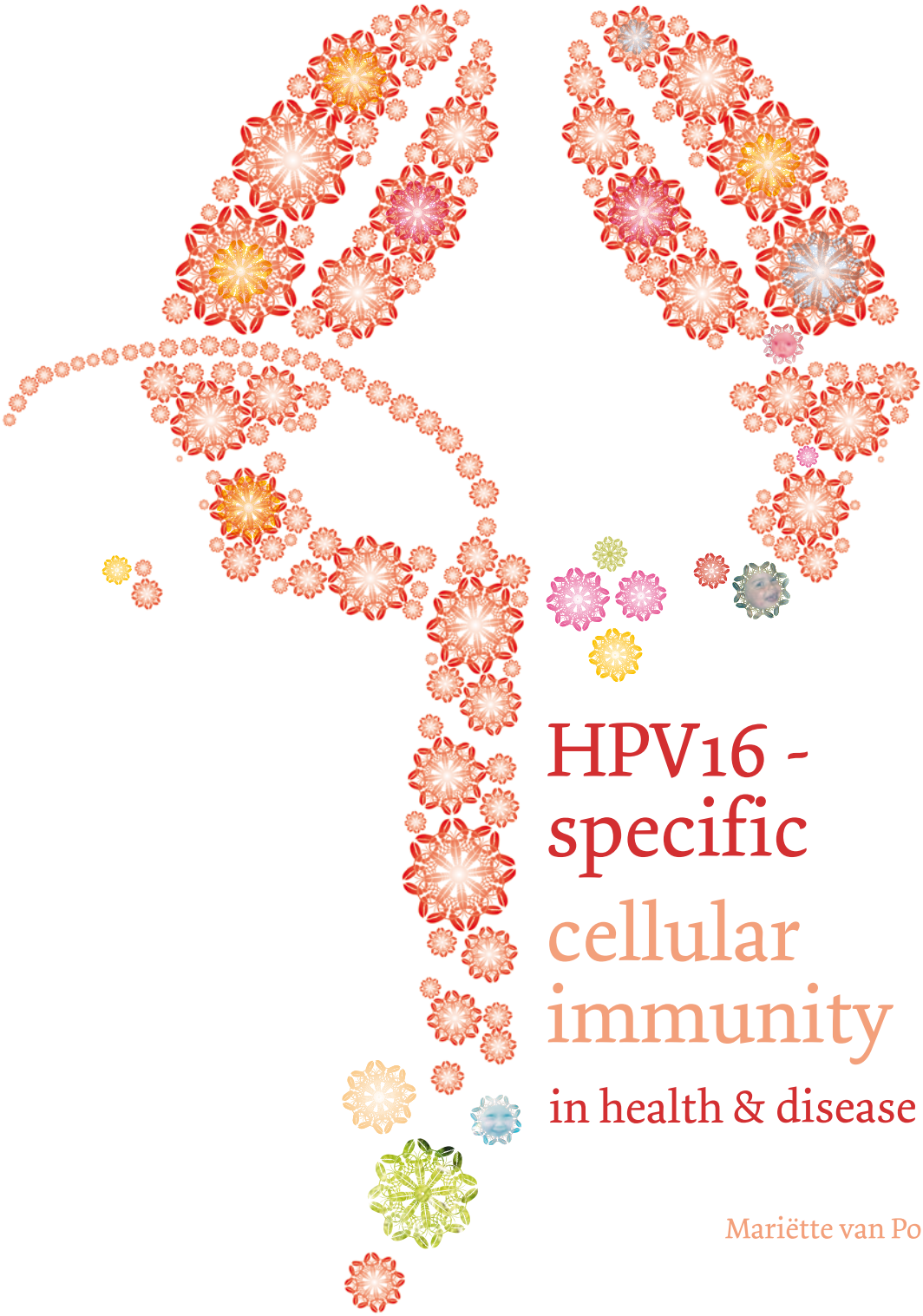
HPV 16-specific cellular immunity in health and disease

Poelgeest, M.I.E. van



HPV16 - specific cellular immunity

in health & disease

Mariëtte van Poelgeest

HPV16-specific cellular immunity in health and disease

Cover design: Eveline Veldt, Den Haag
Printed by Pasmans Offsetdrukkerij BV, Den Haag

ISBN 978-90-9021977-6

Financial support for the publication of this thesis was provided by:
GlaxoSmithKline BV, Sanofi Pasteur MSD, Ethicon Women's Health & Urology,
Will-Pharma, Sanquin and BD Biosciences.

The studies presented in this thesis were supported by the Netherlands Organization for Health Research and Development (ZonMW); grant number 920-03-188.

HPV16-specific cellular immunity in health and disease

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 10 oktober 2007
klokke 13.45 uur

door

Mariëtte Inie Elizabeth van Poelgeest

geboren te 's-Gravenhage
in 1973

Promotiecommissie

Promotores:	Prof. Dr. G.G. Kenter
	Prof. Dr. C.J.M. Melief
Co-promotor:	Dr. S.H. van der Burg
Referent:	Prof. Dr. I.H. Frazer
	(University of Queensland, Australië)
Overige leden:	Prof. Dr. G.J. Fleuren
	Prof. Dr. J.B. Trimbos
	Dr. H.W. Nijman
	(University Medical Center Groningen, Groningen)
	Prof. Dr. P.J.F. Snijders
	(VU University Medical Center, Amsterdam)

Voor papa en mama

opzij, opzij, opzij
want wij zijn haast te laat
we hebben maar een paar minuten tijd
we moeten rennen, springen, vliegen, duiken, vallen, opstaan

en weer doorgaan

Uit: Opzij (Herman van Veen, 1977)

Contents

CHAPTER 1

General introduction

General introduction

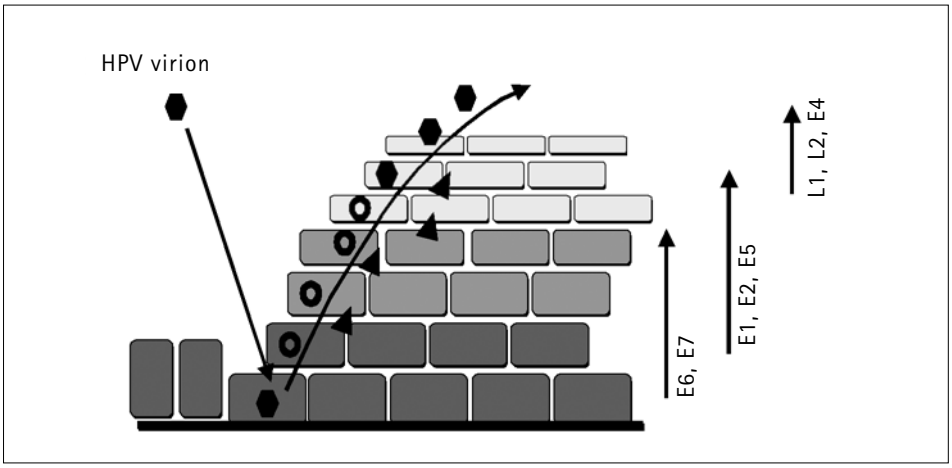
Anogenital intraepithelial neoplasia (AGIN), which includes cervical, vulvar, vaginal and anal intraepithelial neoplasia, is a collective term for a group of precancerous diseases of the female lower genital tract. Successful treatment of these neoplastic diseases can prevent the development of invasive cancer. Recently, it has become clear that human papillomavirus (HPV) is the infectious agent that is responsible for the development of these (pre)malignant disorders.

The first observation that cervical cancer was related to sexual activity was made at the end of the nineteenth century, when the surgeon Rigoni-Stern reported that the appearance of cervical cancer was low in nuns, and high in prostitutes[1]. It was not until 1976 that Zur Hausen suggested that a virus might be involved in the development of cervical cancer, when he made the important observation that the epidemiologic pattern of cervical cancer was the same as for human papillomavirus-induced condylomata acuminata[2]. In the little eighties HPV-DNA was extracted from cervical cancer specimens, and since then the research on HPV as a causal agent in the development of genital cancers and their precursor lesions increased exponentially. Recent data have shown that in 99,7% of cervical cancers HPV is detectable[3]. Cervical cancer is the first cancer that is acknowledged by the World Health Organisation to be virally induced in essentially al cases[4,5].

Human papillomaviruses are small, double-stranded DNA viruses of approximately 8000 base pairs. They are strictly epitheliotropic, which means that they only infect epithelial or mucosal surfaces. At the present, over 100 different HPV types have been described, of which about 40 can infect the anogenital tract[6]. Human papillomaviruses are further divided in the low-risk or non-oncogenic types, which cause benign epithelial proliferations such as genital warts and condylomata acuminata, and the high-risk or oncogenic types, which can cause malignant transformation of infected cells. Human papillomavirus type 16 (HPV16) is found in 60% of cases of cervical cancer[3,7]. The HPV16 genome can be divided in the early (E) genes and the late (L) genes. The early genes encode proteins with regulatory functions engaged in genome persistence and DNA replication. The late genes encode structural proteins that form the capsid of HPV particles. HPVs probably infect the basal cells of the epithelia through breaks or tears in the epithelial layer. After infection, HPV expresses the viral early genes E1, E2, E5, E6 and E7 in the basal layers. Late viral proteins L1, L2 and E4 are made, viral DNA replication takes place, and virus particles are released only

at the superficial terminally differentiated layers (Figure 1). New virus particles may re-infect the host or be transmitted to new hosts by sexual transmission.

Figure 1. The HPV infectious cycle.



HPV infection begins with binding of virions to the basal cells of the epithelium. In this layer the viral genome is amplified to several copies. In the suprabasal layers of the epithelium HPV early (E) proteins are expressed and the viral replication takes place. Only in the most superficial layers of the epithelium, E4 and the late (L) genes L1 and L2 are expressed, HPV DNA is encapsidated, and the virions are released at the epithelial surface.

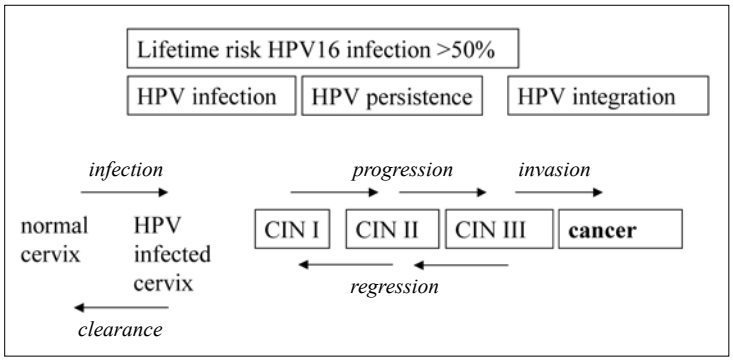
Anogenital HPV infection is believed to be the most common sexual transmitted viral infection, with an estimated 36-month cumulative incidence of more than 40% in young sexually active college women in the United States[8], and an estimated lifetime risk for women of at least 75% for one or more genital HPV infections[9-11]. Fortunately, the vast majority of infections are transient and only a minor fraction of infected subjects develop persistent infections (often defined as infections that are detected more than once in an interval of 6 months or longer) with an oncogenic HPV type, especially HPV16 and 18, that may progress to premalignant lesions or cancer[8,12-14]. The immune system is likely to play an important role in this, because the incidence of HPV infections, HPV-associated warts, CIN lesions and cervical cancer is increased in immunocompromised subjects[15].

HPV-induced anogenital diseases

Cervical intraepithelial neoplasia (CIN) and cervical cancer

Cervical cancer is the second most common cancer in women worldwide, after breast cancer, accounting for over 200.000 deaths each year[16]. The interval between the acquisition of HPV infection and malignant progression usually takes at least 10 years or longer[17,18]. Cervical cancer is therefore very uncommon in women under 25; the incidence rises progressively for women over 25 and is highest for women over 40. In the Netherlands approximately 800 new cases of cervical cancer are reported annually, with an age-standardized incidence rate of 9,5 new cases per 100.000 women. The precancerous stage of cervical cancer, known as cervical intraepithelial neoplasia (CIN), can be detected by cytological screening years before cervical cancer appears. CIN is histopathologically classified as mild (CIN 1), moderate (CIN 2), or severe (CIN 3), depending on the extent to which the epithelial layer is involved in the neoplastic changes. Although patients with CIN are at risk of developing cervical cancer, it is obvious that not all CIN lesions will progress to malignant disease. Studies examining rates of progression for CIN have found that the risk is related to the severity of dysplasia[19,20]. Approximately 57% of CIN 1 lesions may spontaneously regress, while 32% will persist as low-grade and 11% may progress to CIN 2 or 3. The corresponding figures for CIN 2 are 43%, 35% and 22%, respectively. Spontaneous regression, persistence, and progression of CIN 3 to invasive cancer occur in approximately 32%, 56% and 12%, respectively[21]. A model of cervical carcinogenesis in relation to infection with HPV is depicted in figure 2.

