Development of New Human Papillomavirus Vaccines

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1. Introduction

Over the past 35 years, we have observed a remarkable and important increase in the prevalence of HPV infection, both in its clinical forms and appearance of its condyloma acuminate. Colposcopic exploration of this area would be required, with special focus at the regions of introitus and inter-labial folds. Both genital and anal examinations (Guerra-Tapia et al., 2009) are necessary to determine the sub-clinical expression of HPV identified by cytological changes, colposcopy, and/or vulvoscopy and vaginoscopy. Clinical forms of HPV infections generally caused by LR-HPV strains (6, 11) tend to be benign. Sub-clinical forms include benign and pre-malignant lesions, and are generally caused by HR-HPV strains (16 and 18) (Fig. 1) (Rodríguez-Cerdeira C et al., 2008a, 2008b, , 2009a; Walboomers et al., 1999). In a recent study by the International Agency for Cancer Research (IACR) group in 13 areas in 11 countries including Spain, a high prevalence of HPV was seen in both Europe and Sub-Saharan Africa (International Agency for Research on Cancer [IARC], 1995; Muñoz et al., 2003). Furthermore, it was observed that HPV-16 infection was more frequent among European women. We observed the same genotype in a study involving 436 women aged between 16 and 80 years. Three samples from the cervix and vagina of each patient were cytologically examined (Rodríguez-Cerdeira C et al., 2009b).

Thus, epidemiological studies supported by molecular techniques and liquid cytology have confirmed the incidental role of certain strains of HPV in the development of cervical, vulvar, vaginal, anal, and penile cancer (Fig. 2), the risk for which is greatly increased in human immunodeficiency virus (HIV)+ patients (Rodríguez Cerdeira C et al., 2011). An international series with high-sensitivity polymerase chain reaction (PCR) has proven that HPV DNA is present in 90.7% of cervical–uterine carcinomas and is present in all the cases confirmed by exhaustive histological examination. This also occurs in the majority of intraepithelial lesions of the lower genital tract (Walboomers et., 1999).

Persistent HPV infection is considered the principal causative agent of cervical and other anogenital cancers. The finding that HPV DNA is present in practically all cases of cervical

cancer has great importance in developing preventive strategies (Fig. 3) (Rodríguez Cerdeira C et al., 2007; Mougin et al., 2001; Trottier et al., 2006). Integration of the viral genome with the host cell genome does not occur in all cases of cervical cancer and may be explained by mutations in repressive areas such as the region Ying-Yang (YYI), which would maintain the continued expression of E6 and E7 or by the production of a more stable 'chimeric' RNA, thereby permitting greater synthesis of these oncoproteins (Fig. 4) (Alba et al., 2009).

Fig. 1. High-grade vulvar intraepithelial neoplasia in a smoker

Fig. 2. Penile intraepithelial neoplasia developing into invasive carcinoma with extensive exophytic ulcerous mass that invades the gland and projects beyond the preputial-balanic fold in a human papillomavirus-positive patient
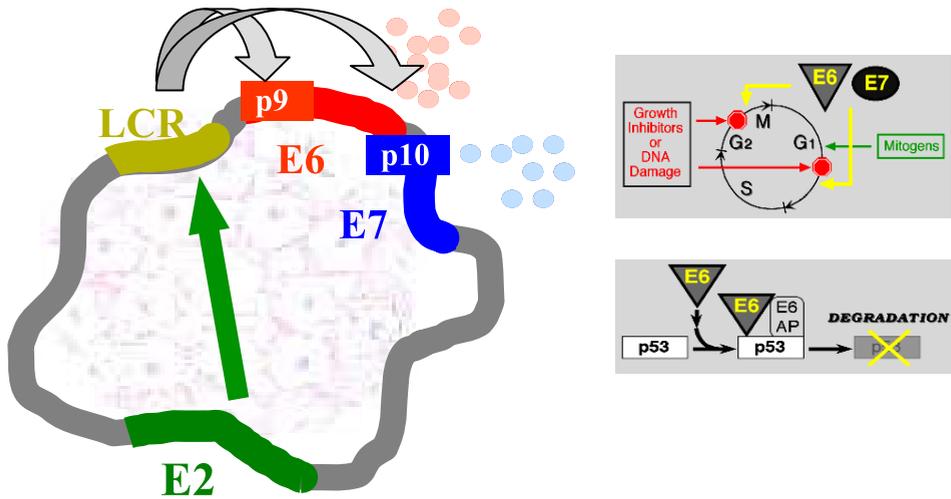
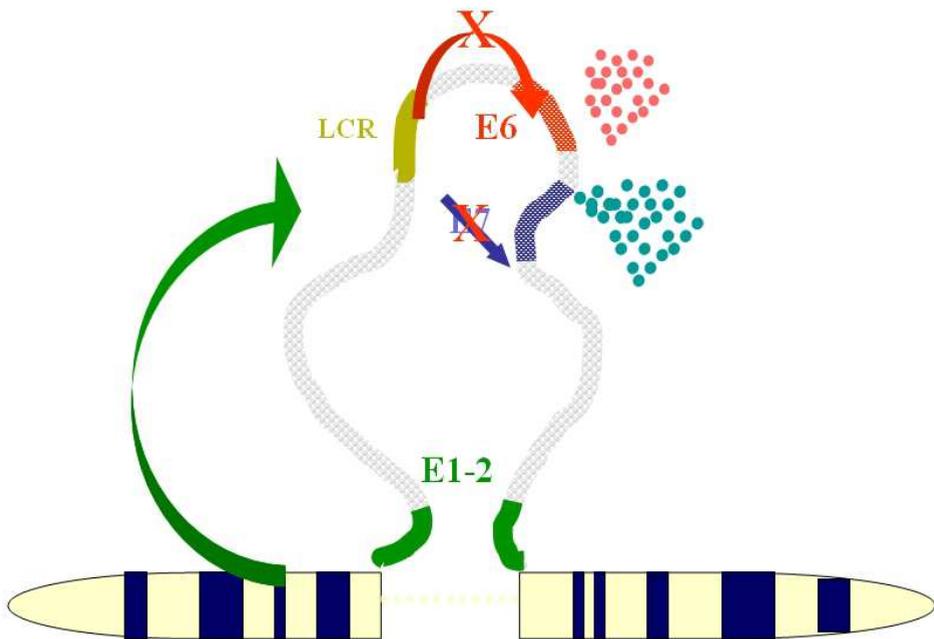Fig. 3. Interaction between the viral protein and the cell cycle

