can be upregulated in epithelial cells in tumor cells [59]. CD4+ T-cell activation is essential for an optimal CD8+ T-cell-mediated immune response [59], either through the classical helper role of CD4+ T-cells that provide cytokine support (IL-2 and IFN-a release) for CD8+ T-cells or by the activation of CD40 expression on APCs which stimulate CD8+ T-cells [60, 61]. CD4+ T-cells can be polarized into multiple different effector T-cell subsets, based on their function and cytokine profile, including type 1 x (x1) helper cells, type 2 x (x2) helper cells, and x17 cells which play an important role in the induction of autoimmunity, but recent evidence suggests that this effector T-cell subset is also involved in tumor immunology by preparing the tumor environment and facilitating tumor-infiltrating CD8+ T-cells and NK-cells [62]. A specialized subtype of CD4+ T-cells distinguished from other subpopulations by their role in immune tolerance is the regulatory T-cell (Treg) subset. Naturally occurring Tregs are directly derived from the thymus, and these highly express CD25 and transcription factor FoxP3. Adaptive Tregs are induced at the periphery and may or may not express FoxP3. Tregs suppress CD8+ CTLs and x1-mediated responses via various known and unknown mechanisms, including the secretion of immunosuppressive cytokines as IL-10 and TGF-β or the consumption of IL-2, thereby inhibiting other T-cells or APCs.

Cancer Immunology

The immune system plays an important role in the development, maintenance, and expansion of cancer. Several numbers of immune cells with different subsets, receptors, cytokines, antibodies, and chemokines contribute to the elimination or promotion of tumor progression. It has been hypothesized that the immune system is able

to recognize, inactivate, and eliminate potentially malignant cells before they establish themselves and form a tumor mass [63–65]. In general, malignant cells are ascribed as the result of genetic changes that occur during cell divisions. Genetic changes may result in the expression of tumor antigens, which make malignant cells immunologically distinguishable from normal cells [66]. There are three interaction processes between tumor cells and immune cells. These three processes include the elimination, equilibrium, and escape phase, representing the fact that the immune system protects the host against tumor development and modulates the immunogenic phenotype of malignant cells (equilibrium phase) and thereby facilitating complete tumor escape from immune attack (escape phase) and uncontrolled tumor growth [67, 68]. Several studies have shown that the nature of tumor-infiltrating T-cells at diagnosis is strongly associated with patient survival in many human cancers [69–73]. The prognostic value of adaptive immune cell infiltration and tumor microenvironment was noted in colorectal cancer, and expressed as an integrated immunoscore, which was based on the type, density, and location of immune cells [74–76]. The role of HPV infections in the development of cervical premalignancies has been recognized [77]. Genital infections with high-risk HPV, particularly HPV type 16 (HPV16), are highly prevalent in young individuals with a lifetime incidence of 80% [78]. The majority of immune competent individuals infected with the virus are able to control and eventually eliminate the viral infection. In most women, an HPV infection is asymptomatic, transient, and cleared within 2 years. Persistent infections with HPV occur in less than 10% of the infected women which increase the risk of development of premalignant cervical lesions [79]. HPV is a non-lytic, circular double-stranded DNA which encodes for six early nonstructural or regulatory genes (E1, E2, and E4–E7) and two late structural proteins (L1 and L2) [80]. These proteins exert specific functions during the different stages of HPV replication which contribute to the development and progression of HPV-associated lesions. Replication of HPV occurs in the supra-basal layer, where E1, E2, and E5 genes are expressed. Oncoproteins E6 and E7 are consistently expressed in the basal cells of the epithelium layer and play an important role in the viral life cycle by modifying the cellular environment and allow viral genome amplification, by driving S-phase reentry in the upper epithelial layers [81, 82]. In case of persistent infection with high-risk HPV, integration of the HPV DNA into the host cell genome might occur and is accompanied with overexpression of E6 and E7 oncoproteins. Persistent high level of expression of E6 and E7 accumulates genetic errors in the host genome, resulting in dysplastic cells which can progress to high-grade intraepithelial lesions or microinvasive carcinoma [83]. Notably, immunosuppressed individuals are known to be at high risk for persistent HPV infections, HPV-associated malignancies, and progression of disease [84, 85]. Undifferentiated keratinocytes at the stratum basale of the epithelium are the primary target for HPV. Keratinocytes express pathogen recognition receptors (PRRs), including the Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors (NLRs), and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), which recognize pathogen-associated molecular patterns (PAMPs) on microbes and viruses [86]. TLRs1–3, TLR5, TLR6, TLR10, RIG-I, protein kinase R (PKR), and

MDA5 are expressed irrespective of the differentiation state of keratinocytes, while the expression of TLR9, the PPR that can recognize viral DNA of HPV, only induced layer terminal differentiation [87]. Moreover, HPV infection downregulates a network of genes encoding for the production and secretion of antivirals such as type I interferon and chemotactic and pro-inflammatory cytokines, including IL-1β, which play a major role in activation of adaptive immunity [87, 88]. HPV also attenuates the effector cytokine reaction of infected cells to the exposure to IFN-a and/or TNF-α, allowing transient escape from immune response [89]. Further, HPVs are able to manipulate Langerhans cells (LCs) and turn them into activated APCs. The functional and phenotypic maturation of LCs and the decrease in number of LCs occur in the HPV-infected epidermis and disturb antigen presentation to T-cells [90–93]. The accumulation of tolerogenic APCs in the microenvironment can be the result of HPV affecting the extent of the CD40 signaling in the infected cells and consequently the production of cytokines and pro-inflammatory signals [94, 95]. HPV interferes with the production of cytokines and suppresses the antigen-presenting pathway, delaying the activation of the adaptive immune system. In adaptive immunity to HPV and escape mechanism, memory B-cells may release HPV capsid type-specific antibodies that can opsonize the virus and protect against subsequent infection with the same HPV type. In Ayer natural infection with HPV, the serum-neutralizing antibody levels are low as the infection is located intraepithelially. Seroconversion is generally detected within 18-month Ayer infection, but the level of Ig antibodies directed against the viral HPV capsids L1 and L2 is low or nonexistent in 30–50% of the patients [96, 97]. Control of HPV is achieved by activation of the HPV-specific interferon-a (IFN-a)-producing CD4+ and CD8+ type 1 T-cell responses to ER 22. The viral protein E2, E6, and E7 responses have been studied and were detected in the peripheral blood mononuclear cells (PBMCs) of healthy, HPV-negative but exposed subjects and in women with regression of their HPV-associated cervical lesions. In the majority of these women, circulating proliferating IFN-a- and IL-5-producing T-cells against E2, E6, and E7 were detected [98, 99]. It has been shown that the infiltration of low-grade squamous intraepithelial lesions by CD8+ cytotoxic cells is related with regression of the lesions, whereas the number of CTLs is substantially lower in patients with persistent low-grade cervical lesions [99, 100]. In patients with persistent HPV infection, this type of immunity is weak, and E6 and E7 are not detectable in the blood [101–105]. At the site of progressive high-grade squamous intraepithelial lesions, the number of infiltrating CD4+ and CD8+ T-cells is reduced and loses their ability to produce IFN-a. [100, 106]. Downregulation of HLA class I and class II molecules on HPV-transformed cells makes the infected cells less visible to the adaptive immune system and evades host immunity. This was shown in patients with cervical dysplasia where allelic loss of HLA-B44 expression showed progression of the lesions, while no downregulation was seen in nonprogressive lesions [107]. These data are consistent with the loss of HLA class I and HLA-A expression in cervical carcinomas [108, 109]. Nonclassical HLA types HLA-G, HLA-E, and MHC class I chain-related molecule A (MICA) are addressed to induce the pertinacity of HPV infections and lesions, as the expression of HLA-G and HLA-E is associated with progression of cervical intraepithelial