Figure 2. Model of cervical carcinogenesis.



The major steps in cervical carcinogenesis are human papillomavirus (HPV) infection (balanced by viral clearance), progression to cervical intraepithelial neoplasia (CIN) (partly offset by regression of low-grade lesions), and invasion. The persistence of oncogenic or high-risk HPV types is necessary for progression and invasion.

Vulvar intraepithelial neoplasia (VIN) and vulvar cancer

Vulvar intraepithelial neoplasia (VIN) is an uncommon chronic skin disorder characterized by histological abnormalities confined to the vulvar epithelium. The importance of VIN lies in its association with vulvar squamous cell carcinoma and impaired quality of life secondary to unpleasant symptoms and deforming treatments. The long-term risk of invasive cancer in women who have previously been treated for VIN 3 is 2,5-7%[22-24], whereas the risk for untreated patients has been estimated up to 80%[23]. High-grade VIN is in over 90% associated with HPV, HPV16 being the type most commonly involved[25-27]. In young women, vulvar cancer is associated with HPV in 60-90% of cases, whereas only the minority of cases in older women (less than 10%) is positive for HPV[28].

Other HPV-associated anogenital disease

The available epidemiological studies indicate that cancers of the vagina and of the anus resemble cancer of the cervix with respect to the role of HPV. In both, HPV-DNA is detected in the great majority of tumors and their precursor lesions. Between 64 and 91% of vaginal cancers and 82 and 100% of cases of high-grade vaginal intraepithelial neoplasia (VAIN 3) lesions are HPV-DNA positive[29-31]. In anal cancers, HPV-DNA is detected in 88-94%[32].

Standard treatment for anogenital intraepithelial neoplasia and cervical cancer

Cervical intraepithelial neoplasia and cervical cancer

The management of pre-invasive disease of the cervix is based on local control and prevention of progression to invasive cervical cancer. According to the Dutch guidelines women with histologically confirmed CIN 2 or CIN 3 should be treated to prevent the development of cervical cancer[33]. Techniques that can be used to remove the transformation zone, which is the area of the cervix where most lesions are located, include surgical excision by large loop excision of the transformation zone (LLETZ) or cone biopsy, and ablation by laser evaporation or cryocoagulation (reviewed in[34]). In general a LLETZ is preferred, as it is a relatively simple and safe procedure that can be performed in an outpatient setting and it provides the availability of tissue for histological examination. Success rates for the different treatment modalities are comparable, exceeding 85-95%[35-38]. Eradication of HPV from the genital mucosa,

associated with over 90% of CIN lesions, is not possible with the currently available techniques. Therefore, cytological follow up is advised 6, 12 and 24 months after primary treatment to detect any residual or recurrent disease[33].

The optimal treatment of cervical cancer depends on the stage at the time of diagnosis. Stage is determined clinically, on the basis mainly of the size of the tumor in the cervix or its extension into the pelvis[39]. Stage I disease is limited to the cervix; stage II disease extends beyond the cervix to the upper two thirds of the vagina or the parametrial tissue but not to the pelvic side wall; stage III tumors have spread to the pelvic side wall, the pelvic nodes, or the lowest third of the vagina; and stage IV tumors have invaded the mucosa of the bladder or rectum or have spread to distant sites of the body. Stage I tumors can be subdivided in stage IA that represents microscopic disease (stage IA1: invasion ≤ 3 mm, stage IA2: invasion 3-5 mm) and stage IB that represents clinically visible lesions confined to the cervix of less (stage IB1) or more (stage IB2) than 4 cm in size. Briefly, stage IA1 tumors can be treated by conization or by a simple hysterectomy. For stage IA2, IB or IIA tumors a radical hysterectomy with pelvic lymph node dissection or chemoradiation are the treatments of choice. Patients with stage IIB-IVA are usually treated by chemoradiation or radiation in combination with hyperthermia. The treatment for stage IVB cervical cancer and for recurrent disease has to be individualized and may be radiotherapy, extensive surgery or chemoradiation, or a combination of these therapies.

Clinical stage is a reliable prognostic indicator for patients with cervical cancer[40,41]. The 5-year survival approaches 100% for patients with stage IA tumors and averages 70-85% for those with stage IB1 and smaller IIA lesions. For stage III and IV, these figures are 30-50% and 5-15%, respectively. After optimal primary treatment of early stage disease local recurrences are found in 15% of patients[42]. The most important prognostic factors in early stage cervical cancer are the presence of lymph node metastasis, tumor size, vaso-invasion, and infiltration depth[43-45].

Vulvar intraepithelial neoplasia

The treatment for VIN 3 has classically been surgical local excision or, in case of multifocal VIN, vulvectomy. Because of the increasing incidence of the disease in younger women the past two decades[24,46] and the major impact of surgical treatments, there has been a gradual trend towards more conservative treatment modalities in patients with VIN 3. Laser treatment as well as different types of medical therapies can be used, especially in younger patients to limit surgical mutilation[47].

New treatment modalities

New surgical techniques

New surgical modalities in the treatment of early-stage cervical cancer include trachelectomy and nerve-sparing surgery. Radical vaginal trachelectomy (amputation of the cervix and surrounding parametrial tissue in combination with pelvic lymphadenectomy) is a fertility-sparing option for selected women with early-stage cervical cancer[48-50]. Recent studies have shown a low incidence of recurrences (0-7,3%) and acceptable cumulative conception rates. The operation is, however, associated with a significant incidence of second trimester miscarriage and premature labour[50].

Radical hysterectomy is associated with great morbidity in terms of serious bowel, bladder, and sexual dysfunction[51-53]. The pelvic autonomic nerves are responsible for the neurogenic control of rectal and bladder function. Nerve-sparing radical hysterectomy, which includes the identification and subsequent preservation of these nerves, is currently under investigation as a new treatment option for early-stage cervical cancer[54,55]. The technique seems feasible and safe and might provide patients with an improved quality of life[56]. Future large clinical trials will have to decide whether it can be implemented as a standard treatment for cervical cancer patients.

Chemotherapy

Chemotherapy for advanced or recurrent cervical cancer has been and continues to be considered palliative. Many agents have been investigated as single or combined regimens. Cisplatin is considered the most active single agent in recurrent disease. As a single agent, cisplatin has been compared with the combination of cisplatin and paclitaxel in a randomized phase 3 study. The combined regimen was superior to single-agent cisplatin in terms of response rate and survival, at a cost of reversible bone-marrow toxic effects[57]. The combination of cisplatin and topotecan was also shown to increase survival and response rates in patients with locally advanced cervical cancer in another phase 3 study[58]. Concomitant chemoradiation appears to improve overall survival and progression-free survival for patients with locally advanced cervical cancer (Cochrane Database Syst Rev 2005). Overall, response rates for chemotherapeutic regimens average 10-40%, with complete responses seen only rarely and for short duration. Therefore, there is need for new therapeutic approaches in the battle against cervical cancer.

Photodynamic therapy

5-Aminolevulinic acid (ALA) photodynamic therapy (PDT) is a new technique that is based on the local or systemic application of the photosensitizer ALA, which is preferentially absorbed in neoplastic tissue. Activation of ALA by light leads to the release of highly reactive oxidants capable of producing local tissue destruction. PDT is a minimal invasive procedure that can be performed in an outpatient setting. Small trials using PDT in patients with CIN showed little efficacy[59]. Clinical responses to PDT in patients with VIN have been described[60,61]. In comparison to laser vaporization and surgery, PDT was shown to have little side effects but higher recurrence rates[62]. This may be explained by the fact that the therapy does not induce HPV-specific immunity aimed at long-term protection against HPV-infected cells but is just aimed at local destruction of tissue.

Immunomodulators

Imiquimod is an immunomodulating agent that directly activates the innate immune system, resulting in cytokine release and costimulatory molecule expression, followed by T-cell activation. It has shown efficacy and safety in clinical trials for the treatment of genital warts[63-65]. It is hypothesized that this topical treatment may be effective in stimulating HPV-specific T-cell immunity and the subsequent regression of HPV-related dysplastic lesions of the vulva. Recently, some trials reported on beneficial effects of topical imiquimod application in the treatment of high-grade VIN[66-69]. A large randomized study on the use of Imiquimod in the treatment of patients with multifocal high grade VIN showed that Imiquimod was safe and highly effective in these patients (Van Seters, unpublished results). We have shown that the presence of IFNγ-associated T-cell immunity against HPV16 is significantly associated with a more favourable clinical response upon treatment with imiquimod in a group of patients with VIN 3 (*chapter 6*).

HPV-specific immunotherapy

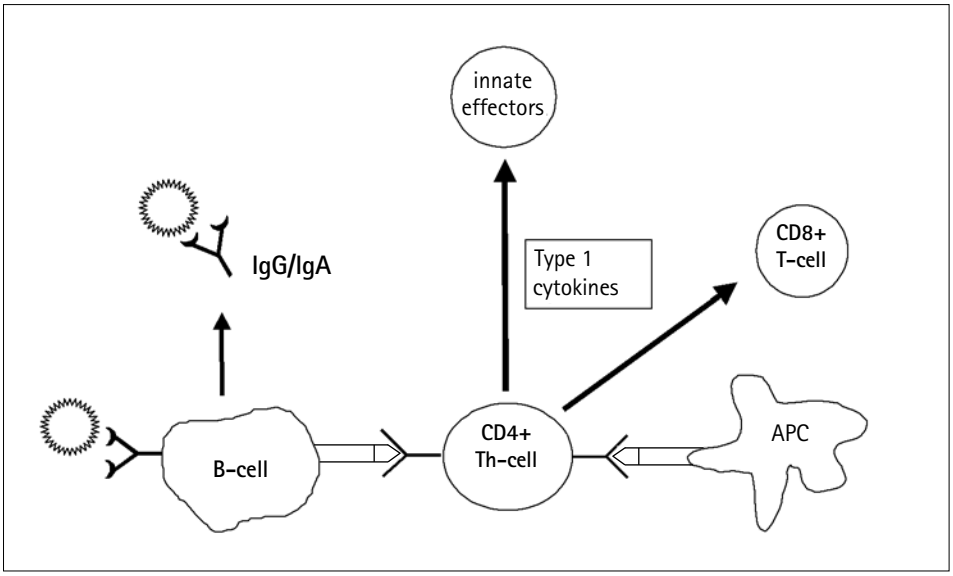
The important role of a viral agent in the pathogenesis of AGIN and cervical cancer offers the potential for the development of vaccination strategies. HPV-specific immunotherapy, aimed at prevention of HPV infection on the one hand and destruction of HPV-infected dysplastic cells on the other, is a promising new treatment regimen. For the design of effective immunotherapeutic strategies against HPV-induced disease, it is important to have detailed knowledge about the natural immune responses against HPV in healthy individuals and patients with HPV-induced disease.

Immune responses in anogenital neoplasia

The immune system can be divided in the non-specific immune system and the antigen-specific immune system. The former functions as a first line of defense and involves the natural barriers (like skin and mucus), bactericidal enzymes, granulocytes and macrophages, and the complement system. The latter consists of the cellular and humoral immune system.

Cell-mediated immunity can be divided in two categories: CD4+ T-cells, and CD8+ T-cells or cytotoxic T-lymphocytes (CTL). CD4+ T-cells can further be divided in T-helper (Th) cells and regulatory T-cells (Treg). CD4+ T-helper cells play a central role in regulating immune responses and are essential in antitumor immunity[70-72] (Figure 3). Based on their pattern of cytokine production, CD4+ Th-cells can be divided into Th1 and Th2 subsets. Th1 cells produce type 1 cytokines like IL-2, IL-12, GM-CSF, TNF-α, and interferon gamma (IFNγ), and are primarily responsible for activating and regulating the development of CTL and innate effector cells. Th2 cells are characterized by the production of type 2 cytokines IL-4, IL-5, IL-10, and TGF-β. Th2-

Figure 3. The central role of CD4+ T-helper cells in protective antiviral immunity.



CD4+ T-helper (Th) cells play a central role in the protection against viral pathogens, by 1) stimulation of the production of antibodies (IgG/IgA) by B-cells, 2) activation and stimulation of innate effector cells, and 3) activation of CD8+ (cytotoxic) T-cells. Presentation of antigen by antigen presenting cells (APC) to Th-cells is necessary for the induction of an effective immune response.

type cytokines are immunoinhibitory for T-cell responses and predominantly induce humoral immune responses, i.e. the formation of antibodies. Recently, it has been shown that regulatory T-cells play a major role in maintaining peripheral tolerance to tumors[73,73]. Based on their cytokine pattern Tregs can be divided in Tr1 cells that secrete IFNγ and IL-10[74] and Tr3 cells that act predominantly via the secretion of TGF-β[75]. CD8+ T-cells play a pivotal role in the clearance of virally infected cells and killing of tumor cells.