Fig. 4. Integration of human papillomavirus in the host genome

Over the recent years, the morbimortality and health costs associated with cervical, vaginal, vulvar, anal, and peneal cancers and their precursory lesions have provoked intense investigation (Kadish et al., 1992; Guerra-Tapia et al., 2009; Rodríguez-Cerdeira C et al., 2008a) to achieve vaccine-based prevention of HPV infections, which would dramatically reduce the risk of these cancers. Experiences with girls or women with current infection

with 1 or more of the vaccine HPV types gained protection from the infections or diseases caused by the remaining vaccine HPV types, and they were also protected against re-infection with the same HPV type after clearance of an infection caused by a vaccine HPV type. High seroconversion rates and high levels of anti-HPV antibodies were observed in all vaccinated individuals of all age ranges from 9 to 45 years. Rechallenge with a quadrivalent HPV vaccine produced a potent anamnestic humoral immune response (Rodríguez Cerdeira C et al., 2009a, 2009b; Alba et al., 2009). The vaccine is generally well tolerated, and is projected to be cost effective in most pharmacoeconomic models. However, there are some questions that we are faced with: Does the intensity of such a humoral immune response correlate with long-term protection? Although a direct correlation between antibody levels and protection may seem intuitively obvious, it is still unclear whether differing antibody titres indicate better disease protection or longer duration of immune protection. Given that virtually all vaccinated women are seroconverted, we may deduce that until now, we do not have any immunological correlates for protection. The question still remains unanswered: Why, when the body's natural antibodies respond so poorly, do the HPV vaccines that generate serum neutralizing antibodies work? The answer is that the quality and quantity of the immune response generated by the vaccine is different from those by natural infections.

Is it stated that vaccines will induce a generation of long-lived memory immune cells that, after re-exposure to the relevant antigen, will generate a potent immune response, thus preventing HPV infections? Time is needed to suitably answer this question. In the opinion of other investigators, preventive HPV vaccination is an expensive practice, and it may be an insufficient tool to tackle cervical cancer worldwide. Therapeutic intervention is seeking for safe/effective vaccines inducing the activation of CD8^{+} cytotoxic T lymphocytes (CTLs) that are required to clear the tumour. Linking a tumour-specific antigen (i.e. E7 oncoprotein of 'high-risk' HPVs) to molecules able to increase its immune 'visibility' represents a strategy to force the immune system to fight cancer. They focussed on plants as sources of innovative immunostimulatory sequences. Thus, a new vaccination route for systemic and mucosal immunity, and other issues will be addressed throughout this chapter.

2. HPV infection and immunity

Any viral infection requires the presence of a cellular receptor that allows for the internalisation of the viral particles. This circumstance supposes the principal barrier to entry and explains the species-specific and even organ-specific nature of viral infections. Some viruses use the major histocompatibility complexes I and II (MHC I and II) as receptors for their internalisation, while others use molecules on the cellular surface (CD4, chemokines, growth factors, and β2 microglobulin). The HPV does not have a specific cellular receptor but rather has a well-conserved surface molecule with vital cellular functions, which makes its use impossible as a target for blocking infection. As opposed to other viruses, it does not appear that the surface receptors are implied in tissue or species specificity or in HPV tropism (Alba et al., 2009).

Infection recognition by the host cell and specific tropism of each viral subgroup determine the cytopathic effects in specific tissues (Rodríguez-Cerdeira C et al., 2008a); this makes it possible to distinguish between latent infections that do not show their effects for long periods and active infections that have practically immediate cytopathic effects. Based on these parameters, it is possible to qualify the antigenic or immunogenic level of each virus to

comprise the knowledge base for the manufacture of therapeutic or prophylactic vaccines (Alba et al., 2009; Rodríguez-Cerdeira et al., 2009c).

2.1 Cellular and humoral immunity to human papillomavirus

Cellular immunity is principally represented by T cells, which act at the local tissue level via close cell-cell contact. The humoral response is measured by B cells according to the instructions from T helper cells, through antibody production. The T cells recognise proteins on the surface of the HPV that are associated with the molecules from the cellular surface (human leukocyte antigen [HLA]), while the antibodies recognise both surface and soluble antigens. In the latter case, this is done with greater specificity. The T-cell receptors recognise specific sequences of small peptides presented by the MHC, while the antibodies recognise steric three-dimensional structures with determined structures. If correct presentation of the antigen is essential for inducing an immunological response, the kinetics of antigen-antibody joining and the number and distribution of these joins would be the factors determining the immunological response level.

In general, after the first infection of the cervical epithelial cells by HPV, a non-specific response is provoked, accompanied by an inflammatory process, neutrophil chemoattraction, macrophage activation, natural killer (NK) cell intervention, production of natural antibodies, and activation of the complement system, which forms the first non-specific yet defensive immunological barrier. Prolongation of the response over time and protection against future infections requires specific immunological mechanisms (Alba et al., 2009; Kadish et al., 1992; Rodríguez-Cerdeira et al., 2009c). T CD4 lymphocyte activation requires recognition of the surface molecules exposed by the presenter cell. The viral peptide along with class II HLA will be recognised in the context of T cell receptor and CD4, but requires a 'safety' mechanism for deactivation process control. Thus, it is necessary that other molecules such as CD40 and B7 that are present on the presenter cell surface be recognised by their receptors (CD40 linking and CD28, respectively) for activation to occur. Each activated T CD4 lymphocyte will be converted into a type 1 or 2 lymphocyte T helper cell depending on a series of local tissue factors fundamentally comprising antigen entry route, processing mechanism, and the presence of different interleukins. The Th1 pathway will induce T CD8+ lymphocyte maturity towards cytotoxic effector cells (Fig. 5) (Alba et al., 2009; Rodríguez-Cerdeira et al., 2009c, 2009d).
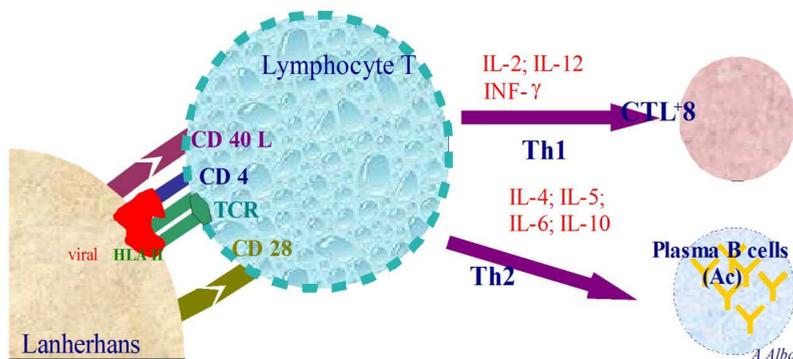