neoplasias to invasive squamous cell carcinoma [110, 111], and low expression of MICA is associated with impaired survival in patients with cervical tumors [109]. The expression of 23x cells and CTLs such as inhibitory molecules may result in suppression of the effector function of T-cells and may counteract migration of these cells to the infected lesions. This was demonstrated in different studies which showed that activated T-cells express inhibitory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), program death 1 (PD-1), and T-cell immunoglobulin mucin-3 (TIM-3). Upon interaction with their ligands (CTLA-4 ligand, PD-ligand 1 and/or PD-ligand 2, and galectin-9), induction of apoptosis of x1 cells and inhibition of functional CTLs and x1 cells occur [112–114]. Also, tumor-associated (M2) macrophages and Tregs are attracted to the tumor site, where they form an immunosuppressive environment [115]. In high-grade lesions, the proliferation and function of effector T-cells are suppressed by Tregs, and it was shown that the ratio of tumor-infiltrating CD4+/CD8+ T-cells and the presence of Tregs in tumors are strongly associated with the prognosis and survival of patients with cervical cancer [109, 115, 116]. It was demonstrated that a strong intraepithelial infiltration of M1 macrophages was associated with a large influx of intraepithelial T lymphocytes, improving disease-specific survival [117]. Vaccination to prevent HPV infection and subsequently preclude HP-related disease is a valid strategy. Prophylactic vaccines aim to prevent an HPV infection by antibodies or humoral immune responses. These prophylactic HPV vaccines have no therapeutic effects as they do not increase viral clearance in subjects already infected with HPV [118]. For patients with progressive disease, multiple therapeutic immunotherapeutic modalities have been developed, of which therapeutic vaccination, non-specific immune stimulation with cytokines and antibodies, and adoptive cell therapy (ACT) are best known. Monoclonal antibodies directly mitigate the tumor-induced immunosuppressive conditions. The blockade of immune inhibitory pathways by targeting CTLA-4 (ipilimumab) and PD-1/PDL-1 (nivolumab) has demonstrated to be successful in preclinical studies and melanoma patients [119–122]. For the treatment of virus-induced malignancies and cancer, various therapeutic immunotherapies have been investigated with the goal to induce notable cell-mediated immunity [123]. In general, specificity is required to prevent destruction of healthy host tissue, and memory is required to prevent recurrences of primary tumors. A study focused on immunotherapy employed reinforcement of antigen-specific T lymphocytes [124]. A model has been proposed which took into account that transforming infection by HPV contributes to deregulation of the DNA methylation machinery, which, upon selection, may give rise to DNA methylation-mediated silencing of tumor suppressor genes [125] (Fig. 7.2).

Immunological Treatment Approaches for Premalignant Lesions

Several studies have shown increased immune activity, in premalignant lesions; however studies to determine the feasibility of immunotherapeutic approaches to treat lesions or to prevent their reoccurrence or progression to cancer have been few.

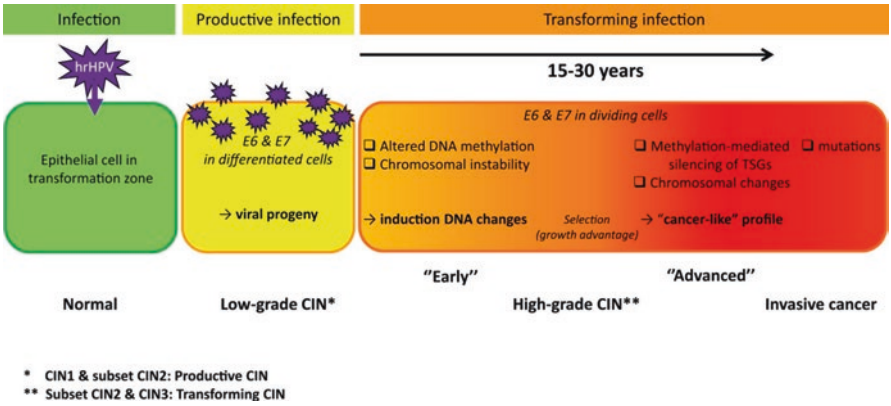

Fig. 7.2 Schematic representation of HPV-mediated cervical carcinogenesis. Progression of a high-grade CIN lesion, characterized by viral oncogene expression in dividing cells (i.e., a transforming infection), to invasive cancer results from the accumulation of DNA changes induced by HPV. High-grade CIN represents a heterogeneous stage of disease with varying duration of existence (up to 30 years). "Advanced" lesions show a cancer-like profile including hypermethylation of tumor suppressor genes and specific chromosomal alterations. Complementary somatic mutations only become detectable at the stage of invasive cancer. *CIN* cervical intraepithelial neoplasia, *TSG* tumor suppressor gene. (From Wilting and Steenbergen [237], with permission)