The cytokine microenvironment in HPV-induced (pre)malignant lesions

Cytokines are secreted by macrophages, granulocytes, T-cells, normal epithelial cells, and tumor cells amongst others, and they regulate cell-mediated immune responses[76-78]. Some studies have indicated that CIN lesions are associated with a decreased expression of the proinflammatory type 1 cytokines IFNγ and TNF-α[79-81] and an increased production of the regulatory cytokine IL-10[80,82]. The local cytokine environment in HPV-infected lesions may determine the outcome of disease. The decreased expression of type 1 cytokines in tumor cells may contribute to a less effective local antitumor response, because these cytokines are involved in augmenting antigen presentation, maturation, and cytotoxicity of Langerhans cells. Indeed, the expression of IFNγ in cervical cancer has a better prognostic value[83]. In contrast, the presence of IL-10 was shown to be associated by more extensive disease in HPV-induced CIN lesions[84]. Furthermore, cervical carcinoma cell lines continue to express TGF-β while the expression of the proinflammatory cytokines TNF-α and GM-CSF is strongly decreased[85]. The production and secretion of TGF-β by cervical cancer cells was associated with an increased amount of intratumoral stroma and a reduction in the number of infiltrating immune cells[86].

Recently, subsets of CD4+CD25+ regulatory T-cells have been described that suppress Th1 responses through the secretion of IL-10 and TGF-β[87]. A recent study showed that in high-grade CIN lesions the infiltrating CD4+CD25+ T-cells produced IFNγ as well as IL-10 and TGF-β, suggesting the presence of such regulatory T-cells in these lesions[88]. The production of another type 2 cytokine IL-4 by CD3+ T-cells in patients with cervical cancer was associated by increased infiltration of eosinophilic granulocytes, which has been shown to correlate with a worse survival[89,90].

Taken together, these data indicate that an imbalance between type 1 and type 2 cytokines in HPV-infected tissue may determine the efficacy of the local antitumor response. Modification of the local cytokine environment in the treatment of patients with HPV-induced AGIN may have strong effects on clinical outcome.

The infiltration of immune cells in HPV-infected (pre)malignant lesions
Infiltration of lymphocytes is correlated with regression of genital warts in both human and animal models[91,92]. Studies investigating the local immune environment in patients with HPV-induced anogenital disease have shown contradictionary results. Some studies reported on a localized immunodeficiency in cervical and vulvar dysplastic tissue in a qualitative or quantitative way. A depletion of intraepithelial CD4+ T-cells was shown in CIN lesions[93,94] and infiltrating T-cells in cervical lesions displayed an immunosuppressed phenotype[95]. Other studies have shown that the numbers of infiltrating T-cells and Langerhans cells (LC) were significantly increased in cervical and vulvar neoplasia compared to healthy control tissue[88,96-99].

In several types of human invasive cancers the clinical significance of a localized immune response has been inferred from the correlation of tumor-infiltrating CD8+ T-cells with outcomes[100-103]. The clinical significance of the local infiltration of immune cells in patients with HPV-induced disease has been suggested in a number of studies. In a group of vaccinated VIN patients clinical responsiveness to treatment was shown to be dependent on the presence of lesion-associated CD4+, CD8+ and CD1a+ immune cells[104]. In another study, it was shown that an increase of CD8+ T-cell infiltration in the lesions of VIN patients was significantly associated with clinical responsiveness to treatment with photodynamic therapy (PDT)[105]. Decreased proportions of tumor-infiltrating CD4+ T-cells, which have been observed in cervical neoplasia[93,94,98], were correlated with rapid tumor growth and lymph node metastasis in patients with cervical cancer[106]. We recently showed that an increased infiltration of intraepithelial CD8+ T-cells was significanty associated with the absence of lymph node metastases in patients with large early-stage cervical cancer (*chapter 7*). So far, the relationship between systemic antitumor immunity, tumor infiltration of T-cells, and clinical outcome in patients with HPV-induced cervical cancer has not yet been studied.

HPV-specific immunity

Anogenital HPV infections are common sexually transmitted diseases. While most infections are transient, persistence is found in a small proportion of infected persons and this may lead to the development of anogenital neoplasia and cancer. The enhanced prevalence of HPV infections and increased incidence of HPV-induced disease in immunocompromised subjects, such as HIV-infected subjects and organ

transplant patients[15,107] suggests that the immune system plays a role in preventing the appearance and inducing the regression of anogenital lesions. Both the late and early viral proteins form a target for the HPV-specific immune response.

Humoral immunity to the structural protein L1

Mucosal IgG and IgA antibodies to HPV16 are present in a proportion of patients with HPV-induced lesions[108-110] and may prevent re-infection of the epithelium by released viral particles. Unlike many other human viruses, human papillomaviruses do not naturally provoke a strong serological response. Although serum IgG antibodies to HPV16 are found in 56-60% of subjects with incident HPV infections within 8-18 months[111-113], the titres of these antibodies are low[111]. Antibodies can persist for decades[114], but in women without HPV-associated lesions they rapidly disappear[115]. Given the high frequency of serum IgG antibodies in patients with CIN[76], VIN[116,117], and cervical cancer[118], these antibodies do not appear to correlate with the prevention or clearance of HPV-induced genital lesions but may serve as a marker for progressive disease. Indeed, the presence of serum HPV antibodies seems to be correlated with the persistence of HPV and the development of premalignant lesions[119,120]. Serum HPV16-specific IgA was shown to be associated with an early appearance[120] and a shorter duration compared to IgG[121]. Serum IgA seems to be a marker for an ungoing HPV infection whereas IgG is a marker for lifetime cumulative exposure to HPV16[110]. In contrast to the naturally occurring humoral response, vaccination with virus-like particles (VLPs) of papillomaviruses has shown to result in high titres of virus-specific serum IgG antibodies and protection against virus-induced (pre)malignant lesions in animals and in humans[122-125].

CD4+ T-cell immunity against HPV16

Studies addressing T-cell immunity against HPV have focused on HPV16 because of the high prevalence in high-grade CIN lesions and in cervical cancers. So far, many studies addressing T-cell responses against the HPV16 early proteins E2, E6 and E7 have been performed. Proliferative responses against HPV16 E6 were more frequent observed in sexually active women without disease than in women with current CIN[126]. Bontkes et al. showed that Th-cell responses against E2 were frequently seen at time of viral clearance in patients with CIN[127]. T-cell responses against HPV16 E6 and E7 were found to be associated with clearance of infection and regression of CIN[128,129]. We have detected frequent HPV16-specific Th immunity against E2 and E6 in the majority of healthy individuals, and also occasionally E7-specific T-cell responses[130-132], suggesting that in humans CD4+ T-cell immunity against the early antigens of

HPV16 protects against progressive disease. HPV-specific immunity in patients with AGIN may differ not so much in the quantitative aspects of the immune response but more in the quality of the response. We have recently found that although patients with cervical cancer display HPV16 E2 and/or E6-specific proliferation, this reactivity is not associated with the production of the type 1 cytokine IFNγ typical for the HPV16-specific response in healthy subjects (*chapter 2*).

Animal models revealed that L1-specific immunity can protect against infection with the cottontail rabbit papilloma virus[133] or the canine oral papilloma virus[124,134,135]. Previous work in humans has shown that patients diagnosed with HPV16+ CIN displayed proliferative responses against HPV16 L1[136,137] and that there was no difference in such proliferative responses (measured by IL-2 production) between patients who cleared their lesions or in whom lesions persisted[76]. Large HPV16 L1-VLP vaccination trials in humans revealed that vaccine-induced immunity could prevent persistent infections and HPV-induced CIN[123,125,138,139]. Immunological evaluation of these trials was predominantly focused on the role of neutralizing antibodies in preventing viral infection. HPV16 L1 vaccination in healthy subjects was shown to induce not only strong antibody responses, but also L1-specific T-cell responses[140]. These T-cell responses against L1 are not likely to protect against the progression of established HPV-induced disease. The large HPV VLP vaccination studies were not designed to evaluate the therapeutic effects of the vaccines on subjects with evidence of prior HPV infections. Recently, HPV VLP vaccination was shown not to speed viral clearance in women with existing HPV infections[141]. We have shown that unlike E2 and E6, HPV-specific type 1 T-cell responses against L1 were present in the majority of patients with HPV16-induced CIN and cervical carcinoma, indicating that T-helper type 1 immunity against the structural protein is not correlated with health or disease (*chapter 3*).

Taken together, studies on HPV16-specific T-helper cell immunity in humans indicate that HPV-specific CD4+ Th1 responses against the early antigens, but not the late antigen L1, are associated with protection against progression of HPV-induced disease.

CD8+ T-cell immunity against HPV16

CD8+ T-cells or cytotoxic T-cells (CTL) play a vital role in the clearance of virally infected cells[142]. The role of naturally occurring CTL in mediating regression of HPV-related disease has not been proven[143], and limited data are available regarding the CTL responses to HPV16 in humans. Some studies have showed the presence of HPV16-specific CTL in the blood of patients with high-grade CIN, VIN and cervical

cancer. CTL responses to E6 and E7 were more commonly detected in HPV16-positive women without CIN than in HPV16-positive patients with CIN[144,145], suggesting a protective effect of HPV-specific CTL in the development of AGIN. In other studies, patients with persistent infections or progressive disease occasionally displayed HPV16-specific CTL responses[146-148]. The impairment of CD4+ T-cell immunity, which is of pivotal importance for the induction and maintenance of CTL immunity[149], may explain why the detection of HPV-specific CTL in all studies was rare, and of low frequency.

Papillomavirus vaccination

Identification of a viral agent such as HPV as a cause of disease implies that succesfull intervention against the viral agent should prevent or cure the disease it causes. There are two types of immunological intervention strategies: prophylactic and therapeutic vaccination.

Prophylactic HPV vaccination

Prophylactic vaccines aim at the induction of antibodies that are capable of preventing viral infection. Efforts to develop a vaccine to prevent HPV infection have focused on eliciting humoral immune responses to the HPV capsid proteins using synthetic empty capsids, termed "virus-like particles" (VLPs)[150]. VLPs are morphologically indistinguishable from the authentic virion, are non-infectious and lack any oncogenic DNA. In animal papillomavirus models, systemic vaccination with L1 VLPs was shown to be capable of inducing high titres of neutralizing antibodies as well as protecting against viral infection after challenge with the homologous virus[122,124]. Early phase human trials have also indicated that L1 VLP vaccines are highly immunogenic in humans[151-153]. Recently, proof-of-principle trials of HPV16 L1[123,139], HPV16 and 18 L1[138] or HPV6, 11,16 and 18 L1[125] VLPs have shown that over 90% of vaccinated subjects were protected against persistent infection with the homologous HPV type, whereas the placebo group had persistent infections with HPV and subsequent HPV-associated cytological abnormalities. Although the results of these studies are highly encouraging, several things should be kept in mind. First, it has not been proven that these vaccines really protect against the development of cervical cancer. It will take several decades before this will become clear. Second, the studies mentioned indicated that protection was associated with the concentration of HPV-specific L1-antibodies. Although high anti-HPV16 titres were observed after

completion of the vaccination regimens, the titres waned over time[125,139]. It is important to examine what titre of antibodies is necessary for optimal protection, and in what frequency booster vaccinations are needed for maintenance of this titre. Other unresolved issues include the most critical groups to vaccinate and if the costs of the vaccines are low enough for widespread implementation in the developing world, where 80% of cervical cancer occurs[154,155]. In conclusion, long-term studies addressing these issues are necessary before large-scale implementation of prophylactic HPV vaccines can take place.

Therapeutic HPV vaccination

VLP vaccination leading to the circulation of antibodies to L1 will not elicit the cell-mediated immune responses required for the therapeutic treatment of established HPV-induced (pre)malignant disease, in which a spectrum of early gene products is expressed and in which at later stages of disease the expression of the late antigen L1 is lost[141,156,157]. We showed that HPV16 L1-specific type 1 T-cell immunity is found in the majority of both healthy subjects and patients with HPV16-induced CIN and cervical cancer, suggesting that this type of immunity does not protect against the development of AGIN (*chapter 3*). Therapeutic vaccines are aimed at eradicating or reducing the number of HPV-infected cells. In many cases HPV-associated tumors only express the E6 and E7 oncoproteins, so it is not surprisingly that most candidate vaccines are aimed at inducing T-cell immunity against these proteins. However, animal studies suggest that targeting the early proteins E1 and E2 can contribute to therapeutic vaccine efficacy. In the cottontail rabbit papillomavirus (CRPV) model, which is a model for high-risk papillomavirus infections in humans, DNA vaccines encoding the early antigens E1, E2, E6 and E7 were shown to prevent persistent infections and associated dysplasia[158,159]. Immunization of rabbits with the E1 and E2 proteins induced a CD4+ T-cell response, fewer papillomas developed and they regressed more rapidly than in non-vaccinated animals[160]. Similarly, therapeutic vaccination with long E6 and E7 peptides was able to control wart growth in immunized rabbits and resolve latent infections[161]. In view of the strong immune responses to the E2, E6 and E7 early proteins that are readily detected in sexually active healthy individuals[130,132], these animal models strengthen the notion that the same HPV proteins may form an important target for therapeutic vaccines.