Fig. 5. Lymphocyte activation towards cytotoxic effector cells

Specific cells existing in the cervical epithelium are capable of acting as antigen presenters. Although some keratinocytes develop this ability, Langerhans reticular cells are the true antigen presentation specialists in the cervical epithelium. These cells absorb the viral particles to digest them into endosomes and start an activation process that includes presentation on the antigen surface together with the presenter cell HLA. These activated cells will be recognised by the T CD4 lymphocytes in the case that they recognise every molecule in the correct environment, after which, they evolve into lymphocyte helpers (Th) in the local context of the expression of certain interleukins (IL). Depending on IL type, it will advance to differentiation towards a Th1 pathway that will induce the activation and proliferation of T CD8+ cytotoxins with specific immunity (CTL+8) or towards a Th2 pathway that will induce the activation and expansion of B lymphocytes, which differentiate towards plasma-antibody producer cells for the viral base proteins of non-specific immunity that we could identify as prophylactic (Alba et al., 2009). CTL+8 would have the ability to act against the established viral infection, while the plasma B cells produce antibodies act against the external viral antigens that are exposed during this and successive HPV infections.

The nature of antibody responses and duration following HPV vaccination plays a key role in long-term protection against HPV infection. The importance of vigorous and prolonged immune protection is also very important. In addition, it provides maximum benefit against cervical cancer and other HPV-related cancers (Alba et al., 2009; Rodríguez-Cerdeira et al., 2008a, 2009c, 2009d). Nevertheless, it should also be highlighted that long-term protection is not fully predictable at the introduction of any vaccine, because it varies according to many variables (e.g. cohort target, coverage, acceptance, catch-up) that are not strictly related to immune response. Although some researchers have developed a model to predict long-term immunity, it remains an ongoing and challenging issue. HPV is a family of many different genotypes. Ideally, a vaccine should cover at least the majority of the genotypes that are linked to tumour development, i.e. those that are considered HR-HPV. Nevertheless, the large number of different genotypes among the HPV viruses raises the question about the number of HPV viruses that must be included in the vaccine preparation process. Thus, the possibility of developing second-generation cross-reacting vaccines covering a larger portion of the HPV family must be considered in the HPV investigation (Mariani & Venuti , 2010) biological evolution is concerned, HPV strains are successful infectious agents. They induce persistent infections without causing frequent or serious complications for the host and shed virions for transmission to other naive individuals. To achieve this lifestyle and maintain a state of equilibrium, HPV must avoid the host's defence system. Many factors contribute to evading immune pools, particularly, the following:

- Virus capsid entry is usually an activating signal for dendritic cells (DCs).
- Free virus particles are shed from the surface of the squamous epithelia with poor access to the vascular and lymphatic channels and to the lymph nodes where immune responses are initiated.
- Most DNA viruses have mechanisms for inhibiting interferon (IFN) synthesis and receptor signalling, and papillomaviruses are no exception.

Despite HPV's ability to evade the host's immune system and to down-regulate innate immunity, a primary HPV infection is cleared naturally in approximately 90% of the cases, thus indicating the central role of immunity in the resolution of cervical and anogenital

HPV-associated diseases. Innate immunity acts as the first line of non-specific defence against any pathogen (DCs, IFN-α, cytokines, neutrophils, and macrophages), and attacks by HPV should be detected by the intraepithelial DCs. There is evidence indicating that DCs are not activated by the uptake of HPV capsids, suggesting a limited role in the host's response to HPV infection (Mariani & Venuti , 2010; Fausch et al, 2002, 2003).

Other critical point is in regards to the long-term clinical significance of immunity evoked by natural infection. Certain studies have showed that some women in the placebo group developed the disease despite consuming antibodies against the offending HPV types at enrolment, thus confirming, as stated in the recent WHO position paper, that host antibodies directed against the viral L1 protein do not necessarily protect against subsequent infection by the same HPV genotype (Fausch et al, 2002; Olsson et al., 2009; World Health Organization [WHO], 2009).

3. Prophylactic vaccines against human papillomavirus

Prophylactic vaccines against HPV infection, which are currently in the advanced stage of development and evaluation, seem to give more hope than the therapeutic ones, whose objective is to prevent new infections. Various approaches have been examined, namely, recombinant live vector vaccines, protein and peptide vaccines, vaccines without DNA, and innocuous vaccines. The most advanced vaccines against HPV consist of particles that are similar to the virus called virus-like particles (VLP). These substances are created by L1 and L2 proteins. These particles do not contain DNA and are synthesised through self-assembly of the proteins of the upper antigen of the L1 capsid. VLPs, constructed by genetic engineering, are structures that are identical to the native virus but do not have an infectious capacity. The lower structural protein L2 can assemble with L1 and form an even more stable VLP. Their antigenic similarities with the genuine HPV virions explain why VLPs introduce a powerful humoral response with neutralising antibodies (Rodríguez-Cerdeira et al., 2009c, 2009d).

3.1 Do those vaccines activate the immune memory system?

The WHO explicitly stated that the induction of immune memory should be assessed by means of evaluating immune responses to additional doses of vaccine administered at planned intervals following the completion of the primary series. Subsequently, the immune-memory anamnestic response using an antigen challenge has been reported for the quadrivalent vaccine. Nevertheless, the questions in vaccinated women include the following: Does natural re-exposure to the same HPV type vaccine significantly boost antibody levels, which contributes to the long-term persistence of anti-HPV responses, and consequently, does it improve protection over the next few decades? Time is needed to suitably answer this question (WHO, 2006; Olsson et al., 2007)

Einstein *et al.* while comparing the immune response and reactogenicity of 2 vaccines by using the same pseudovirion-based neutralisation assay, stated that for any age strata, the positivity rates for the anti-HPV 16 and 18 neutralising antibodies in the cervicovaginal secretions and circulating HPV 16- and 18-specific memory B cell frequencies were higher after vaccination with the bivalent vaccine compared to the quadrivalent vaccine (Einstein et al., 2009). Regan *et al*. (24) considered transient infection in estimating the impact of an HPV

16 vaccine in Australia and showed that it has significant implications on patient immunity and overall vaccine effectiveness (Regan et al., 2007).