Squamous dysplasias have been shown to express some of the same tumor antigens as digestive tract carcinomas, namely, esophageal squamous cell carcinoma [126]. Similarly, tumor antigens were noted to be expressed by premalignant oral lesion of patients as are seen on head and neck squamous cell carcinomas [127]. Sharing of tumor antigens between premalignant oral lesions of a carcinogen-induced tongue lesion mouse model and the tongue cancers that developed from these lesions was also noted [128]. Some studies that have utilized immunotherapy for treatment of premalignant lesions and to prevent their progression to cancer have had varied results. Topical application of the agents, imiquimod and diclofenac, stimulates cytokine production and can trigger regression of premalignant skin actinic keratosis lesions [129, 130]. It was demonstrated that administration of selective inhibitors of cyclooxygenase-2 (COX-2) to rats diminishes the carcinogen-induced inflammatory NF-kB signaling pathways and slows the development of colonic tumors [131]. Also, administration of the select COX-2 inhibitor celecoxib to a mouse model of helicobacter-associated precancerous lesions tempered the immune inhibitory effects of PGE2 on expression of the Th1 cytokine IFN-γ and, consequently, accelerated the development of the premalignant lesions [132]. In a population-based, case-controlled study on the effectiveness of nonsteroidal anti-inflammatory drugs in subjects with Barrett's esophagus, whose progression to esophageal adenocarcinoma has been strongly shown to be inflammation-associated, no protective effects of the anti-inflammatory treatment on the incidence of cancer development were shown [133]. Treatment with anti-inflammatory compounds was found not to diminish the development of Barrett's esophagus in subjects with gastroesophageal

reflux disease [134]. There have been varied results of analyses of the effectiveness of nonsteroidal anti-inflammatory compounds and aspirin on the development of cancer in subjects with Barrett's esophagus [135]. A vaccination study in which patients with low-grade premalignant cervical abnormalities were vaccinated with a HPV16 synthetic long-peptide vaccine representing the E6 and E7 oncoprotein sequences showed HPV16-specific IFN-γ T-cell responses [136]. In another study using a mouse HPV tumor model to assess both immunological and clinical responses, peptide mixtures of the HPV E7 oncogene were shown to stimulate both antibody and cellular immune responses reactive to HPV constructs and to limit progression to malignancy [137]. Administration of a premalignant lesion-pulsed dendritic cell vaccine increased Th1 and Th17 immune reactivities and slowed progression to cancer [138]. In addition, insulin-like growth factor (IGF)-binding protein 2 and IGF receptor-I were used to test a vaccine consisting of peptides derived from these proteins in a TgMMTV-neu mouse model [138].

Targeted Immunotherapy of High-Grade Uterine Cervical Intraepithelial Neoplasia

Cervical cancer is the third most common cancer in women and the fifth most common overall cancer worldwide as age-standardized incidence rate in both sexes combined [139, 140]. The prime causal factor of the disease is a persistent infection with high-risk human papillomavirus (HPV), with individuals failing to mount adequate immune response against the virus. The high-risk HPV genome encodes three oncoproteins, E5, E6, and E7; the last two oncoproteins are constitutively expressed in high-grade lesions and cancer. These are required for the onset and maintenance of the malignant phenotype. About 170 HPV genotypes have been identified, and 40 can infect the anogenital area: the uterine cervix, vulva, vaginal wall, penis, and anus. HPVs are classified as high-risk types, commonly associated with cancer, and low-risk types, mostly identified in condyloma acuminatum. The International Agency for Research on Cancer (IARC) conducted a study on over 30,000 cervical cancers that showed HPV-16, HPV-18, HPV-58, HPV-33, HPV-45, HPV-31, HPV-52, HPV-35, HPV-59, HPV-39, HPV-51, and HPV-56 to be the most common types associated with invasive cervical cancer with HPV-16 accounting for over 50% and HPV-16 and HPV-18 for >70% worldwide [141]. Epidemiological data report that HPV infection occurs at least once during lifespan in about 75% of US women [142], and natural history shows that most HPV infections resolve spontaneously, while in some women, infection persists and progresses to cervical cancer. The incidence of high-grade cervical intraepithelial neoplasia 3 (CIN 3) is about one to two per ten females with low-grade CIN, and without treatment, about one third progresses to cervical cancer [143, 144]. Studies in HIV women or in patients treated with immunosuppressive agents reported an increased incidence of CIN lesions, suggesting an important role of cell-mediated immune response against HPV antigens [145, 146]. The role of systemic and local mucosal immune responses to HPV antigens is controversial. Some studies suggest a positive association

between systemic cell-mediated immune responses and the regression of CIN [147]. Moreover, antibody responses to the major viral capsid protein, L1, can be detected by about 6 months after infection and may be observed up to 5 years later in women who have been cleared from infection. Type-specific L1 antibody responses have also been detected in persistent disease and cancer in about half of the patients [148, 149]. The number of escape factors may affect the natural immune response against HPV proteins, together with the loss of correct signals from immune system to activate adaptive immune system. Indeed, optimal activation of adaptive immunity and generation of specific CD4 T-helper 1 type immunity supporting development of CD8 cytotoxic T-cells against viral early proteins, like E2, E6, and E7, are critical for virus clearance in basal epithelial cells. T-helper cells also support optimal activation of B-cells, with secreting HPV capsid type-specific neutralizing antibodies, which can protect against subsequent infections at mucosal and systemic levels [101]. Spontaneous regression occurs in lesions infiltrated by CD4+ and cytotoxic CD8+ T-cells, and it is also associated with circulating HPV early antigen-specific CD4+ and CD8+ T-cells [150–153]. The three oncogenes of the virus, E5, E6, and E7, play a notable role in immune evasion. The E5 protein [154] appears to facilitate the virus-induced immune escape by downregulating MHC/HLA class I and II [155, 156] and inducing a reduction in recognizing CD8+ T-cells [157]. This downregulation does not affect the HLA molecules (HLA-C/E) [158, 159]. Also, it has been shown that E5 selectively inhibits surface expression of HLA-A and HLA-B [155]. E6 and E7 still play an essential role: (i) high-risk E6 reduces the surface expression of CDH1 by epithelial cells; (ii) E6 and E7 inhibit the transcription of Toll-like receptor (TLR) 9, necessary to activate antigen-presenting cells as part of innate immune response; (iii) E7 reduces expression of transporter associated with antigen processing 1 (TAP1), a component of the presentation and processing pathway; and (iv) high-risk HPVs downregulates the expression of pro-inflammatory cytokines [160]. In addition, therapeutic T-cell effector mechanisms are limited due to the following: changes in local immunity, the production of cytokines such as interleukin (IL)-10, and increased number of regulatory T-cells (Tregs) and to immunosuppressive myeloid cells. Moreover, frequent mutational events in cancer include HLA loss of expression, with subsequent escape of tumor cells [161, 162]. To summarize, HPV-related tumors usually present MHC class I downregulation, impaired antigen-processing ability, avoidance of T-cell-mediated killing, increased immunosuppression due to Treg infiltration, and secretion of immunosuppressive cytokines [163]. These are obstacles faced when achieving a valid immunotherapy against HPV-related pathologies where a number of different strategies have been developed to overcome them including adjuvants. Certain adjuvants have recently been demonstrated to be able to induce cellular immunity which are summarized in Table 7.1 according to their mechanism of action [164].