At present, several vaccines have been tested in phase 1/2 clinical trials in humans (reviewed in[162,163]). A recombinant vaccinia virus expressing the HPV16 and HPV18 E6 and E7 genes (TA-HPV) in patients with cervical cancer was shown to be safe,

well-tolerated[164], and immunogenic in a proportion of the patients tested[165]. This vaccine was also tested in a group of patients with high-grade VIN. In the first series of 18 patients with VIN 3, there were T-cell responses against HPV16 in 13/18 cases. Furthermore, a 50% decrease in lesion size was reported in 8/18 patients and a reduction or loss of viral load in 12/18 patients[104]. In a group of women with high-grade VIN, the TA-HPV vaccine induced a systemic HPV-specific T-cell response in 6/10 patients[166]. All these 6 patients showed a clinical response during treatment. Although there was no overt correlation between the clinical change and the T-cell responses, this was a remarkable observation.

Peptide vaccines are attractive because peptides are relatively inexpensive and well tolerated in humans. In a phase 1 trial of 15 HPV16-positive patients with late-stage cervical cancer, vaccination with 2 HPV16 E7 CTL epitopes and a universal Th epitope did not result in the induction of HPV16-specific CTL nor was there evidence of clinical benefit, although there were strong immune responses induced against the non-specific CD4+ component of the vaccine[167]. Vaccination with a similar E7 vaccine resulted in E7-specific T-cell responses in a fraction of patients with high-grade anogenital neoplasia[168]. In women with CIN an HPV16 E6E7 fusion protein in Isomatrix adjuvant resulted in cellular immune responses in a substantial part of vaccinated subjects[169]. Recently, a phase 1/ 2 trial involving vaccination with 32-35 aminoacid long overlapping synthetic peptides spanning HPV16 E6 and E7 has shown that strong HPV16-specific IFNγ-associated CD4+ and CD8+ T-cell responses are induced, even in patients with advanced cervical cancer (Kenter, van der Burg and Melief, unpublished observations).

In summary, potential therapeutic vaccine candidates are being tested in human clinical trials. They have proven to be safe and well tolerated in patients and are immunogenic in most cases. Although some encouraging clinical responses have been reported, the relation between the induction of systemic HPV-specific T-cell responses and clinical efficacy is not clear and needs to be examined in future studies.

Outline of this thesis

The aim of this study was to gain further insight into the natural HPV-specific T-cell responses in healthy individuals and in patients with HPV-induced disease of the female genital tract. Furthermore, the T-cell responses were studied in relation to clinical responses upon immunomodulatory treatment of anogenital intraepithelial neoplasia.

In *chapters 2 and 3* we describe the HPV-specific immune responses that spontaneously develop during viral infection and persistence. We found that systemic CD4+ T-cell responses against HPV16 E2 and E6 were frequently found in healthy subjects and were predominantly of a mixed Th1/Th2 character. HPV16-specific T-cell responses in patients with cervical cancer lacked the strong proinflammatory cytokine profiles generally associated with HPV16-specific responses in healthy individuals, suggesting immune failure in patients with HPV16+ cervical cancer. Unlike E2 and E6, type 1 T-cell immunity against the structural protein L1 was not correlated with health or disease, as we found HPV16-specific IFNγ-associated T-cell immunity to L1 in healthy individuals and in patients with HPV16- positive cervical neoplasia with similar magnitude. *Chapter 4* reports on a pilot study of the use of a skin test as a safe and simple method to detect HPV16-specific T-cell responses in vivo. The skin test results and correlation with immunological responses in a group of eleven women with histories of cervical neoplasia and a group of nine healthy controls are evaluated. In *chapters 5 and 6* we studied the immune responses against HPV16 during immunomodulation in women with HPV16-associated anogenital intraepithelial neoplasia (AGIN). An HPV oncogene vaccination protocol was shown to be highly immunogenic in a group of patients with AGIN and in some cases these HPV-specific type 1 immune responses were associated with an objective regression of lesions. The presence of IFNγ-associated HPV16-specific T-cell responses was associated with a more favorable clinical response upon treatment with imiquimod in a group of patients with high-grade VIN. *Chapter 7* describes the results of a detailed analysis of the systemic immune response and local tumor infiltration in a group of patients with HPV16- or 18- positive cervical cancer. Strong CD8+ T-cell tumor infiltration was found in patients without lymph node metastases and this may be associated with better prognosis.

A discussion of the overall results is presented in *chapter 8*, as well as directions for future research.

References

1. Griffiths M. 'Nuns, virgins, and spinsters'. Rigoni-Stern and cervical cancer revisited. Br J Obstet Gynaecol 1991; 98: 797-802.
2. zur Hausen H. Condylomata acuminata and human genital cancer. Cancer Res 1976; 36: 794.
3. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999; 189: 12-19.
4. Bosch FX, Lorincz A, Munoz N, et al. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002; 55: 244-265.
5. zur Hausen H. Papillomavirus infections - a major cause of human cancers. Biochim Biophys Acta 1996; 1288: F55-F78.
6. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002; 2: 342-350.
7. Bosch FX, Manos, MM, Munoz N, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995; 87: 796-802.
8. Ho GY, Bierman R, Beardsley L, et al. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998; 338: 423-428.
9. Helmerhorst TJ and Meijer CJ. Cervical cancer should be considered as a rare complication of oncogenic HPV infection rather than a STD. Int J Gynecol Cancer 2002; 12: 235-236.
10. Jenkins D, Sherlaw-Johnson C, and Gallivan S. Can papilloma virus testing be used to improve cervical cancer screening? Int J Cancer 1996; 65: 768-773.
11. Van Ham MA, Melchers WJ, Hanselaar AG, et al. Fluctuations in prevalence of cervical human papillomavirus in women frequently sampled during a single menstrual cycle. Br J Cancer 2002; 87: 373-376.
12. Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis 171: 1026-1030, 1995.
13. Koutsky L. Epidemiology of genital human papillomavirus infection. Am J Med 1997; 102: 3-8.
14. Khan MJ, Castle PE, Lorincz AT, et al. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst 2005; 97: 1072-1079.
15. Palefsky JM and Holly EA. Chapter 6: Immunosuppression and co-infection with HIV. J Natl Cancer Inst Monogr 2003; 41-46.
16. Parkin DM, Pisani P, and Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993; 54: 594-606.
17. Schiffman M and Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. J Natl Cancer Inst Monogr 2003; 14-19.
18. Snijders PJ, Steenbergen RD, Heideman DA, et al. HPV-mediated cervical carcinogenesis: concepts and clinical implications. J Pathol 2006; 208: 152-164.
19. Melnikow J, Nuovo J, Willan AR, et al. Natural history of cervical squamous intraepithelial lesions: a meta-analysis. Obstet Gynecol 1998; 92: 727-735.
20. Ostor AG. Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol 1993; 12: 186-192.
21. Gad C. The management and natural history of severe dysplasia and carcinoma in situ of the uterine cervix. Br J Obstet Gynaecol 1976; 83: 554-559.
22. Iversen T. and Tretli S. Intraepithelial and invasive squamous cell neoplasia of the vulva: trends in incidence, recurrence, and survival rate in Norway. Obstet Gynecol 1998; 91: 969-972.
23. Jones RW and Rowan DM. Vulvar intraepithelial neoplasia III: a clinical study of the outcome in 113 cases with relation to the later development of invasive vulvar carcinoma. Obstet Gynecol1994; 84: 741-745.
24. van Seters M, van Beurden M, and de Craen AJ. Is the assumed natural history of vulvar intraepithelial neoplasia III based on enough evidence? A systematic review of 3322 published patients. Gynecol Oncol 2005; 97: 645-651.
25. Buscema J, Naghashfar Z, Sawada E, et al. The predominance of human papillomavirus type 16 in vulvar neoplasia. Obstet Gynecol 1988; 71: 601-606.
26. Hording U, Junge J, Poulsen H, et al. Vulvar intraepithelial neoplasia III: a viral disease of undetermined progressive potential. Gynecol Oncol 1995; 56: 276-279.
27. van Beurden M, ten Kate FJ, Smits HL, et al. Multifocal vulvar intraepithelial neoplasia grade III and multicentric lower genital tract neoplasia is associated with transcriptionally active human papillomavirus. Cancer 1995; 75: 2879-2884.

28. Munoz N, Castellsague X, de Gonzalez AB, et al. Chapter 1: HPV in the etiology of human cancer. Vaccine 2006; 24S3: S1-S10.
29. Minucci D, Cinel A, Insacco E, et al. Epidemiological aspects of vaginal intraepithelial neoplasia (VAIN). Clin Exp Obstet Gynecol 1995; 22: 36-42.
30. Sugase M and Matsukura T. Distinct manifestations of human papillomaviruses in the vagina. Int J Cancer 1997; 72: 412-415.
31. Srodon M, Stoler MH, Baber GB, et al. The distribution of low and high-risk HPV types in vulvar and vaginal intraepithelial neoplasia (VIN and VaIN). Am J Surg Pathol 2006; 30: 1513-1518.
32. Frisch M, Fenger C, van den Brule AJ, et al. Variants of squamous cell carcinoma of the anal canal and perianal skin and their relation to human papillomaviruses. Cancer Res 1999; 59: 753-757.
33. Helmerhorst T and Wijnen JA. Richtlijnen bevolkingsonderzoek baarmoederhalskanker. Nederlands Tijdschrift voor Gynaecologie en Obstetrie,1998; 111: 264-265.
34. Cirisano FD. Management of pre-invasive disease of the cervix. Semin Surg Oncol 1999; 16: 222-227.
35. Alvarez RD, Helm CW, Edwards RP, et al. Prospective randomized trial of LLETZ versus laser ablation in patients with cervical intraepithelial neoplasia. Gynecol Oncol 1994; 52: 175-179.
36. Bigrigg A, Haffenden DK, Sheehan AL, et al. Efficacy and safety of large-loop excision of the transformation zone. Lancet 1994; 343: 32-34.
37. Mitchell MF, Tortolero-Luna G, Cook E, et al. A randomized clinical trial of cryotherapy, laser vaporization, and loop electrosurgical excision for treatment of squamous intraepithelial lesions of the cervix. Obstet Gynecol 1998; 92: 737-744.
38. Prendiville W and Turner M. Large loop excision of the transformation zone. Lancet 1991; 337: 618.
39. Creasman WT. New gynecologic cancer staging. Gynecol Oncol 1995; 58: 157-158.
40. Cannistra SA and Niloff JM. Cancer of the uterine cervix. N Engl J Med 1996; 334: 1030-1038.
41. Waggoner SE. Cervical cancer. Lancet 2003; 361: 2217-2225.
42. Larson DM, Copeland LJ, Stringer CA, et al. Recurrent cervical carcinoma after radical hysterectomy. Gynecol Oncol 1998; 30: 381-387.
43. Hellebrekers BW, Zwinderman AH, Kenter GG, et al. Surgically-treated early cervical cancer: Prognostic factors and the significance of depth of tumor invasion. Int J Gynecol Cancer 1999; 9: 212-219.
44. Suprasert P, Srisomboon J, and Kasamatsu T. Radical hysterectomy for stage IIB cervical cancer: a review. Int J Gynecol Cancer 2005; 15: 995-1001.
45. Takeda N, Sakuragi, N, Takeda, M, et al. Multivariate analysis of histopathologic prognostic factors for invasive cervical cancer treated with radical hysterectomy and systematic retroperitoneal lymphadenectomy. Acta Obstet Gynecol Scand 2002; 81: 1144-1151.
46. Jones RW, Baranyai J, and Stables S. Trends in squamous cell carcinoma of the vulva: the influence of vulvar intraepithelial neoplasia. Obstet Gynecol 1997; 90: 448-452.
47. Preti M, van Seters M, Sideri, M, et al. Squamous vulvar intraepithelial neoplasia. Clin Obstet Gynecol 2005; 48: 845-861.
48. Abu-Rustum NR, Sonoda Y, Black D, et al. Fertility-sparing radical abdominal trachelectomy for cervical carcinoma: technique and review of the literature. Gynecol Oncol 2006; 103: 807-813.
49. Plante M, Renaud MC, Hoskins IA, et al. Vaginal radical trachelectomy: a valuable fertility-preserving option in the management of early-stage cervical cancer. A series of 50 pregnancies and review of the literature. Gynecol Oncol 2005; 98: 3-10.
50. Shepherd JH, Spencer C, Herod J, et al. Radical vaginal trachelectomy as a fertility-sparing procedure in women with early-stage cervical cancer-cumulative pregnancy rate in a series of 123 women. BJOG 2006; 113: 719-724.
51. Pieterse QD, Maas CP, ter Kuile MM, et al. An observational longitudinal study to evaluate miction, defecation, and sexual function after radical hysterectomy with pelvic lymphadenectomy for early-stage cervical cancer. Int J Gynecol Cancer 2006; 16: 1119-1129.
52. Samlal RA, van der Velden J, Van Eerden T, et al. Recurrent cervical carcinoma after radical hysterectomy: an analysis of clinical aspects and prognosis. Int J Gynecol Cancer 1998; 8: 78-84.
53. Bergmark K, Avall-Lundqvist E, Dickman P, et al. Vaginal changes and sexuality in women with a history of cervical cancer. N Engl J Med 1999; 340: 1383-1389.
54. Landoni F. Nerve sparing surgery in cervical cancer. Gynecol Oncol 2005; 99: S150-S151.
55. Trimbos JB, Maas CP, de Ruiter MC, et al. A nerve-sparing radical hysterectomy: guidelines and feasibility in Western patients. Int J Gynecol Cancer 2001; 11: 180-186, 2001.
56. Maas CP, Trimbos JB, de Ruiter MC, et al. Nerve