In our experience, the vaccines induced very high concentrations of neutralising antibodies, much higher than those for natural infection, and the seroconversion rates in the trials approached 100%. In a recent study, we evaluated whether women with naturally acquired HPV antibodies who were HPV DNA-negative at the baseline were less likely to develop new infection with the same HPV type than the HPV antibody-negative and DNA-negative women were. HPV infection rates were assessed over a year. New infections were detected by type-specific PCR according to the baseline HPV 6/11/16/18 serostatus. Our findings suggest that natural immunity does not reliably protect against new infections with HR-HPV types. Only a small proportion of women with naturally acquired antibodies had limited protection against incidental and persistent infection with 1 HPV type. Therefore, our results support vaccination of all women regardless of the naturally acquired-HPV antibody status (Rodríguez-Cerdeira C et al., 2009b). The medium-term objective of these vaccines is the prevention of precursory lesions of cervical cancer and cervical intraepithelial neoplasia (CIN), especially CIN 3. Furthermore, many studies have been published in favour of protection against vulvar and vaginal neoplasias by using the tetravalent vaccine (WHO, 2006). The final long-term objective of prophylactic vaccines for HPV is the prevention of invasive cervical carcinoma. Associated objectives include the prevention of other forms of HPV-related cancer, namely, those that target the vulva, vagina, anus, penis, and oropharynx (Olsson et al., 2007).

Some researchers are much more sceptical about the effectiveness of trying to establish vaccines and new models of transmission of HPV infection. They postulate that the future aim of globally eradicating HPV-associated pathologies will be achieved by the local production of antigens with cross-reactivity among the different HPV types. In addition, they state that while sensitive methods are available for the detection of viral genetic material (DNA and RNA), we do not have a definitive method for determining the infectious state of an individual, from the perspective of transmission of infections. Furthermore, currently, as we do not have any reliable correlates of immune protection against infection, we are unable to precisely report whether an individual has acquired immunity to infection, either through exposure to infection or vaccination; what level of protection has been conferred or how long it will last; or whether this protection will prevent further transmission (Olsson et al., 2007).

Clinical trials have shown that quadrivalent vaccine effective against anogenital warts but they have not ruled out the possibility that transient infection may lead to transmission. We have discussed several areas of uncertainty that are less important. The extent to which condoms and circumcision are protective against infections has not been firmly established. To date, modellers have assumed that transmission occurs via heterosexual penile-vaginal intercourse, because the greatest disease burden is cervical cancer and this mode of transmission is both the most studied and the best understood. However, other modes of transmission must be considered more closely if their role in other diseases such as anal and oropharyngeal cancers is to be studied. Unfortunately, for modellers, most studies of HPV natural history have not been designed to improve models but rather to answer broader questions. Studies of transmission in couples have not been carried out on a large enough scale or with sufficient sampling to clearly observe and measure transmission. It is our hope

that as the demand for accurate quantitative modelling studies to evaluate the impact of vaccination programs increases, studies will increasingly be designed with model parameterisation in mind (WHO, 2006; Olsson et al., 2007; Regan et al., 2007). Other research regarding prevention indicates that oncogenic virus-mediated cell fusion induces chromosomal instability and tumours (Gao & Zheng, 2011).

An expanding body of work including tissue culture studies, mouse models, and human patients suggests that tetraploidy is a precursor of the chromosomal instability state and the diploid-tetraploid-aneuploid sequence. It has also been reported that tetraploid cells tend to activate a p53 response that leads to G1 arrest and ultimately senescence or apoptosis. Thus, deregulation of the cell cycle checkpoint and apoptosis will provide tetraploid cells an opportunity to undergo dysplasia and become oncogenic aneuploid cells, which has been verified by both *in vivo* and *in vitro* experiments. It is remarkable that all human oncogenic viruses can express proteins that have the ability to inhibit pRb and p53, two critical regulators of both the cell cycle and apoptosis (Storchova & Kuffer, 2008; Castedo et al., 2006).

Therefore, multicellular organisms need to be equipped with tools that allow them to detect and remove those cells. Multiple lines of evidence suggest that p53-dependent apoptosis is the major tool for eliminating accidental tetraploid cells, which cannot undergo normal mitosis and will trigger cell cycle checkpoints. Both p53 and pRb may function as tumour suppressors in this context. Nonetheless, tetraploid cells produced by oncogenic virus-mediated cell fusion can sometimes overcome this arrest and continuously proliferate as human oncogenic viruses, expressing oncoproteins and having the ability to perturb pRb, p53, and/or apoptotic proteins. For example, HR-HPV E6 and E7 can inhibit the functions of p53 and pRb, respectively (Castedo et al., 2006; Narisawa-Saito & Kiyono, 2007). Once the tetraploid cells resulting from oncogenic virus-mediated cell fusion survive and proliferate, they may undergo dysplasia, a hallmark of most malignant tumours (Holland & Cleveland, 2009).

Chromosome stability is related to mitosis. The oncoprotein HPV-16 E5 was recently determined to have fusogenic activity and to lead to increased incidence of CIN, particularly in the presence of p53 and pRb inhibitors HPV 16 E6 and E7, respectively. Moreover, it should be noted that HPV-16 E5 must be expressed on both cells for cell fusion to occur. In this model, 2 cervical cells both expressing E5 fuse at a high rate and the resulting tetraploid cell undergoes CIN with the help of E6 and E7 to ultimately become an aneuploid cervical cancer cell. Of course, accumulation of deleterious mutations in the fused cells may also lead to the extinction of pre-malignant lesions before they become cancerous (Hu & Ceresa, 2009).

However, this model is challenged by 2 facts. First, either HR-HPV E6 or E7 alone can also contribute to tetraploid cell formation by inducing cytokinesis failure (Incassati et al., 2006; Heilman et al., 2009; Duensing et al., 2001a). Second, it is widely accepted that increasingly deregulated expression of E6 and E7 has been identified as the major transforming factor in the pathogenesis of cervical dysplasia and derived cancers.

However, our model and these 2 facts are not mutually exclusive. First, *in vitro* and clinic studies have revealed that CIN and aneuploidisation seem to precede and favour HPV genome integration, prior to which the expression of E6 and E7 is low for tight restriction in

host cells. Second, E5 is thought to play a role only in the early stage because E5 expression is inhibited by HPV genome integration, but E6 and E7 act throughout carcinogenesis, especially after integration. This model is also supported by a study showing that the formation of tetraploid cells is primarily attributed to E5 and E5-induced cell fusion rather than E6/E7 and cytokinesis failure. Therefore, cytokinesis failure induced by E6 or E7 in an over-expression system may only occur in the late stage, whereas E5-mediated cell fusion may play a key role in initial cell transformation (Duensing et al., 2001a, 2001b; Melsheimer et al., 2004).