Immunity can be utilized in a therapeutic setting in two ways: first, by using specific natural or synthetic antibodies against defined targets or, second, by inducing an immune response in the organism against specific antigens (preventive and therapeutic vaccines). Particularly, HPV-induced lesions and cancer viral antigens and/or virus-induced host antigens can be targeted by these approaches. Indeed,

Table 7.1 List of adjuvants by their dominant mechanism of action

Antigen delivery systems	Immunopotentiators
Electroporation	Alternative pathogen-associated molecular patterns (PAMPs), e.g., cholera enterotoxin, liquenase
Gene gun	Heat-shock proteins
Liposomes	Lysosome and endocellular reticulum (ER)-targeting agents
Virosomes™	Saponins (Quils, QS-21)
ISCOMS®	TLRs agonists, e.g., imiquimod, oligonucleotides (CpG, etc.), double-stranded RNA (dsRNA)
Micro/nanoparticles, e.g., microparticles of poly(lactide-co-glycolide) (PLG)	Cytokines and chemokines, e.g., IL2, IL12, and GM-CSF
Emulsions, e.g., MF59, Montanides	Treg inactivators, e.g., anti-apoptotic molecules, low-dose cyclophosphamide, antibodies anti-CD 25, anti-CTLA, anti-IL10, or anti-PDL-1
Viruslike particles and viral/bacterial vectors	Monophosphoryl lipid A (MPL) and synthetic derivatives
	Muramyl dipeptide (MDP) and derivatives

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once a patient is infected with HPV, there is no effective way to cure persistent HPV infection which is the first step toward the development of precancerous lesions. It was estimated that with mass vaccination through highly effective preventive quadrivalent or bivalent HPV vaccines [165–169], it will take about 20 years or more before the prevalence of cervical cancer significantly decreases. As existing treatments [170–172] are partially effective in premalignant and malignant lesions, and invalid in persistent infections, immune therapies may offer a valid therapeutic modality. Table 7.2 focuses on the clinical trials of already established viral infections causing premalignant lesions of the uterine cervix [165].

The following are the therapeutic modalities of developing immunotherapeutic agents for premalignant uterine cervical lesions.

Therapeutic Antibodies

Intracellular antibodies (intrabodies) to inhibit protein function are valid pathways for the treatment of human diseases. This modality is effective and specific as it combats intracellular parasites like HPV viruses. Infected cells and transformed cells require the continuing of E6 and E7 oncogenes. This has been demonstrated in Hela cells, derived from an HPV-associated malignancy [173, 174]. Intrabodies against the E6 [175] and E7 [176] of HPV have been produced and proved effective in in vitro cancer cell models. An intrabody against the E7 of HPV-16 has been shown to inhibit tumor growth in animal models [177]. Intrabodies are thought to be useful inhibitors of viral protein-protein interactions and appropriate for the treatment of HPV-associated diseases. The utilization of monoclonal antibodies against membrane-expressed antigens may be induced by the HPV, i.e., epidermal growth

Table 7.2 Clinical trials for HPV-associated pre-neoplastic cervical lesions

Vaccine	Antigen(s)	Phase	Lesions
ADXS11–001:	HPV-16 E7	II	CIN 2/3
Lm secreting fusion/LLO-HPV-16 E7 protein (Lm-LLO-E7)	HPV-16 and HPV-18 E7	I/II	High-risk HPV infections before CIN appearance
ProCervix: adenylate cyclase protein vector delivering HPV16 and HPV18 E7 antigens			
MVA E2: recombinant modified vaccinia Ankara (MVA) encoding E2 from BPV	Bovine papillomavirus E2	I/II	CIN1–3
		II	High-grade CIN
TG4001/R3484:	HPV-16 E6/E7	IIa	CIN 2/3
Recombinant MVA expressing E6–E7of HPV-16 and IL-2		IIb	
Peptides: HPV E7 (aa 12–20) plus E7 lipopeptide (PADRE helper peptide, linker peptide, and E7 peptide, aa 86–93) and Montanide ISA-51 adjuvant	HPV-16 E7	I	High-grade CIN and
			HSIL
HPV-16 E6/E7 fusion protein plus ISCOMATRIX adjuvant	HPV-16 E6 and E7	I	CIN 1–3, HPV-associated AIN in HIV-positive male
PD-E7: Modified HPV-16 E7/Hib protein D fusion protein and AS02B adjuvant	HPV-16 E7	I/II	CIN 1, CIN 3
SGN-00101: HPV-16 E7/*M. bovis*, Hsp65 fusion protein		II	ASCUS and LSIL, high-grade CIN
SGN-00101 in poly-ICLC adjuvant	HPV-16 E7	I	CIN 1–3
ZYC101: Recombinant HPV-16 E7 DNA plasmid encapsulated in poly-microparticles	HPV-16 E7	I	CIN 2/3
ZYC101a: Recombinant HPV-16 and HPV-18 E6–E7 DNA plasmid encapsulated in poly-microparticles	HPV-16 and HPV-18 E6 and E7	II/III	High-grade CIN
pNGVL4a-Sig/E7/Hsp70: DNA plasmid expressing mutated HPV-16 E7 fused to Sig and Hsp70	HPV-16 E7	I	CIN 2/3
pNGVL4a-CRT/E7: DNA plasmid expressing mutated HPV-16 E7 fused to calreticulin	HPV-16 E7	I	CIN 2/3
VGX-3100: DNA plasmid expressing HPV-16 and HPV-18 E6 and E7 proteins	HPV-16 and HPV-18 E6 and E7	I	CIN 2/3 (after surgery or fourth dose)
		II	CIN 2/3
TA-CIN/TA-HPV prime/boost	HPV-16 and HPV-18 E6 and E7 and HPV-16 L2	II	CIN 2/3
TA-HPV/TA-CIN prime/boost	HPV-16 and HPV-18 E6 and E7 and HPV-	II	CIN 2/3