sparing radical hysterectomy: latest developments and historical perspective. Crit Rev Oncol Hematol 2003; 48: 271-279.
57. Moore DH, Blessing JA, McQuellon RP, et al. Phase III study of cisplatin with or without paclitaxel in stage IVB, recurrent, or persistent squamous cell carcinoma of the cervix: a gynecologic oncology group study. J Clin Oncol 2004; 22: 3113-3119.
58. Long HJ, Bundy BN, Grendys EC Jr, et al. Randomized phase III trial of cisplatin with or without topotecan in carcinoma of the uterine cervix: a Gynecologic Oncology Group Study. J Clin Oncol 2005; 23: 4626-4633.
59. Wierrani F, Kubin A, Jindra R, et al. 5-aminolevulinic acid-mediated photodynamic therapy of intraepithelial neoplasia and human papillomavirus of the uterine cervix--a new experimental approach. Cancer Detect Prev 1999; 23: 351-355.
60. Fehr MK, Hornung R, Schwarz VA, et al. Photodynamic therapy of vulvar intraepithelial neoplasia III using topically applied 5-aminolevulinic acid. Gynecol Oncol 2001; 80: 62-66.
61. Hillemanns P, Untch M, Dannecker C, et al. Photo-dynamic therapy of vulvar intraepithelial neoplasia using 5-aminolevulinic acid. Int J Cancer 2000; 85: 649-653.
62. Hillemanns P, Wang X, Staehle S, et al. Evaluation of different treatment modalities for vulvar intraepithelial neoplasia (VIN): CO(2) laser vaporization, photodynamic therapy, excision and vulvectomy. Gynecol Oncol 2006; 100: 271-275.
63. Arican O, Guneri F, Bilgic K, et al. Topical imiquimod 5% cream in external anogenital warts: a randomized, double-blind, placebo-controlled study. J Dermatol 2004; 31: 627-631.
64. Sauder DN, Skinner RB, Fox TL, et al. Topical imiquimod 5% cream as an effective treatment for external genital and perianal warts in different patient populations. Sex Transm Dis 2003; 30: 124-128.
65. Tyring SK, Arany I, Stanley MA, et al. A randomized, controlled, molecular study of condylomata acuminata clearance during treatment with imiquimod. J Infect Dis 1998; 178: 551-555.
66. Todd RW, Etherington IJ, and Luesley DM. The effects of 5% imiquimod cream on high-grade vulval intraepithelial neoplasia. Gynecol Oncol 2002; 85: 67-70.
67. van Seters M, Fons G, and van Beurden M. Imiquimod in the treatment of multifocal vulvar intraepithelial neoplasia 2/3. Results of a pilot study. J Reprod Med 2002; 47: 701-705.
68. Marchitelli C, Secco G, Perrotta M, et al. Treatment of bowenoid and basaloid vulvar intraepithelial neoplasia 2/3 with imiquimod 5% cream. J Reprod Med 2004; 49: 876-882.
69. Wendling J, Saiag P, Berville-Levy S, et al. Treatment of undifferentiated vulvar intraepithelial neoplasia with 5% imiquimod cream: a prospective study of 12 cases. Arch Dermatol 2004; 140: 1220-1224.
70. Ossendorp F, Toes RE, Offringa R, et al. Importance of CD4(+) T helper cell responses in tumor immunity. Immunol Lett 2000; 74: 75-79.
71. Pardoll DM and Topalian SL. The role of CD4+ T cell responses in antitumor immunity. Curr Opin Immunol 1998; 10: 588-594.
72. Toes RE, Ossendorp F, Offringa R, et al. CD4 T cells and their role in antitumor immune responses. J Exp Med 1999; 189: 753-756.
73. Tanaka H, Tanaka J, Kjaergaard J, et al. Depletion of CD4+ CD25+ regulatory cells augments the generation of specific immune T cells in tumor-draining lymph nodes. J Immunother 2002; 25: 207-217.
74. Roncarolo MG, Bacchetta R, Bordignon C, et al. Type 1 T regulatory cells. Immunol Rev 2001; 182: 68-79.
75. Kitani A, Chua K, Nakamura K, et al. Activated self-MHC-reactive T cells have the cytokine phenotype of Th3/T regulatory cell 1 T cells. J Immunol 2000; 165: 691-702.
76. de Gruijl TD, Bontkes HJ, Walboomers JM, et al. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. I. Differential T-helper and IgG responses in relation to HPV infection and disease outcome. J Gen Virol 1999; 80 (Pt 2): 399-408.
77. Mosmann TR, Li L, and Sad S. Functions of CD8 T-cell subsets secreting different cytokine patterns. Semin Immunol 1997; 9: 87-92.
78. Stadnyk AW. Cytokine production by epithelial cells. FASEB J, 1994; 8: 1041-1047.
79. Giannini SL, Hubert P, Doyen J, et al. Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development of squamous intraepithelial lesions of the cervix. Int J Cancer 2002; 97: 654-659.
80. Mota F, Rayment N, Chong S, et al. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. Clin Exp Immunol 1999; 116: 33-40.
81. Pao CC, Lin CY, Yao DS, et al. Differential expression of cytokine genes in cervical cancer tissues. Biochem Biophys Res Commun 1995; 214: 1146-1151.
82. El Sherif AM, Seth R, Tighe PJ, et al. Quantitative

analysis of IL-10 and IFN-gamma mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. J Pathol 2001; 195: 179-185.
83. Tartour E, Gey A, Sastre-Garau X, et al. Prognostic value of intratumoral interferon gamma messenger RNA expression in invasive cervical carcinomas. J Natl Cancer Inst 1998; 90: 287-294.
84. Clerici M, Merola M, Ferrario E, et al. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. J Natl Cancer Inst 1997; 89: 245-250.
85. Hazelbag S, Fleuren GJ, Baelde JJ, et al. Cytokine profile of cervical cancer cells. Gynecol Oncol 2001; 83: 235-243.
86. Hazelbag S, Gorter A, Kenter GG, et al. Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer. Hum Pathol 2002; 33: 1193-1199.
87. Seo N, Hayakawa S, Takigawa, M, et al. Interleukin-10 expressed at early tumour sites induces subsequent generation of CD4(+) T-regulatory cells and systemic collapse of antitumour immunity. Immunology 2001; 103: 449-457.
88. Kobayashi A, Greenblatt RM, Anastos K, et al. Functional attributes of mucosal immunity in cervical intraepithelial neoplasia and effects of HIV infection. Cancer Res 2004; 64: 6766-6774.
89. van Driel WJ, Hogendoorn PC, Jansen FW, et al. Tumor-associated eosinophilic infiltrate of cervical cancer is indicative for a less effective immune response. Hum Pathol 1996; 27: 904-911.
90. van Driel WJ, Kievit-Tyson P, van den Broek LC, et al. Presence of an eosinophilic infiltrate in cervical squamous carcinoma results from a type 2 immune response. Gynecol Oncol 1999; 74: 188-195.
91. Coleman N, Birley HD, Renton AM, et al. Immunological events in regressing genital warts. Am J Clin Pathol 1994; 102: 768-774.
92. Selvakumar R, Schmitt A, Iftner T, et al. Regression of papillomas induced by cottontail rabbit papillomavirus is associated with infiltration of CD8+ cells and persistence of viral DNA after regression. J Virol 1997; 71: 5540-5548.
93. Bell MC, Edwards RP, Partridge EE, et al. CD8+ T lymphocytes are recruited to neoplastic cervix. J Clin Immunol 1995; 15: 130-136.
94. Tay SK, Jenkins D, Maddox P, et al. Lymphocyte phenotypes in cervical intraepithelial neoplasia and human papillomavirus infection. Br J Obstet Gynaecol 1987; 94: 16-21.
95. de Gruijl TD, Bontkes HJ, Peccatori F, et al. Expression of CD3-zeta on T-cells in primary cervical carcinoma and in metastasis-positive and -negative pelvic lymph nodes. Br J Cancer 1999; 79: 1127-1132.
96. Gul N, Ganesan R, and Luesley DM. Characterizing T-cell response in low-grade and high-grade vulval intraepithelial neoplasia, study of CD3, CD4 and CD8 expressions. Gynecol Oncol 2004; 94: 48-53.
97. Caorsi I and Figueroa CD. Langerhans' cell density in the normal exocervical epithelium and in the cervical intraepithelial neoplasia. Br J Obstet Gynaecol 1986; 93: 993-998.
98. Edwards RP, Kuykendall K, Crowley-Nowick P, et al. T lymphocytes infiltrating advanced grades of cervical neoplasia. CD8- positive cells are recruited to invasion. Cancer 1995; 76: 1411-1415.
99. Morris HH, Gatter KC, Sykes G, et al. Langerhans' cells in human cervical epithelium: effects of wart virus infection and intraepithelial neoplasia. Br J Obstet Gynaecol 1983; 90: 412-420.
100. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348: 203-213.
101. Schumacher K, Haensch W, Roefzaad C, et al. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res 2001; 61: 3932-3936.
102. Kondratiev S, Sabo E, Yakirevich E, et al. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin Cancer Res 2004; 10: 4450-4456.
103. Menon AG, Fleuren GJ, Alphenaar EA, et al. A basal membrane-like structure surrounding tumour nodules may prevent intraepithelial leucocyte infiltration in colorectal cancer. Cancer Immunol Immunother 2003; 52: 121-126.
104. Davidson EJ, Boswell CM, Sehr P, et al. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003; 63: 6032-6041.
105. Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, et al. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. Cancer Res 2001; 61: 192-196.
106. Sheu BC, Hsu SM, Ho HN, et al. Reversed CD4/CD8 ratios of tumor-infiltrating lymphocytes are correlated with the progression of human cervical carcinoma. Cancer 1999; 86: 1537-1543.
107. Palefsky JM, Minkoff H, Kalish LA, et al. Cervicovaginal human papillomavirus infection in

human immunodeficiency virus-1 (HIV)-positive and high-risk HIV-negative women. J Natl Cancer Inst 1999; 91: 226-236.
108. Hagensee ME, Koutsky LA, Lee SK, et al. Detection of cervical antibodies to human papillomavirus type 16 (HPV- 16) capsid antigens in relation to detection of HPV-16 DNA and cervical lesions. J Infect Dis 2000; 181: 1234-1239.
109. Rocha-Zavaleta L, Pereira-Suarez AL, Yescas G, et al. Mucosal IgG and IgA responses to human papillomavirus type 16 capsid proteins in HPV16-infected women without visible pathology. Viral Immunol 2003; 16: 159-168.
110. Sasagawa T, Rose RC, Azar KK, et al. Mucosal immunoglobulin-A and -G responses to oncogenic human papilloma virus capsids. Int J Cancer 2003; 104: 328-335.
111. Carter JJ, Koutsky LA, Wipf GC, et al. The natural history of human papillomavirus type 16 capsid antibodies among a cohort of university women. J Infect Dis 1996; 174: 927-936.
112. Carter JJ, Koutsky LA, Hughes JP, et al. Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection. J Infect Dis 2000; 181: 1911-1919.
113. Ho GY, Studentsov YY, Bierman R, et al. Natural history of human papillomavirus type 16 virus-like particle antibodies in young women. Cancer Epidemiol Biomarkers Prev 2004; 13: 110-116.
114. af Geijersstam V, Kibur M, Wang Z, et al. Stability over time of serum antibody levels to human papillomavirus type 16. J Infect Dis 1998; 177: 1710-1714.
115. Carter JJ, Madeleine MM, Shera K, et al. Human papillomavirus 16 and 18 L1 serology compared across anogenital cancer sites. Cancer Res 2001; 61: 1934-1940.
116. Davidson EJ, Sehr P, Faulkner RL, et al. Human papillomavirus type 16 E2- and L1-specific serological and T-cell responses in women with vulval intraepithelial neoplasia. J Gen Virol 2003; 84: 2089-2097.
117. Sun Y, Hildesheim A, Brinton LA, et al. Human papillomavirus-specific serologic response in vulvar neoplasia. Gynecol Oncol 1996; 63: 200-203.
118. Park JS, Park DC, Kim CJ, et al. HPV-16-related proteins as the serologic markers in cervical neoplasia. Gynecol Oncol 1998; 69: 47-55.
119. Wideroff L, Schiffman MH, Nonnenmacher B, et al. Evaluation of seroreactivity to human papillomavirus type 16 virus-like particles in an incident case-control study of cervical neoplasia. J Infect Dis 1995; 172: 1425-1430.
120. Sasagawa T, Yamazaki H, Dong YZ, et al. Immunoglobulin-A and -G responses against virus-like particles (VLP) of human papillomavirus type 16 in women with cervical cancer and cervical intra-epithelial lesions. Int J Cancer 1998; 75: 529-535.
121. Onda T, Carter JJ, Koutsky LA, et al. Characterization of IgA response among women with incident HPV 16 infection. Virology 2003; 312: 213-221.
122. Breitburd F, Kirnbauer R, Hubbert NL, et al. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. J Virol 1995; 69: 3959-3963.
123. Koutsky LA, Ault KA, Wheeler CM, et al. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 2002; 347: 1645-1651.
124. Suzich JA, Ghim SJ, Palmer-Hill FJ, et al. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci USA1995; 92: 11553-11557.
125. Villa LL, Costa RL, Petta CA, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol 2005; 6: 271-278.
126. Nakagawa M, Stites DP, Farhat S, et al. T-cell proliferative response to human papillomavirus type 16 peptides: relationship to cervical intraepithelial neoplasia. Clin Diagn Lab Immunol 1996; 3: 205-210.
127. Bontkes HJ, de Gruijl TD, Bijl A, et al. Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia. J Gen Virol 1999; 80 (Pt 9): 2453-2459.
128. de Gruijl TD, Bontkes HJ, Walboomers JM, et al. Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study. Cancer Res 1998; 58: 1700-1706.
129. Kadish AS, Ho GY, Burk RD, et al. Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia. J Natl Cancer Inst 1997; 89: 1285-1293.
130. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.

131. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II- restricted epitopes. Int J Cancer 2001; 91: 612-618.
132. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory T-helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.
133. Lin YL, Borenstein LA, Selvakumar R, et al. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. Virology 1992; 187: 612-619.
134. Bell JA, Sundberg JP, Ghim SJ, et al. A formalin-inactivated vaccine protects against mucosal papillomavirus infection: a canine model. Pathobiology 1994; 62: 194-198.
135. Yuan H, Estes PA, Chen Y, et al. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. J Virol 2001; 75: 7848-7853.
136. Luxton JC, Nath R, Derias N, et al. Human papillomavirus type 16-specific T cell responses and their association with recurrence of cervical disease following treatment. J Gen Virol 2003; 84: 1063-1070.
137. Shepherd PS, Rowe AJ, Cridland JC, et al. Proliferative T cell responses to human papillomavirus type 16 L1 peptides in patients with cervical dysplasia. J Gen Virol 1996; 77 (Pt 4): 593-602.
138. Harper DM, Franco EL, Wheeler C, et al. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 2004; 364: 1757-1765.
139. Mao C, Koutsky LA, Ault KA, et al. Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial. Obstet Gynecol 2006; 107: 18-27.
140. Pinto LA, Edwards J, Castle PE, et al. Cellular immune responses to human papillomavirus (HPV)-16 L1 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles. J Infect Dis 2003; 188: 327-338.
141. Hildesheim A and Herrero R. Effect of a HPV16/18 vaccine on resolution of infections in women with pre-existing HPV. 23rd International Papillomavirus Conference & Clinical workshop 2006.
142. Zinkernagel RM. Immunology taught by viruses. Science, 1996; 271: 173-178.
143. Steele JC, Mann CH, Rookes S, et al. T-cell responses to human papillomavirus type 16 among women with different grades of cervical neoplasia. Br J Cancer 2005; 93: 248-259.
144. Nakagawa M, Stites DP, Farhat S, et al. Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia. J Infect Dis 1997; 175: 927-931.
145. Nakagawa M, Stites DP, Patel S, et al. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. J Infect Dis 2000; 182: 595-598.
146. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. Int J Cancer 2000; 88: 92-98.
147. Nimako M, Fiander AN, Wilkinson GW, et al. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. Cancer Res 1997; 57: 4855-4861.
148. Ressing ME, van Driel WJ, Celis E, et al. Occasional memory cytotoxic T-cell responses of patients with human papillomavirus type 16-positive cervical lesions against a human leukocyte antigen-A *0201-restricted E7-encoded epitope. Cancer Res 1996; 56: 582-588.
149. Melief CJ, van der Burg SH, Toes RE, et al. Effective therapeutic anticancer vaccines based on precision guiding of cytolytic T lymphocytes. Immunol Rev 2002; 188: 177-182.
150. Schiller JT and Lowy DR. Papillomavirus-like particle based vaccines: cervical cancer and beyond. Expert Opin Biol Ther 2001; 1: 571-581.
151. Ault KA, Giuliano AR, Edwards RP, et al. A phase I study to evaluate a human papillomavirus (HPV) type 18 L1 VLP vaccine. Vaccine 2004; 22: 3004-3007.
152. Evans TG, Bonnez W, Rose RC, et al. A Phase 1 study of a recombinant viruslike particle vaccine against human papillomavirus type 11 in healthy adult volunteers. J Infect Dis 2001; 183: 1485-1493.
153. Harro CD, Pang YY, Roden RB, et al. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. J Natl Cancer Inst 2001; 93: 284-292.
154. Lowy DR and Frazer IH. Chapter 16: Prophylactic human papillomavirus vaccines. J Natl Cancer Inst Monogr 2003; 111-116.
155. Lowy DR and Schiller JT. Prophylactic human

papillomavirus vaccines. J Clin Invest 2006; 116: 1167-1173.
156. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond) 2006; 110: 525-541.
157. Baker CC, Phelps WC, Lindgren V, et al. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol 1987; 61: 962-971.
158. Han R, Cladel NM, Reed CA, et al. Protection of rabbits from viral challenge by gene gun-based intracutaneous vaccination with a combination of cottontail rabbit papillomavirus E1, E2, E6, and E7 genes. J Virol 1999; 73: 7039-7043.
159. Han R, Cladel NM, Reed CA, et al. DNA vaccination prevents and/or delays carcinoma development of papillomavirus-induced skin papillomas on rabbits. J Virol 2000; 74: 9712-9716.
160. Selvakumar R, Borenstein LA, Lin YL, et al. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. J Virol 1995; 69: 602-605.
161. Vambutas A, Devoti J, Nouri M, et al. Therapeutic vaccination with papillomavirus E6 and E7 long peptides results in the control of both established virus-induced lesions and latently infected sites in a pre-clinical cottontail rabbit papillomavirus model. Vaccine 2005; 23: 5271-5280.
162. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. Nat Rev Immunol 2004; 4: 46-54.
163. Galloway DA. Papillomavirus vaccines in clinical trials. Lancet Infect Dis 2003; 3: 469-475.
164. Borysiewicz LK, Fiander A, Nimako M, et al. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet 1996; 347: 1523-1527.
165. Kaufmann AM, Stern PL, Rankin EM, et al. Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 E6 and E7 genes, in women with progressive cervical cancer. Clin Cancer Res 2002; 8: 3676-3685.
166. Baldwin PJ, van der Burg SH, Boswell CM, et al. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003; 9: 5205-5213.
167. Ressing ME, van Driel WJ, Brandt RM, et al. Detection of T helper responses, but not of human papillomavirus- specific cytotoxic T lymphocyte responses, after peptide vaccination of patients with cervical carcinoma. J Immunother 2000; 23: 255-266.
168. Muderspach L, Wilczynski S, Roman L, et al. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. Clin Cancer Res 2000; 6: 3406-3416.
169. Frazer IH, Quinn M, Nicklin JL, et al. Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIXtrade mark adjuvant in women with cervical intraepithelial neoplasia. Vaccine 2004; 23: 172-181.

CHAPTER 2

HPV16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6

Annemieke de Jong
Mariëtte I.E. van Poelgeest
Jeanette M. van der Hulst
Jan Wouter Drijfhout
Gert Jan Fleuren
Cornelis J.M. Melief
Gemma G. Kenter
Rienk Offringa
Sjoerd H. van der Burg

Cancer Research 2004; 64: 5449-5455

Abstract

Cervical cancer is the possible outcome of genital infection with high-risk human papillomavirus (HPV) and is preceded by a phase of persistent HPV infection during which the host immune system fails to eliminate the virus. Fortunately, the majority of genital HPV infections are cleared before the development of (pre)malignant lesions. Analysis of CD4+ T-helper (Th) immunity against the E2, E6 and E7 antigens of HPV type 16 (HPV16) in healthy women revealed strong proliferative E2- and E6-specific responses associated with prominent IFNγ and IL-5 secretion. This indicates that the naturally arising virus-induced immune response displays a mixed Th1/Th2 cytokine profile. Of all HPV16+ cervical cancer patients, approximately half failed to mount a detectable immune response against the HPV16-derived peptides. The other half of the patients showed impaired HPV16-specific proliferative responses, which generally lacked both IFNγ and IL-5. This indicates that the HPV16-specific CD4+ T-cell response in cervical cancer patients is either absent or severely impaired, despite a relatively good immune status of the patients, as indicated by intact responses against recall antigens. It is highly conceivable that proper CD4+ T-cell help is important for launching an effective immune attack against HPV because infection of cervical epithelia by this virus is, at least initially, not accompanied by gross disturbance of this tissue and/or strong proinflammatory stimuli. Therefore, our observations concerning the lack of functional HPV16-specific CD4+ T-cell immunity in patients with cervical cancer offer a possible explanation for the development of this disease.

Introduction

Cervical encounter with human papillomavirus (HPV) generally results in a transient infection, with the majority of individuals showing clearance of the virus within 1 year of detection[1-4]. Factors underlying the lag time between HPV infection and clearance are related to the minimal disturbance of the epithelial layer initially caused by the virus, lack of HPV-induced Langerhans cell activation, and the capacity of viral proteins to evade innate immune recognition by physically inhibiting specific components of the innate immune system, such as interference with the type I interferon pathway[5-10]. Once the process of regression of HPV-induced dysplasia is initiated, it is characterized by an influx of macrophages and T lymphocytes, resembling a delayed-type hypersensitivity response[11]. Indirect evidence that the adaptive cellular immune system plays a major role in the protection against HPV-induced lesions is given by the high incidence of persistent HPV infections and subsequent HPV-related dysplasia in both immunosuppressed transplant patients and HIV-infected individuals[12,13]. An underlying cause of the failing anti-HPV immune response in immunocompetent individuals with persistent infection was suggested to be a locally altered cytokine environment with an increase in IL-10 production and a decrease in proinflammatory cytokines[14-16]. Others have shown that a so-called T-helper (Th)2 type cytokine bias in the peripheral blood of CIN patients was associated with more extensive cervical disease[17], but the implication of such altered cytokine balance on HPV-specific immunity has not been determined.

Studies analyzing T-cell responses against viral antigens from the high-risk type HPV16 in the healthy population and several stages of disease do not present an unequivocal relationship between protection against HPV16-induced (pre)malignant lesions and virus-specific T-cell immunity[18-21]. We have previously described the presence of IFNγ-producing memory CD45RO+CD4+ Th cells specific for HPV16 E2 and E6 proteins in the peripheral blood of the majority of healthy individuals[22,23], suggesting a role for these Th cells in protection against HPV16-induced progressive disease. In order to fully appreciate the importance of HPV16 E2- and E6-specific Th responses in managing infection and subsequent progressive (pre)malignant lesions, we have performed a detailed analysis with respect to the magnitude and cytokine polarization of this immunity and compared this between healthy subjects and HPV16+ patients.

Materials and methods

Healthy blood donors and patients

A selected group of twenty sexually active young females within the age of 19-31 years (median 23 years) participated in this study after informed consent. It was expected that a large fraction of these individuals had experienced previous transient HPV infection because most anogenital HPV infections are acquired soon after sexual debut and are also cleared at an early age. Older individuals were excluded because women who are HPV DNA positive at ages 35 and above may represent failure of viral clearance[24]. A Pap smear was performed for cytological examination, and an additional cervical swab specimen was obtained for HPV DNA analysis. Women presenting with histologically proven cervical carcinoma or CIN at the department of gynecology of the Leiden University Medical Center (LUMC) were enrolled in this study after informed consent. Blood was drawn at day of treatment prior to surgery. Carcinoma subjects enrolled were staged FIGO IB/IIA and were treated by radical hysterectomy. The age of the cervical cancer patients (n=17) ranged from 34-72 years old (median 45 years), whereas the age of the CIN III patients (n=13) ranged from 29 – 42 years (median 31 years) We preferred to use CIN III for analyses of cases in which the naturally occurring immune response has not been able to control the infection because there is a substantial heterogeneity in the microscopic diagnosis and biologic meaning of CIN II lesions in particular. In general, the majority of CIN III lesions are incipient precancers that are destined to persist[2]. PBMC and serum were obtained for the analysis of HPV16-specific T-cell reactivity and VLP L1-specific antibodies.