According to the mechanisms discussed above, cell fusion is also a potential and necessary mechanism for cancer progression since tumour cells would degenerate and become extinct for fusion among cancer cells with distinct potency and may also accelerate cancer evolution. This conjecture has been confirmed in a study that showed that *in vitro* or *in vivo* spontaneous fusion between the bone- and lung-tropic sub lines of human breast cancer cell line MDA-MB-231 can produce hybrids with dual metastasis organotropism. Cell fusion with metastatic cancer cells can also endow primary cancer cells the ability to resist the cytolytic activity of cytotoxic T lymphocytes. Given these considerations, the development of fusion inhibitors would be beneficial for cancer prevention and treatment of virus-associated cancers, since they would inhibit the entry and spread of the virus and affect the oncogenic role (Lu & Kang, 2009; Lee et al., 2000).

4. Therapeutic vaccines

The investigation of therapeutic vaccines capable of providing specific cell-mediated immunity is justified. The possible indications for the therapeutic vaccine include: (1) post-exposure, (2) diagnosis of low-grade squamous intraepithelial lesion (L-SIL), and (3) diagnosis of high-grade squamous intraepithelial lesion (H-SIL) or invasive cancer (15,-17). Data from dog and rabbit models hint that vaccines with *E1* and *E2* genes as targets would be suitable for both post-exposed and L-SIL women. Nonetheless, in women affected by CIN 2/3 or cancer, continuous expression of *E6* and *E7* oncogenes is essential for progression and maintenance of the malignant phenotype. The experimental E6 and E7 vaccines have shown immunogenicity and effectiveness in transplantable tumour models in rodents. Nevertheless, human trials have demonstrated immunogenicity and safety but very limited effectiveness (Rodríguez-Cerdeira et al., 2009d; Fausch et al, 2002).

Therapeutic vaccines should be able to induce specific immunity mediated by the cells capable of preventing lesion development or eliminating existing lesions or even malignant tumours by using recombinant peptides derived from *E6* and *E7* oncogenes (minigenes) (Rodríguez-Cerdeira et al., 2009d). Various vaccine approaches based on the E7 protein or peptides representing the T cell epitopes have been developed and tested in preventive and therapeutic pre-clinical tumour models. Although effective in preventing the growth of transplantable E7-expressing tumours in mice, these vaccines have demonstrated only moderate efficacy in therapeutic settings. Although the exact mechanism of the vaccine failure is yet to be defined and is probably complex, the active immune evasion mechanisms employed by the tumour may play a critical role. The success of E7 TAA-based therapeutic vaccines against cervical cancer, therefore, may require vaccine formulations containing adjuvants that not only generate E7-specific potent immune responses but also overcome the tumour-mediated immune evasion mechanisms.

Co-stimulation plays a critical role for the generation of adaptive immune responses. We recently proposed that vaccine formulations containing co-stimulatory ligands may have efficacy in therapeutic cancer settings. We particularly focussed on the 4-1BBL, a co-stimulatory member of the tumour necrosis factor (TNF) family, because of the critical role played by 4-1BB signalling in the generation and maintenance of CD8+ T cell memory, which is critical for tumour eradication (Uno et al., 2006). Although 4-1BBL has no function as a soluble trimeric molecule, we generated a chimeric recombinant SA-4-1BBL in which the extracellular portion of this molecule was cloned at the C-terminus to the core streptavidin (SA). This chimeric molecule exists as tetramers and oligomers and has potent co-stimulatory activity on the CD4+ and CD8+ T cells in the soluble form. Vaccination with SA-4-1BBL and E7 peptide representing the dominant CD8+ T cell epitope resulted in effective eradication of the E7-expressing TC-1 tumours. The therapeutic efficacy of the vaccine was superior to other vaccine formulations containing an agonistic antibody (Ab) to the 4-1BB receptor or toll-like receptor agonists such as lipopolysaccharide, monophosphoryl lipid A, and CpG (Rabu et al., 2001; Elpek et al., 2007; Schabowsky et al., 2009).

Sharma *et al.* tested the efficacy of SA-4-1BBL as the immunomodulatory component of an E7 protein-based vaccine in the TC-1 tumour model as a prelude to phase I cancer. Use of whole E7 protein as the antigenic component of the vaccine alleviates the concerns related to the use of a single peptide representing a CD8+ T cell dominant epitope that includes the following: (i) saturation of immune response due to antigen exhaustion; (ii) lack of CD4+ T cell help, which can limit the vaccine's anti-tumour efficacy; (iii) lesser magnitude and duration of the immune response towards a single epitope compared to the collective responses to multiple epitopes; (iv) higher possibility of immune-edited escape variants; and (v) requirement for HLA compatibility that will limit the target patient populations (Sharma et al., 2010). .

We herein showed that single vaccination with SA-4-1BBL and a recombinant whole E7 protein resulted in the eradication of the established tumours in 70% of the test mice. The therapeutic efficacy of the vaccine was associated with robust primary and memory T cell responses, Th1 cytokines, enhanced intra-tumoural CD4+ and CD8+ T cell infiltration, and NK cell function. Taken together, these data corroborate the utility of SA-4-1BBL as a novel multifunctional immunomodulatory component of therapeutic vaccines and justify testing of the E7 protein-based vaccine formulation in human clinical trials. Sharma *et al.* in there studies concluded that the therapeutic efficacy of whole E7 protein and SA-4-1BBL vaccines in the TC-1 tumour model was comparable to the efficacy obtained using a synthetic peptide representing the dominant CD8+ T cell epitope for E7 protein as the antigenic component of the vaccine. The comparable therapeutic efficacies of the peptide and whole E7 protein-based vaccines may be due to both the vaccine formulation and the tumour model used in these studies. Both the vaccine formulations included equal amounts of antigen and therefore, more molar amounts of the peptide. The excess peptide amount and its faster kinetics of presentation by MHC class I molecules may allow robust generation of a CD8+ T cell response that curbs tumour growth (Sharma et al., 2010, Yan et al., 2009).

As much as the TC-1 tumour model uses transplantable tumours and has fast growth kinetics, antigen escape variants due to immunological pressure may not occur during the experiment. However, this may be different in a spontaneous tumour setting wherein

immunological pressure may possibly give rise to antigen-loss variants. Therefore, use of either whole E7 protein-based vaccine or those with both and E7 proteins may have better efficacy in a spontaneous setting due to the availability of not only multiple CD8+ but also CD4+ T cell epitopes (44). Consistent with this notion, we demonstrated potent primary and memory CD4+ and CD8+ T cell responses in mice vaccinated with E7 protein and SA-4-1BBL. Although CD4+ T cells seem unnecessary for primary immune responses, it is critical for the generation and maintenance of long-term memory and recall responses (Gunn et al., 2001; Kumaraguru et al., 2005).