(continued)

Table 7.2 (continued)

Vaccine	Antigen(s)	Phase	Lesions
pNGVL4a-Sig/E7 /Hsp70 and TA-HPV prime/boost plus TLR agonist imiquimod	HPV-16 and HPV-18 E6 and E7	II	CIN 2/3

From Vici et al. [165], with permission

factor receptor (EGFR). Monoclonal antibodies anti EGFR are currently clinically utilized [170]. Other membrane-associated antigens can be found in transformed cervical cells and may be targeted by monoclonal antibodies. Adecatumumab (MT201), a humanized monoclonal antibody targeting epithelial cell adhesion molecules, is an example of these antibodies. It has shown some activity in cervical cancer cell lines overexpressing epithelial cell adhesion molecule (EpCAM) [178].

Therapeutic Vaccines

Therapeutic vaccines aim to kill or reduce infected cells by stimulating cytotoxic T-cells against target infected cells and upregulating MHC class I expression. Vaccine-mediated immune strategies have two stages of the oncogenic infection: firstly, infection and then, secondly, the established infection. By eliciting neutralizing antibody responses, the prophylactic vaccines challenge the first infection by inhibiting the HPV to bind to the cell or the early phases of viral entry. The therapeutic vaccines could be tailored based on the presence of episomal replicating virus or integrated viral sequences. In the first case, the vaccine targets early proteins; in the second case, it targets E6–E7 proteins [179]. Effective immunotherapy administered before tumor challenge includes an antigen-specific component, whereas an effective immunotherapy after tumor challenge can be achieved through the enhancement of either innate or adaptive immunity. Immunotherapy in patients with HPV-associated premalignancy is more effective than in cancer patients, as the impaired antigen presentation by cervical cancer cells due to mutations in MHC and TAP genes may render the immunotherapy less effective. However, there are potential immune-evasive mechanisms that are attributed to the HPV infection [180]. Examples of those therapeutic vaccines are as follows:

Dendritic Cell (DC)-Based Vaccines

The immune response to infection causes inflammatory responses that trigger innate effector cells, such as NK and NKT cells. This inflammatory response, driving the innate immunity, is initiated through pathogen-associated molecular pattern (PAMP) sensors including TLRs 1–9. These receptors in response to specific bacterial or viral components activate APCs via the transcription factor nuclear factor-KB (NF-KB). Also, infection may alter the local metabolic and cellular microenvironment activating danger-associated molecular pattern (DAMP) sensors, specially nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), inducing maturation and releasing members of the IL-1 family. The produced IL-1b and

IL-18 mediate repair responses such as angiogenesis and, via upregulation of cytokines and chemokines, induce the recruitment of inflammatory cells to the site of infection. The slow clearance of HPV infection and weak immune responses to viral proteins are consequences of the nonlytic nature of HPV infection and a consequent delay in induction of PAMP- and DAMP-induced inflammatory responses through TLRs and the inflammasomes. In the absence of inflammation, IL-10 production by Th cells and mast cells, IFN-gamma production by CD-1d-activated NKT cells, and increased TGF-beta occur inducing negative signals that change the state of the APC by altering co-stimulatory molecule expression, thus inhibiting induction of cytotoxic effector T-cells. Consequently, a therapy aimed to reactivate these APCs could be a valid tool for clinical intervention. DCs are the most potent APC as they express high levels of MHC and co-stimulatory molecules. A variety of methods have been established for generating DCs, loading them with tumor antigens, and administering them to patients. Provenge, a DC vaccine incorporating prostatic acid phosphatase, has been studied in patients with advanced prostate cancer [181, 182]. In a study, autologous DCs were pulsed with HPV-16 or HPV-18 E7 recombinant proteins, and E7-specific CD8+ T-cell responses were observed in 4 out of 11 late-stage cervical cancer patients [183]. In another study, stage IB or IIA cervical cancer patients were vaccinated with autologous DC pulsed with recombinant HPV-16/HPV-18 E7 antigens and keyhole limpet hemocyanin 1 (KLH1). This vaccine generated E7-specific T-cell responses in eight out of ten patients and antibody responses in all patients [184].

Nucleic Acid-Based Vaccines

DNA vaccines have been used to elicit antigen-specific immune responses. They have several advantages; mainly naked DNA is relatively safe, stable, cost-efficient, and able to sustain reasonable levels of antigen expression within cells. DNA-based plasmid vectors remain stable in a wide range of conditions over a long time, and they can be delivered with slight risk to individuals who are immunosuppressed. Also, they can be repeatedly administered with similar efficacy. Many strategies have been employed to produce an efficient delivery of targeted antigen-to-antigen-presenting cells (APC) such as dendritic cells (DCs), an enhancement of antigen processing and presentation in DCs, and an augmentation of DC and T-cell interaction [185]. It has been reported that the fusion of the E7 gene of HPV-16 with a plant virus coat protein produced a strong antitumor activity in a mouse model activating both CD4+ and CD8+ T-cells [186–188] and a fusion of E7 gene to a gene encoding a mutated form of the immunotoxin from *Saponaria officinalis*, the saporin [187]. A dose-escalation trial of plasmid DNA encoding a transgene that produced E7 linked to Hsp70 showed a limited efficacy at the highest dose, with low induction of responses in the IFN-gamma ELISPOT assay and a resolution rate of 33% [188]. A plasmid DNA encoding a 13-amino acid sequence of E7 encapsulated in biodegradable poly(D, L-lactide-co-glycolide) microparticles was utilized to develop the ZYC101 vaccine expressing HPV-16 E7 HLA-A2-restricted peptide. Another two different phase I clinical trials examining the potential treatment of patients with anaplasia and with high-grade CIN, respectively, showed a high number of

immunological responses, circulating HPV-specific T-cells and histological regression/improvement in 1/3 of the patients [189, 190]. Version ZYC101a that includes the HPV-encoding sequences of HPV-16 E7, the regions encoding segments of HPV-16 and HPV-18 E6 and E7 viral proteins, has reached phase II/III clinical trials involving patients with high-grade CIN. In a population of women younger than 25 years, CIN resolution was significantly higher in the ZYC101a groups compared to placebo [191]. In addition, it was evaluated in the treatment of patients with CIN 2/3 where half of 21 patients receiving the vaccine showed HPV-16-/HPV-18-specific T-cell responses, but only 6 patients recovered from high-grade CIN [192]. The methodologies for production and delivery of HPV therapeutic vaccines are shown in Fig. 7.3 [193].