The subjects were typed for HPV16 using HPV16-specific primers on DNA isolated from cervical swab specimens, paraffin-embedded sections of biopsies, or surgical resection specimens[25]. The study design was approved by the Medical Ethical Committee of the LUMC. Two of twenty healthy females were HPV positive (one type 62 and the other type 31), but no HPV16 was detected. Of the CIN III and cervical carcinoma patients, only the HPV16 positive subjects were included in the immunological analyses.

Antigens

A set of peptides spanning the whole HPV16 E2, E6, and E7 protein were used in pools of 2 peptides for the T-cell proliferation assays. The E2 peptides consisted of 22 30-mer peptides with 15 amino acids overlap and the C-terminal peptide with a length of 35 amino acids. The length of the E6 and E7 peptides was 32 and 35 amino acids, respectively, with an overlap of 14 amino acids. The peptides were synthesized

and dissolved as described previously[26]. The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (*e.g.* $E2_{1-45}$, residues 1-30 + 16-45). Memory Response Mix (MRM), consisting of a mixture of tetanus toxoid (0.75 LF/ml; National Institute of Public Health and Environment, Bilthoven, The Netherlands), Mycobacterium tuberculosis sonicate (2.5 µg/ml; generously donated by Dr.P. Klatser, Royal Tropical institute, Amsterdam, The Netherlands) and Candida albicans (0.005%, HAL Allergenen Lab, Haarlem, The Netherlands), was used to confirm the capacity of PBMC to proliferate and produce cytokine in response to common recall antigens.

HPV16 VLP ELISA

For the detection of HPV16-specific antibodies in serum we used an ELISA method previously described by Kirnbauer *et al.*[27]. Each serum was tested for reactivity against HPV16 virus-like particles (VLP, baculovirus-expressed capsids comprising the L1 protein) and against bovine papillomavirus (BPV) capsids, the latter disrupted by treatment with 0.1M carbonate buffer to serve as a negative control. Both VLP and BPV were kindly provided by Prof. dr. J. Dillner (LUNDS University, Sweden). The patients (all proven HPV16-positive by PCR) were tested for HPV16-specific IgG only, whereas the healthy controls were tested for both IgG and IgA because it has been described that transient infections more frequently lead to specific serum IgA antibodies than IgG. Overall, HPV16-specific antibodies can only be detected in a small fraction of individuals experiencing a transient HPV16 infection and only for a limited time period after infection[28]. Therefore, only the presence, but not the absence of HPV16-specific antibodies allows conclusions as to whether or not an individual has experienced a transient infection in the past.

Short-term T-cell proliferation assay

Freshly isolated PBMC were incubated with 12 pools of HPV16 E2-derived 30-mer peptides, 4 pools of E6 32-mer peptides, and 2 pools of E7 35-mer peptides (each pool containing two overlapping peptides). PBMC were seeded at a density of 1.5 x 10^5 cells/well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 150 µl of ISCOVE's medium (Bio-Whittaker) supplemented with 10% autologous serum. HPV16 E2-, E6- and E7-derived peptides were added at a concentration of 10 µg/ml/peptide. Medium alone was taken along as a negative control, and MRM (dilution 1:50) served as a positive control. For each peptide pool, 8 parallel microcultures were incubated. 50 µl of supernatant from the microcultures was taken at day 5 after incubation and stored at –20°C until cytokine analysis. Peptide-specific proliferation was measured

at day 7 by [^{3}H]thymidine incorporation. Cultures were scored positive when the proliferation of ≥ 75% of the test wells exceeded the mean proliferation plus 3 x SD of the control wells containing medium only, and the stimulation index (SI), defined as the mean of all test wells divided by the mean of the control wells, was ≥ 3. Previously performed short-term proliferation assays with immunologically naïve cord blood cells (10 samples) revealed no specific proliferation against the E6 or E7 oncoprotein-derived peptides, indicating that the 7-day stimulation with peptides does not result in the *in vitro* induction of HPV16-specific proliferative responses.

CFSE labeling

Freshly isolated PBMC were washed in cold PBS and incubated with 0.5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) in PBS at 37°C for 30 min; 5% autologous serum was added, and the PBMC were washed twice in PBS. The CFSE-labeled PBMC were then resuspended in IMDM supplemented with 10% autologous serum and incubated with the HPV16-derived peptides as described for the short-term proliferation assays.

Analysis of cytokines associated with HPV16-specific proliferative responses

The detection of cytokines in the supernatants of the short-term proliferation assays was performed using the cytometric bead array (Becton Dickinson). Analysis of the cytokine kinetics revealed that at day 5, antigen-specific production of all cytokines could be detected. IL-2 and IL-4 can both be consumed by proliferating cells; therefore, levels of these cytokines in this assay may be underestimated. Supernatants from the individual microcultures harvested at day 5 were pooled per peptide pool and 50 µl of this supernatant was used for cytokine analysis. The cytometric bead array was performed according to manufacturer's instruction. Cutoff values were based on the standard curves of the different cytokines (50 pg/ml for IFNγ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and > 2x the concentration of the medium control[29].

Analysis of PHA-induced proliferation and cytokine production

Liquid nitrogen-stored PBMC were used for these assays, enabling the analysis of all the samples at the same time for optimal comparison. The cervical carcinoma and the healthy control group consisted of the same individuals as those analyzed in the short-term proliferation assays, minus those from whom an insufficient amount of PBMC was available (healthy subjects n=17, cervical carcinoma patients n=13). The

CIN group included additional samples, which had been collected previously and stored in liquid nitrogen (CIN patients n=13). PBMC were thawed and resuspended at a concentration of 10^6/ml in serum-free x-vivo15 medium (BioWhittaker). 100 µl of cell suspension was seeded per well of a 96-well round-bottom plate, in total 24 wells per subject. 50 µl of 1,5 µg/ml PHA (Murex Diagnostics, Dartford, UK) in x-vivo15 was added to 12 wells, whereas 50 µl of plain x-vivo15 medium served as a negative control in the remaining 12 wells. After 48 hours 50 µl of supernatant per well was harvested, and the supernatants from the PHA-stimulated wells were pooled, as were the controls. Supernatants were stored at –20°C until cytokine analysis by ELISA. PHA-induced proliferation was measured by [^{3}H]thymidine incorporation 48 hours after stimulation. IFNγ was measured by ELISA as described previously[26]. IL-4 and IL-10 (CLB, Amsterdam) and IL-5 (Pharmingen) ELISA's were performed according to the instructions of the manufacturer.

Statistical analysis

Statistical analysis of the HPV16-specific proliferative responses associated with cytokine production was performed using Fisher's exact test. For the statistical analysis of the mitogen-induced proliferation and the cytokine levels in response to recall antigens, an unpaired two-tailed t-test was applied, and an unpaired two-tailed t-test with Welch correction for the analysis of the mitogen-induced cytokine ratios.

Results

HPV16 E2- and E6-specific CD4+ T-cell responses in healthy individuals are associated with Th1 and Th2 type cytokines

Previously, we have demonstrated the presence of HPV16 E2- and E6-specific memory CD4+ Th cells in the CD45RO+ fraction of peripheral blood of the majority of healthy individuals[22,23]. These memory Th cells produced IFNγ upon stimulation, indicating that they are of a Th1 type. So far, it has not been determined whether the HPV16-specific responses in the healthy population consist solely of Th1 type cytokines or whether other cytokines are also involved. We therefore analyzed the cytokine profiles of the HPV16-specific T-cells in a cohort of 20 young, sexually active, healthy women. All women had a normal cytology and were proven HPV16 negative by PCR and VLP ELISA at the time of analysis, therefore, the presence of responses against the HPV16 antigens are expected to represent memory T-cells induced upon a transient HPV16 infection in the past. Short-term proliferation assays were per-

formed against peptides derived from the HPV16 proteins E2, E6, and E7 as well as a mix of common recall antigens (Memory Response Mix, MRM). In accordance with our previous data[22,23], half of the individuals showed proliferative responses against E2 (10/20), and an even larger fraction showed responses against the E6-derived peptides (13/20). E7-specific responses were detected in only a minority of subjects (2/20) (Figure 1a). CFSE labeling of PBMC demonstrated that exclusively CD4+ Th cells contributed to the HPV16-specific proliferation, as upon stimulation with E2 and E6 peptides, cell division could only be detected in the CD4+ fraction of PBMC (Figure 2). This is in line with our previous notion that HPV16-specific IFNγ secretion was solely derived from CD4+ T-cells[22,23] (and data not shown).

Analysis of the supernatants of these T-cell cultures for the presence of IFNγ, TNFα, IL-2, IL-4, IL-5, and IL-10 revealed the secretion of both Th1- and Th2-type cytokines in response to HPV16-derived peptides (Figure 1b). The most predominantly secreted cytokines were IFNγ and IL-5 (Figure 1b and Figure 3), often accompanied by IL-2 and, to a lesser extent, by TNFα and IL-10. Antigen-specific secretion of IL-4 was rarely observed. In response to recall antigens (MRM), the majority of Th1- and Th2-type cytokines for which we analyzed were detected. Taken together, the consistent HPV16-specific secretion of IFNγ and IL-5 in healthy individuals suggests that both Th1 and Th2 type immunity play a role in the effective control of HPV16 infection.

Impaired HPV16-specific Th immunity in patients with HPV16+ (pre)malignant lesions

Having characterized the HPV16-specific Th responses in healthy individuals, a fraction of whom have apparently succeeded in clearing HPV16 infection, we questioned how these compare to patients who have evidently failed to establish an effective immune response against this virus. A group of HPV16-positive patients, consisting of 8 subjects diagnosed with CIN III and 17 subjects with cervical carcinoma (CxCa), was analyzed for E2, E6 and E7 reactivity by short-term proliferation assay. In only 1 out of 8 CIN III patients HPV16-specific proliferation was observed (Figure 1c), which was associated with the production of both IFNγ and IL-5 (Figure 1d). The remaining 7 patients with high-grade CIN did not show any proliferative reactivity against the E2, E6, or E7 antigens, whereas all 8 patients did respond to the recall antigens. In contrast to the near absence of HPV16-specific responses in high-grade CIN patients, cervical carcinoma patients showed a response frequency that resembled that of the healthy subjects (Figure 1c), with half of the patients showing proliferative responses against E2 (8/16; patient 9 was not tested against E2), and less frequent responses

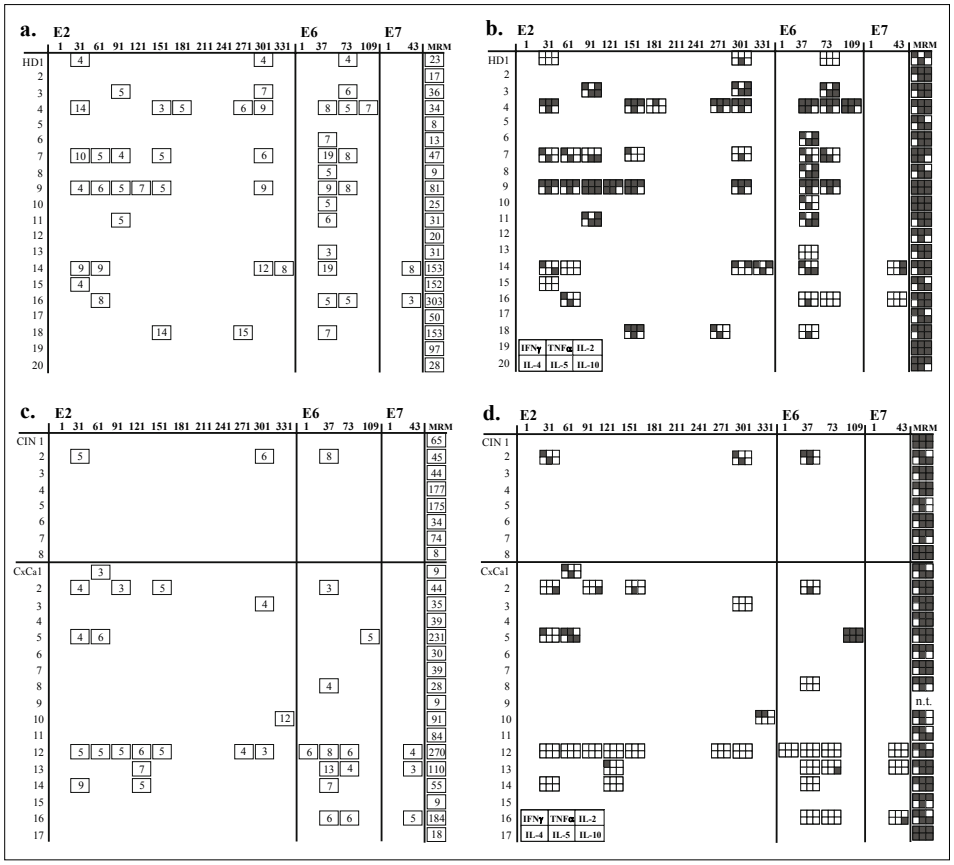


Figure 1.