The development of non-toxic adjuvants that not only activate the effector arm of the immune system against tumours but also overcome various immune evasion mechanisms employed by the vaccines against cancer is important. Importantly, we previously reported the pleiotropic effects of SA-4-1BBL on the cells of innate, adaptive, and regulatory immunity (Alba et al., 2009), and treatment with SA-4-1BBL at therapeutic doses did not result in detectable signs of acute toxicity that were recently reported for agonistic antibodies to 4-1BB and were assessed by lymphadenopathy, lymphocyte proliferation, systemic cytokine response, and gross pathology (Fausch et al, 2002, 2003). Taken together, our findings support the notion that SA-4-1BBL is a potentially effective and safe adjuvant that can serve as a component of therapeutic vaccines. Testing its therapeutic efficacy in clinical trials will be important; if effective, this molecule may serve as a safe and effective platform for the development of therapeutic vaccines against cancer and chronic infections (Sharma et al., 2010).

In order to evaluate the therapeutic potential of the responses of L1-specific CD4+ and CD8+ T lymphocytes in cervical cancer patients, L1 VLP-loaded DCs were used to stimulate peripheral blood lymphocytes from the cervical cancer patients, and such responses were compared to those elicited by the E7 oncoprotein. We showed by reverse transcriptase (RT)-PCR that all the flash-frozen cervical biopsy samples collected from HPV 16-positive cervical cancer patients harboured L1 in addition to E6 and E7 RNA. The E7 RNA copy number (mean, 176.2) was significantly higher than those of E6 RNA (mean, 47.3) and L1 (mean, 58.3) in HPV 16-positive cervical cancers (P < 0.0001 and P < 0.001, respectively). However, no significant differences between the levels of expression of E6 and L1 were noted. Kinetic studies of E6, E7, and L1 RNA and protein expression levels in primary tumours showed sharp reductions in L1 expression versus E6 and E7 expression after multiple *in vitro* passages. Autologous DCs pulsed with HPV 16 VLPs or recombinant full-length E7 elicited strong type 1 L1- and E7-specific responses in CD4+ and CD8+ T cells from cervical cancer patients. Importantly, L1 VLP-specific CD8+ T lymphocytes expressed strong cytolytic activity against autologous tumour cells and were as effective as E7-specific cytotoxic T lymphocytes in lysing autologous tumour cells naturally infected by HPV 16. Taken together, these data demonstrate consistent expression of L1 in primary cervical tumours and the possibility of inducing effective L1/tumour-specific CD4+ and CD8+ T-lymphocyte responses in patients harbouring HPV-infected cervical cancer. These results may have important implications for the treatment of patients harbouring established HPV-infected lesions with L1 VLPs or combined E7/L1 DC-based vaccinations (Shedlock & Shen, 2003; Bellone et al, 2009).

One novel cancer therapy involves using the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid (DMXAA). In the current study, we aimed to test the

combination of DMXAA treatment and HPV-16 E7 DNA vaccination to enhance the anti-tumour effects and E7-specific CD8+ T cell immune responses in treated mice. We determined that DMXAA treatment generates significant therapeutic effects against TC-1 tumours but does not enhance antigen-specific immune responses in tumour-bearing mice. We then found that the combination of DMXAA treatment and E7 DNA vaccination generates potent anti-tumour effects and E7-specific CD8+ T cell immune responses in the splenocytes of the tumour-bearing mice. Furthermore, the DMXAA-mediated enhancement or suppression of E7-specific CD8+ T cell immune responses generated by CRT/E7 DNA vaccination depended on the time of DMXAA administration and was also applicable to other antigen-specific vaccines. DMXAA is a synthetic flavonoid that induces the production of local cytokines, including TNF-α. DMXAA has been shown to induce anti-tumour effects in animal models, especially in combination with established anti-cancer agents. It has also demonstrated a good safety profile and looks promising after phase I clinical trials (Peng et al., 2001 Shedlock & Shen, 2003; Silk & Finn, 2007).

Mice were immunised with 2 µg of various DNA vaccines and received boosters with the same regimen as indicated in the figure legends. For vaccinia encoding SigE7LAMP1 vaccination, 1×10^7 plaque-forming units (pfu) viruses were intraperitoneally injected in a volume of 100 µL. Splenocytes were harvested 1 week after the last vaccination. DMXAA treatment generates significant therapeutic effects against TC-1 tumours but does not enhance antigen-specific immune responses in tumour-bearing mice. To determine the anti-tumour effects of DMXAA treatment, we first challenged groups of C57BL/6 mice (5 per group) with TC-1 tumour cells and treated them with a single dose of DMXAA that was administered on day 13 after tumour challenge via intraperitoneal injection, and monitored the tumour size over time. Tumour-bearing mice treated with DMXAA showed significantly lower tumour volumes over time compared to tumour-bearing mice not treated with DMXAA. We also characterised the E7-specific CD8+ T cell immune responses in these mice. One week after DMXAA treatment, the splenocytes from tumour-bearing mice were harvested and characterised for E7-specific CD8+ T cells by using intracellular IFN-γ staining followed by flow cytometry analysis.

Combination of DMXAA treatment with E7 DNA vaccination generates potent anti-tumour effects and E7-specific CD8+ T cell immune responses in the splenocytes of tumour-bearing mice. In order to determine the therapeutic anti-tumour effects and E7-specific CD8+ T cell immune responses in TC-1 tumour-bearing mice treated with DMXAA and CRT/E7 DNA vaccination, we first challenged groups of C57BL/6 mice (5 per group) with TC-1 tumour cells and then treated them with CRT/E7 DNA vaccine with or without DMXAA. Seven days after the last vaccination, we harvested the splenocytes from the vaccinated mice and characterised them for the presence of E7-specific CD8+ T cells by using intracellular cytokine staining for IFN-γ followed by flow cytometry analysis. The tumour-bearing mice that were treated with CRT/E7 DNA vaccine in combination with DMXAA experienced better therapeutic anti-tumour effects than the mice treated with any other regimens. Furthermore, mice treated with the DNA vaccine in combination with DMXAA also generated the highest number of E7-specific CD8+ T cells compared to the mice treated with any of the other regimens.