VGX-3100, a DNA vaccine incorporating plasmids targeting HPV-16 and HPV-18 E6 and E7 proteins, was utilized in a phase I clinical trial; 78% of the VGX-3100-vaccinated high-grade CIN subjects showed T-cell and antibody responses [194]. Other DNA vaccines have also been associated with other adjuvating treatments, namely, the TLR7 agonist, imiquimod, promoting the activation of antigen-presenting cells and leading to the production of cytokines IFN-alpha, IL-6, and TNF-alpha [195] which was shown to be active in mouse models [196]. Notably, the imiquimod treatment affected the tumor microenvironment by reducing the number of myeloid-derived suppressor cells that have an immunosuppressive role and increasing natural killer (NK) and NKT cells that may play a role in tumor volume reduction. Moreover, the use of RNA replicons is a potentially valid strategy for HPV vaccination. RNA replicons are naked RNA molecules derived from alphaviruses, such as Sindbis virus [194, 195], Semliki Forest virus [196, 197], and Venezuelan equine encephalitis (VEE) virus [198]. These RNA vaccines are self-replicating and self-limiting and may be administered as either RNA or DNA, which is then transcribed into RNA replicons. RNA replicon-based vectors can replicate in a wide range of cell types and can be used to produce sustained levels of antigen expression in cells, making them more immunogenic than conventional DNA vaccines. Notably, RNA replicons are less stable than DNA. To combine the benefits of DNA and RNA replicon, DNA-launched RNA replicon was utilized for HPV vaccine development in preclinical models [199, 200]. This DNA-launched RNA replicon is transcribed into RNA within the transfected cell and provides an efficient way to express tumor antigen, but it induced cellular apoptosis. Another replicon system is derived from the flavivirus Kunjin (KUN) which has been utilized [201]. The new generation of KUN replicon vectors did not induce cellular apoptosis, and it elicited specific T-cell responses [202]. Another mRNA-based vaccine is the RNActive® vaccine platform which is based on a more stable modified mRNA sequence with increased immunogenicity by complexation with protamine. This mRNA vaccine exploits both the antigenic and the adjuvant properties of mRNAs to activate the adaptive and innate immune system.

Live Vector-Based Vaccines

Bacteria, such as *Listeria monocytogenes* (LM), *Lactococcus lactis*, *Lactobacillus casei*, *Salmonella*, and bacillus Calmette-Guerin, and several viral vectors, including vaccinia virus (VV), adenovirus, adeno-associated virus, alphavirus, and its

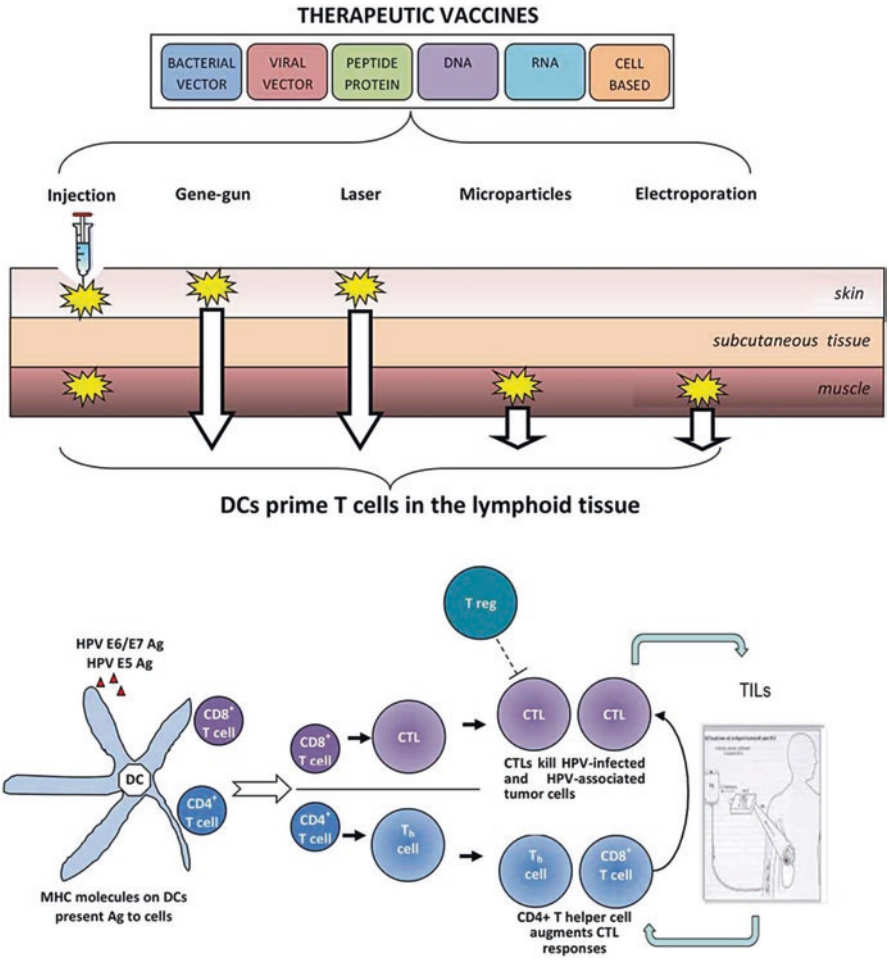

Fig. 7.3 Methodologies for production and delivery of HPV therapeutic vaccines and their immunological activity. Abbreviations: *Ag* antigen, *DCs* dendritic cells, *Treg* regulatory T-cell, *Th* T-helper cell. (From Vici et al. [194], with permission)