In summary, the authors tried to show that the combination of DMXAA treatment and HPV-16 E7 DNA vaccination can enhance or suppress the anti-tumour effects and E7-

specific CD8+ T cell immune responses in treated mice depending on the time of DMXAA administration. These results may have potential implications for future clinical translation. Therefore, further work needs to be done to complete this study (Bellone et al, 2009; Peng et al., 2001; Shedlock & Shen, 2003; Silk & Finn, 2007).

5. Vaccines, prophylactics, and therapeutics

In the study by Petrone et al. (Petrone et al, 2011) that aimed to obtain a highly immunogenic E7 preparation, they did not focus on obtaining identical particles since particles of different sizes can be taken up by different types of antigen-presenting cells such as the DCs, macrophages, and polymorphonuclear leukocytes, sustaining a more potent immune response (Uno et al., 2006; Rabu et al., 2001). However, we standardised the different preparations by using semi-quantitative counting of the particles on EM micrographs (not shown). The immunogenicity of *Escherichia coli*-derived E7 fused through the N-terminus to either HPV16 E6 or GST was also investigated in mice. An antigen-specific immune response of Th2 polarity was obtained when the fusion proteins were administered to the mice without an adjuvant (data not shown). However, we were unable to observe the typical micro- and nanoparticles in these E7-fusion proteins prepared from *E. Coli*. Recently, the cytosolic accumulation of E7 oligomers shown in HPV 16 cervical cancer cell lines and in clinical samples by indirect methods supports a new hypothesis regarding the presence of E7 isoforms and their role in different cell compartments (Dantur et al., 2009; Knapp, et al. 2009). The presence of E7 in different aggregation forms and cell compartments could affect E7 processing and presentation by MHC I and II molecules, assessing both the strength as well as the quality of the host's anti-HPV immunity. More studies on recombinant *E. coli*-derived E7 assembled in different forms would contribute to explaining the stimulation of the different branches of the immune system in the HPV16 mouse tumour model (Knapp et al., 2009).

Significant differences exist between the HPV 16 mouse tumour model and human HPV 16-dependent diseases. However, studies on the immunoglobulin G (IgG) subclasses and their FcgR receptors between mice and humans are comparable. HPV 16 E7 immunogenicity studies in mouse will provide insights into the understanding of the protective immunity against human HPV 16 infections as well.

Commercial preventive HPV vaccines have high production costs, which has prevented the implementation of widespread vaccination programs. Recently combined preventive and therapeutic HPV vaccines produced in *E. coli* have been described (Knapp, et al. 2009; Schädlich et al., 2009; Bian et al., 2008), and the data presented here suggest a possible use of *E. coli*-derived E7 in the particle form in subunit vaccines. The *E. coli*-expressed proteins represent a well-studied and cost-effective means for vaccine production. These methods require reduced time, cost, and labour, and can be easily scaled up to industrial-scale production. Generation of new low-cost HPV vaccines could represent the only possibility for women living in developing countries to gain access to HPV vaccination programs in order to prevent or treat pre-cancerous lesions and cancer (Rubio I et al., 2009). In this paper, the author describes, for the first time, the use of recombinant HPV 16 E7 assembled *in vitro* into particulate form to induce protective immunity against an HPV 16-

related tumour in an HPV-16 mouse tumour model. Data show that E7 particles used without adjuvant are excellent stimulators of the immune system. In C57BL/6 mice, the E7 preparation induces anti-tumour immunity sustained by both humoral and cell-mediated immune responses. This E7 protein (derived from *E. coli*) that does not require an adjuvant could represent, along with the recently proposed *E. coli*-derived HPV antigens ((Knapp, et al. 2009; Yan et al., 2009), a low-cost constituent for the development of a new generation of HPV 16 vaccines that combines prophylactic and therapeutic activities.

Numerous methods have been developed to introduce foreign genes into mammalian cells, including chemical-based procedures, electroporation, gene gun, and mammalian viral vector-based systems. These methods have the following advantages: ease of use, gene capacity, cell specificity, cytotoxicity, efficiency, safety, and reproducibility. High cost of these methods can be the only limitation. To overcome this problem, recombinant baculoviruses have been widely developed using baculovirus/mammalian expression systems. These recombinant baculoviruses will be used extensively for gene therapy and vaccines, nevertheless, the authors did not observe substantial spot numbers in any of the mice treated with wild-type baculovirus. However, when we performed immunostaining for the splenocytes harvested from the mice intramuscularly injected with wild-type baculovirus, we observed non-specific IFN-γ production in 2 of the 6 mice. Several recent studies indicated that baculoviruses may induce innate immune responses (Strauss et al, 2007; Li et al., 2009; Abe et al, 2003, 2005; Huang et al.,2009). Although baculoviruses do not replicate in mammalian cells and thus can serve as safe DNA vaccine vectors, additional information is required on the pre-existing anti-vector immunity from the use of live baculoviruses for vaccine development.

In another study (Liao et al., 2008), a recombinant pseudotype baculovirus (BV-G-E) was generated by inserting the JEV E gene fragment into the pFastBac-VSV/G vector. The authors demonstrated that BV-G-E could elicit high protective immunity in mice. The amounts of BV-G-E injected into mice were 1×10^8, 1×10^9, and 1×10^{10} pfu. In yet another study, without the use of recombinant baculovirus, mice were inoculated intramuscularly with a single dose of 5×10^6 pfu of recombinant attenuated vesicular stomatitis virus (rVSV) expressing HPV16E7 protein. The authors suggested that rVSV-based vaccination should be explored as a therapeutic strategy for cervical carcinoma. Other recherche group (Lee et al., 2010) developed a novel DNA vaccine for HPV; a recombinant baculovirus-bearing human endogenous retrovirus (HERV) envelope protein, which cannot replicate in mammals, was used as a nano-carrier for the HPV-16L1 DNA vaccine (AcHERV-HP16L1). For the *in vivo* test, mice were injected intramuscularly with 107 particles of the constructs, with 2 boosters given at 2-week intervals. Compared to Gardasil® (25 µl/dose), the AcHERV-HP16L1-immunised mice showed similar high levels of humoral immunity in IgG/IgA and in HPV pseudovirion neutralisation. Combined immunisation (AcHERV-HP16L1 primer and Gardasil® booster) induced slightly higher neutralising activity. Compared to the group treated with Gardasil®, the mice immunised with AcHERV-HP16L1 showed 450- and 490-fold increases in IFN-γ at 5 and 20 weeks after the first priming, respectively. The safety levels were comparable. The combined immunisation conferred lower T cell immunity than AcHERV-HP16L1 treatment. The

advantages of our novel AcHERVHP16L1 vaccine over Gardasil® include higher cellular immunogenicity and considerably lower production costs.

The recombinant baculovirus used for HPV16L1 gene delivery does not replicate in the host. Its advantages include safety, ease of processing, and most importantly, the ability to induce both humoral and cellular immunity. Therefore, we suggest that the AcHERV-HPV DNA vaccine be developed as an efficient prophylactic and therapeutic vaccine. On the basis of its advantages, we anticipate that the developed AcHERV- HP16L1 will contribute to global HPV prevention (Chen et al., 2000; Velders et al, 2001; Mahdavi & Monk, 2005).

Acceptance of the vaccine among adolescents and parents, protection against sexually transmitted infections (STIs), and adequate health support and recommendation are the keys to the success of HPV vaccines. Recent studies have shown that although some parents find the vaccine acceptable, others believed that their children are not at real risk of contracting an STI or expressed concern that vaccination may encourage the practice of risky sexual behaviours. In a recent survey, mothers of children aged between 8 and 14 years expressed a willingness to vaccinate their daughters (67%) or children (66%). Those who refused the vaccine cited the risk of unknown side effects, lack of sexual activity at the time, and the lack of a direct benefit in male children. In another parent survey, vaccination rates of 10–15-year-old children increased from 55% to 75% after the parents read a newsletter. Therefore, it is essential that accurate information about the disease and the vaccine be distributed by health professionals to ensure broad participation in the vaccination programs (Roberts et al., 2010; Tsakiroglou et al., 2011; Tan et al., 2010).

6. Future directions

In the development of therapeutic HPV vaccines, we have focused on identifying and targeting the most relevant antigens associated with cervical cancer, the E6 and E7 HPV oncoproteins, which represent tumor-specific antigens and potentially ideal targets for therapeutic HPV vaccines. It is important to consider using strategies such as prime-boost regimens and/or combinations strategies using molecules that are capable of blocking the negative regulators on T cells to further enhance the T cell immune responses. Furthermore, increasing understanding of the molecular mechanisms that hinder immune attack in the tumor microenvironment will lead to the identification of novel molecular targets that can be blocked in order to enhance the therapeutic effect of HPV vaccines. It is conceivable that effective therapy against HPV-associated malignancies will probably require a combination of therapeutic HPV vaccines with the employment of innovative agents that are capable of eliminating the suppressive factors present in the tumor microenvironment. With continued endeavor in the development of HPV therapeutic vaccines, it can foresee that HPV therapeutic vaccines will become an important approach that can be combined with existing forms of therapy such as chemotherapy and radiation therapy to generate better control of HPV-associated malignancies (Hung et al., 2008)

Neutralizing IL10 at the time of PV VLPs immunization increases cytotoxic T cell responses. PV VLPs incorporating PV early protein E2, 6 and 7, together with immune stimulator that promote strong type 1 responses, and at the same time blocking the effect of IL10 may have

therapeutic effect against HPV infection related diseases and are worth further basic and clinical investigation (Chen et al., 2011).

After a long period of scepticism and disbelief, tumour viruses are today recognized as a significant cancer risk factor for humans. Much has been learned about the viral transforming mechanisms and prophylactic vaccines have been developed against tumour viruses' HPV. Yet, many important issues of tumour virology remain unresolved and exciting new ones are emerging from recent discoveries. They define future research directions for the field and include (Hoppe-Seyler et al., 2011):

- Novel strategies for tumour virus hunting.
- Tumour viruses as experimental tools to study human carcinogenesis.
- The interplay between viruses and the world of small non coding RNAs.
- Epigenetic interactions between tumour viruses and the host cell.
- The role of virus/virus interactions for viral carcinogenesis
- Novel strategies for prevention and therapy of virus-associated cancers.

Further study into the tumour microenvironment and molecular mechanisms impeding immune attack against HPV will lead to novel targets for therapeutic intervention in the future. Discovery of such targets, development of new adjuvants, and improved understanding of tumour biology will allow HPV vaccines to be used in combinational therapies in a synergistic manner in the future.

7. Conclusion

Doubts do remain unresolved, and therefore, the existence of cross-protection evidence in the bivalent vaccine against other subtypes of 16/18 capsular structure very similar protein suggested that the cause of this cross-immunity would be an extra-immune response induced by adjuvant use in the vaccine is unknown. Additional benefit that would represent this phenomenon in clinical practice, but how they behave subtypes prevalent highly uncertain risk to cause protection from the vaccine subtypes prevalent now? Will it be replaced? Because it has been reported that HPV 16/18-positive women are at 5–7-times greater risk of acquiring a subsequent infection with HPV 5/7 than the uninfected women, can infection be prevented using subtype vaccination?

We continue with the questions such as whether to vaccinate at-risk populations and what type of vaccine to use. We know that the vast majority of HPV infections are diagnosed in people at medium risk without appreciable risky behaviours. Protection of these people would be very effective because it would disrupt the chain of transmission. One option under discussion would be to vaccinate the gay population, which currently has relatively high rates of anal cancer linked to HPV and could greatly benefit from the preventive effects of the vaccine. Nonetheless, assuming that the vaccine does, in fact, prevent HPV-related anogenital and head and neck cancers in males and prevents male-to-female and male-to-male viral transmission, compelling arguments exist for a gender-neutral approach to vaccination. These arguments include the following: (1) female-only vaccination will not protect men who have sex with men from contracting HPV and HPV-related diseases and (2) the fastest way to achieve the greatest protection for females from cervical cancer and its precursors is to vaccinate both females and males.

Finally, the economic evaluation of any HPV vaccination strategy requires the measurement of clinical benefits and the economic benefits associated with an effective intervention. As newer vaccines targeting morbidity rather than mortality are being launched in the market, quantification of disease burden and modelling of the cost-effectiveness of intervention options are becoming more important when determining the best way to allocate scarce health care funds.

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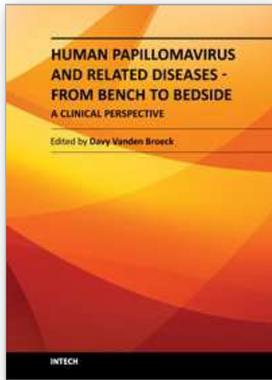
Human Papillomavirus and Related Diseases - From Bench to Bedside - A Clinical Perspective

Edited by Dr. Davy Vanden Broeck

Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on the clinical aspects of HPV and related disease, highlighting the latest developments in this field.

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