derivative vectors, have been used to deliver genes to elicit antigen-specific immunotherapy.[203–208] LM has emerged as a promising vector, as it is able to induce both CD8+ and CD4+ immune responses, to elicit regression of established tumors, and to overcome central tolerance by expanding low-avidity CD8+ T-cells specific for E7 [209, 210]. DXS11–001 a live, attenuated LM bacterial vector secreting HPV-16 E7 fused to listeriolysin O (LLO) was utilized in clinical trials [211, 212]. Several trials are ongoing involving women with persistent or recurrent cervical carcinoma (NCT01266460), with CIN 2/3 with surgical indication (NCT01116245) [213], and patients (including male) with HPV-associated oropharyngeal cancer (NCT01598792). Viral vectors are employed for the expression of HPV antigens, like adenoviruses [214], alphaviruses [215–217], and VV [218–220]. VV vaccines

were the first viral vectors employed in clinical trials on therapeutic vaccines against HPV-associated cancer [221]. Recently avipox viruses have been developed as novel vectors for the development of vaccines. Avipox viruses have been shown to inhibit the growth of HPV16 E7-expressing tumor in C57 Bl6 mice with a HPV16 E7 DNA-prime/Fowlpox HPV16 E7-boost schedule [222]. Several VV vaccines have been employed in clinical trials to deliver genes and antigens of interest efficiently. Phase I/II clinical trials in patients with vulvar, vaginal, and early- and late -stage cervical cancer are conducted with a vaccinia vector encoding HPV-16 and HPV-18 E6 and E7 antigen (TA-HPV) recombinant VV [223–225]. In a phase II clinical trial, 29 patients with stage I or II cervical cancer were vaccinated twice via scarification with TA-HPV; induction of CTL responses were detected in a number of patients in the form of target cell lysis by isolated peripheral bone marrow cells (PBMCs) [226]. In another study, a recombinant VV expressing E6 and E7 antigen together with IL-2 (TG4001/R3484) was administered to CIN 2/3 patients. Ten patients (48%) were evaluated as clinical responders at month 6. At month 12, 7 out of 8 patients without conization reported neither suspicion of CIN 2/3 relapse nor HPV-16 infection [227]. Another phase IIb trial on patients with HPV-related CIN 2/3 lesions demonstrated the activity of vaccine in monotherapy [228]. A recombinant modified vaccinia Ankara vector was also utilized to express bovine papillomavirus E2 (MVA-E2). E2 is a transcriptional repressor of E6 and E7 oncogenes. There is no evidence for E2 expression direct contribution to the therapeutic effect seen in patients with CIN [229, 230] and genital wart [231] response. Synthetic viral vectors like viruslike particle (VLP) can be utilized as they have the capacity for compacting DNA and targeting specific cell receptors. The same technology used for producing anti-HPV prophylactic vaccines was employed for producing chimeric VLPs. An L1–E7 fusion protein has been shown to self-assemble into chimeric VLPs (CVLP) that can induce E7-specific cellular immunity in mice [232]. A randomized, double-blind, placebo-controlled clinical trial has been conducted in CIN 2/3 patients with CVLP. Antibodies with high titers against HPV-16 L1 and low titers against HPV-16 E7 and cellular immune responses against both proteins were induced. A histological improvement to CIN I or normal histology was observed in 39% of the patients [233].

Plant-Derived/Produced Vaccines

Plant molecular biotechnology includes the production of protein biopharmaceuticals such as enzymes, hormones, antibodies, and vaccine antigens in plant systems. The plant platforms present several drawbacks: time-consuming in generating stable transgenic lines, nonhomogeneous protein production in different tissues, impact of pests and diseases, and growth in non-sterile conditions [185–187]. Plant production of prophylactic and therapeutic HPV vaccines is proven, with evidence of efficacy in animals. There are data showing that an adjuvant-like effect was obtained in immunizations with crude tobacco plant extracts containing the E7 protein of HPV-16 [215, 216]. The recombinant plant-derived vaccines without adjuvants were able to elicit also a protective Th1 cell response in mice. A similar adjuvating activity was seen in another tobacco plant-produced fusion protein of the

HPV-16 E7; this preparation was able to induce a specific CD8+ T stimulation that elicited a therapeutic effect on experimental tumor models [188, 189]. The possibility to produce E7 with high immunological activity in microalgae opens the way to producing antigens at affordable price, retaining the adjuvating activity of these plant-derived antigens [217]. An FDA-approved clinical trial for non-Hodgkin's lymphoma with plant-produced single-chain variable fragment (scFv) was able to establish the safety and immunogenicity of plant-made human vaccines [218, 219]; this could be a feasible approach for human anticancer therapies.

Protein-/Peptide-Based Vaccines

There are several protein-/peptide-based vaccines undergoing clinical evaluation. A major limitation to peptide-based vaccines is the HLA restriction that can be overcome by whole protein-based vaccines, which harbor multiple immunogenic epitopes, binding various allelic HLA molecules. A majority of studies were focused on the co-administration of adjuvant immune-enhancing agents such as chemokines, cytokines, and co-stimulatory molecules to enhance the potency of the vaccine. Particularly, saponin-based [152] or liposome-based (LPD) formulations [153] or TLR agonists [154] were employed as adjuvants for protein vaccines. Recently, the fusion of the beta-1,3-1,4-glucanase (LicKM) of *Clostridium thermocellum* bacterial protein to the HPV E7 protein produced an antigen with strong intrinsic adjuvating activity, indicating that it may lead to elicit some functions [155, 156]. Many other fusion proteins were reported to elicit some adjuvating activities such as *Mycobacteria*-derived heat-shock proteins (Hsp) [157, 158], truncated *Pseudomonas aeruginosa* exotoxin A [159], *Bordetella pertussis* adenylate cyclase [160], and the cell-penetrating peptide *Limulus polyphemus* protein [161]. TLR agonists have been explored as adjuvants for peptide-based HPV vaccines because of their capability to activate both innate and adaptive immunities. Vaccines consisting in CTL and/or TH epitope adjuvated with TLR 9 [162]; TLR4 [163] and/or TLR3 [164] agonists demonstrated their efficacy in mouse models. This activity was demonstrated also by utilizing a CTL epitope fused to a T-helper epitope, pan-DR epitope (PADRE) [165]. Adjuvants targeting dendritic cells are useful in peptide-based vaccines. A strategy based on the administration of co-stimulatory anti-CD40 monoclonal, TLR agonist polyinosinic-polycytidylic acid [poly(I:C)] and CD8+ T-cell epitope HPV-16 E7 (aa49–57) was able to induce tumor clearance in two HPV-induced murine cancer models [166]. SGN-00101 vaccine, a fusion protein consisting of Hsp from *Mycobacterium bovis* and HPV-16 E7, has shown that it was able to induce regression of lesions in anal high-grade squamous intraepithelial lesions [167], recurrent respiratory papillomatosis [168], and CIN 2/3 [169–171]. Phase II clinical trial with TA-CIN, a fusion protein-based vaccine expressing HPV-16 L2-E6–E7-conjugated proteins, in conjunction with topical application of TLR agonist imiquimod showed high levels of CD4+ and CD8+ T-cells locally in patients with high-grade vulvar intraepithelial neoplasia (VIN) [172]. The PADRE universal T-helper peptide was utilized to increase the activity of CTL epitopes encoding HPV-16 E7 that was presented by HLA-A*0201. These vaccines failed to achieve a valid immune response in women with late-stage cervical cancer [168–170]. More promising results were

obtained in HLA-A2-positive patients with CIN/VIN 2/3 [176], where HPV E7 lipopeptide (aa 86–93)/PADRE was able to stimulate an immune response and led to complete regression of CIN lesions in 3 of 17 valuable patients. In resected cervical cancer patients, the use of immunization with 13 overlapping long peptides spanning the entire sequence of HPV-16 E6 and E7 mixed with Montanide ISA 51 clearly revealed immunization-driven IFN-gamma production in enzyme-linked immunospot (ELISPOT) assay after completing the protocol [176]. The same platform was tested in immunizing cervical cancer patients and showed that both CD4+ and CD8+ T-cell IFN-gamma responses were detected toward both antigens [178]. Significant increases in proliferative capacity were also noted in responding T-cells [178]. Phase II clinical trials of this vaccine in histologically confirmed HPV-16-positive high-grade VIN patients had a complete regression of their lesion after three or four vaccinations with HPV-16 E6/E7 overlapping peptide vaccine [179]. In non-responders to the vaccine, an increased number of HPV-16-specific CD4 + CD25 + Foxp3+ Treg cells were noted [180]. The presence of these Foxp3+ T-cells is linked to impaired immunity in malignancies. The efficacy of this vaccine was also shown in a phase II study that noted an increased number of HPV-16-specific T-cells in patients with HPV-16+ high squamous intraepithelial lesion (HSIL) [181].

Combinational Immunotherapy

Strategies aiming to alter local immunity have shown positive results; thus therapeutic HPV vaccine strategies have shifted toward combinatorial approaches with radiotherapy and chemotherapy. Low-dose radiation in combination with HPV vaccination was effective in the treatment of tumors in preclinical models [220]. Radiation therapy seems to be a useful method in stabilizing tumor cell growth when applied with immunotherapy by inducing apoptosis in tumor cells. A chemotherapeutic agent in combination with DNA-based vaccines was shown to be an effective HPV therapy in preclinical models [221, 222]. Low-dose cyclophosphamide produced positive effects in persistent low-risk HPV lesions [223]. A randomized study was carried out in 110 recurrent/refractory cervical cancer patients with cisplatin and different doses of HPV bacterial vector-based vaccine ADXS11–001, and results showed efficacy and manageable toxicity [224]. Other compounds affecting the immunological environment like COX-2 inhibitors, through the prevention of the production of prostaglandin E2 or antibodies to IL-6 [225] or IL-10 [226] or the TLR agonist imiquimod, could be a valid therapeutic agent. Imiquimod is currently in clinical use against warts stimulating local innate immunity and potentiating adaptive immune response by activating tissue antigen-presenting cells. Several studies with topical imiquimod have been reported with favorable results in vulvar intraepithelial neoplasia (VIN) lesions [227, 234]. Cytokine-based therapies in combination with HPV therapeutic vaccine showed promising results in preclinical models. Treatment with IL-12 gene, administered as gene therapy, as viral gene therapy, by adenovirus, and in combination with E6–E7 oncogenes,

determined tumor growth suppression [228, 229]. An anti-PD-1 antibody (CT-011) with Treg-cell depletion by low-dose cyclophosphamide (CPM), combined with HPV-16 E7 peptide vaccine, produced antigen-specific immune responses inducing complete regression of established tumors in a notable percentage of treated animals, with prolonging survival [230]. Expanded phase I clinical studies with anti-PD-1 and anti-PDL-1 showed objective clinical responses in renal cell carcinoma, melanoma, and non-small cell lung cancer and a relationship between tumor cell surface PD-L1 expression and objective responses to anti-PD1 therapy [231, 232]. In addition, a recent study showed that PD-1/PDL-1 pathway may create an "immune-privileged" site for initial viral infection in the tonsils and subsequent adaptive immune resistance once tumors are established suggesting a rationale for therapeutic blockade of this pathway in patients with HPV + oropharyngeal squamous cell carcinoma [233]. Other strategies utilize monoclonal antibodies such as ipilimumab. This antibody is a fully human monoclonal antibody against the cytotoxic T-lymphocyte antigen-4 (CTLA-4), an immune inhibitory molecule expressed in activated T-cells and in suppressor T-regulatory cells. The interaction between the monoclonal antibody and CTLA-4 blocks inhibitory signals and enhances T-cell activation, leading to increased antitumor responses [235].

Conclusion

Human cancer has a number of unique features. Immune infiltration into the tumor has been demonstrated, but tumor evasion and subversion of these immune defenses were noted. In the immunosuppressive environment of the tumor, achieving immune reactivity through immunotherapeutic approaches is difficult. There are a number of precancerous lesions that pose a high risk of developing into cancer. It is difficult to determine if the precancerous lesion environment would be less immune subversive than the one for cancer and would be better suited for immunotherapeutic treatment approaches. However, immunotherapy is a promising strategy for cancer treatment. In cervical cancer and its precursors, the use of therapeutic vaccines was associated with the regression of premalignant lesions and some clinical benefit in cancer patients. Current data suggest that vaccines for pre-neoplasia and cancer of the uterine cervix are valid therapeutic modalities. The improvement of all therapeutic strategies and the identification of their optimal combination open an efficient scenario in the treatment of uterine cervical cancer and its premalignant lesions. As the role of immunotherapy for the treatment of patients with precancerous lesions and uterine cervical cancer continues to evolve, further studies on immune cellular and molecular mechanisms of action and on preclinical models are needed to better understand immunological background and to explore the optimal integration among treatments and combination immunotherapies.

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