a. Freshly isolated PBMC from 20 healthy female individuals (HD) were tested in short-term proliferation assays using a complete set of HPV16 E2-, E6- and E7-derived peptide pools (indicated by the first amino acid of the region in the protein covered by the peptides). Responses were scored positive when the proliferation (cpm) of ≥ 6 of 8 test wells exceeded the mean proliferation + 3 x SD of the control (medium only) wells, and the mean stimulation index (SI) of all test wells over control wells was ≥ 3. Memory Response Mix (MRM), consisting of a mixture of recall antigens, was used as a positive control. Indicated are the stimulation indices (SI) of responses scored positive.

b. Supernatants of the positive proliferative responses indicated in (a) were analyzed for the presence of IFNγ, TNFα, IL-2, IL-4, IL-5, and IL-10 by Cytometric Bead Array. The indicated layout is used for the six measured cytokines; a filled square represents antigen-specific cytokine production. Cutoff values were based on the standard curves of the different cytokines (50 pg/ml for IFNγ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and > 2 x the concentration of the medium control.

c. 8 CIN III and 17 cervical cancer (CxCa) patients (all HPV16-positive) were tested in short-term proliferation assays as described in (a).

d. Supernatants of the positive proliferative responses of the patient group were subjected to cytokine analysis as described in (b).
n.t. = not tested

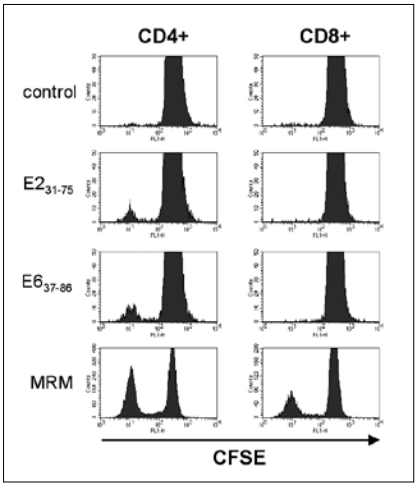
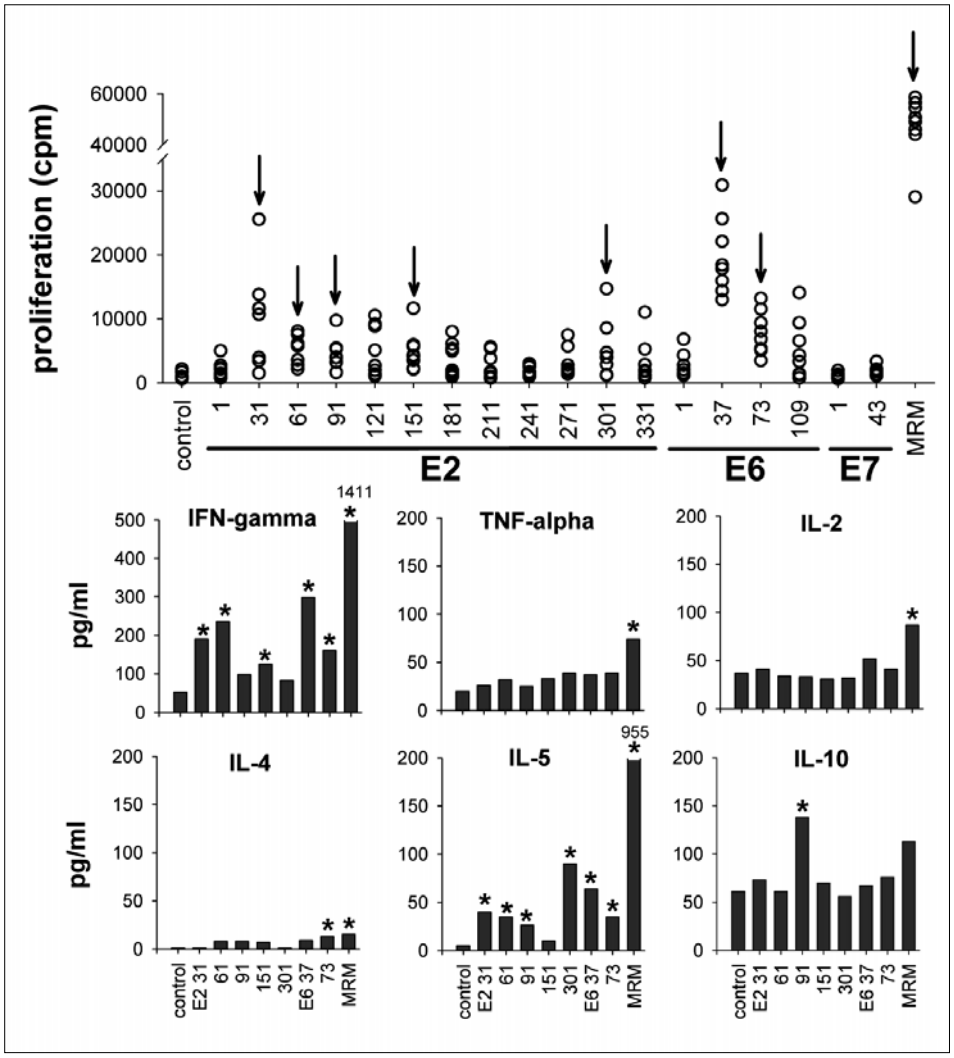


Figure 2. Detection of HPV16-specific T-cell proliferation in the CD4+ fraction of PBMC. Freshly isolated PBMC derived from 4 healthy subjects were labeled with CFSE and incubated with the most immunogenic pools of peptides derived from E2 and E6 ($E2_{31-75}$ and $E6_{37-86}$, respectively) and the mix of recall antigens (MRM). One representative example is shown. At day 6, the cells were counterstained with CD4 and CD8 antibodies, and the division in both T-cell subsets was determined by flow cytometric analysis.

against the E7 antigen (3/17). The number of cervical carcinoma patients showing a proliferative response against HPV16 E6 was lower (7/17) than of healthy individuals (13/20), but this difference was not statistically significant.

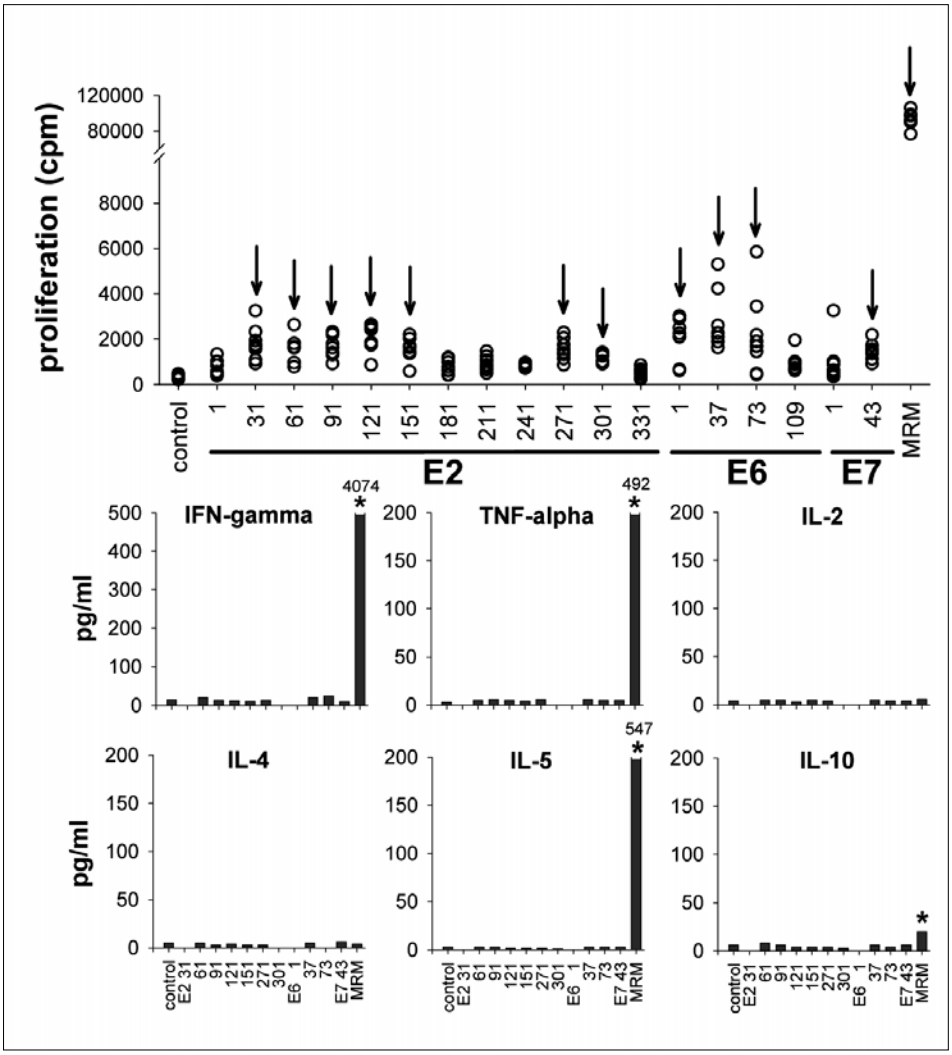
Despite this resemblance of the healthy female group in terms of frequency of proliferative responses against HPV16 antigens, the corresponding cytokine production showed a completely different picture (Figure 1d). In the group of cervical carcinoma patients, only a minor fraction of the proliferative responses was associated with cytokine production. In fact, in most cases, none of the cytokines were detected, despite the occasional broad proliferative response against all three HPV16 antigens (Figure 1d and 4). In a large set of supernatants derived from microcultures lacking antigen-specific proliferation, we were not able to detect specific cytokine production, indicating that selection of supernatant samples based on proliferation does not result in an underestimation of the cytokine responses. Overall, compared with the healthy subjects, the responses against both E2 and E6 in cervical carcinoma patients are characterized by lack of IFNγ and IL-5, as reflected by a significantly reduced fraction of total responses associated with either cytokine (IFNγ: $p<0.05$ IL-5: $p\leq0.001$, Fisher's exact test). The secretion of other cytokines besides IFNγ and IL-5 is also reduced in cervical carcinoma patients, but the antigen-specific secretion of IL-10 appears to be less affected. As a consequence, HPV16 responses associated solely with the immunoregulatory cytokine IL-10 can be observed. HPV16-specific IL-10 production was accompanied by IFNγ and /or IL-5 in 11 out of 12 responses in healthy individuals but in only 2 out of 6 responses in cervical carcinoma patients

Figure 3. HPV16-specific T-cell responses in healthy individuals are predominantly associated with IFNγ and IL-5.



Strong proliferative Th responses against the HPV16 E2- and E6-derived peptides were observed in healthy female-derived PBMC. One representative example (H7) displaying such proliferative responses is depicted in the upper panel. Each open circle represents the proliferation of an individual microculture; supernatants of the positive responses (arrows) were subjected to cytokine analysis. The corresponding cytokine levels are depicted in the lower panels, indicating the predominant secretion of IFNγ and IL-5. Antigen-specific cytokine secretion, as defined in the legend of figure 1, is indicated with an asterisk (*).

Figure 4. Absence of detectable cytokine levels despite HPV16-specific proliferation in cervical cancer patients.



Approximately half of the HPV16-positive cervical cancer patients revealed HPV16-specific proliferation. One representative example is shown (CxCa12, upper panel). HPV16-specific proliferative responses were generally not associated with detectable cytokine production (lower panels), whereas the recall response (MRM) was associated with high levels of IFNγ, TNFα, and IL-5. For explanatory notes, see figure 3 legend.

($p<0.05$, Fisher's exact test). The low number of responses in CIN III patients and low number of E7 responses in all groups precluded these parameters from statistical analysis. Importantly, no significant differences in the magnitude and cytokine profiles of the recall antigen (MRM) responses were observed between cervical carcinoma patients and healthy individuals. The mean IFNγ and IL-5 levels in response to MRM were lower in cervical cancer patients than in healthy women; however, this difference was not significant (Figure 1. IFNγ: healthy subjects mean = 3110 pg/ml range 307-10500 pg/ml, CxCa patients mean = 2229 pg/ml range 150-8250 pg/ml. IL-5: healthy subjects mean = 466 pg/ml range 11-1091 pg/ml, CxCa patients 280 pg/ml 10- 1276 pg/ml, two-tailed t-test). This indicates that the recall antigen-specific cytokine production was not significantly impaired in the patient population, which is in accordance with the fact that these patients had low-stage (mainly FIGO IB) disease.

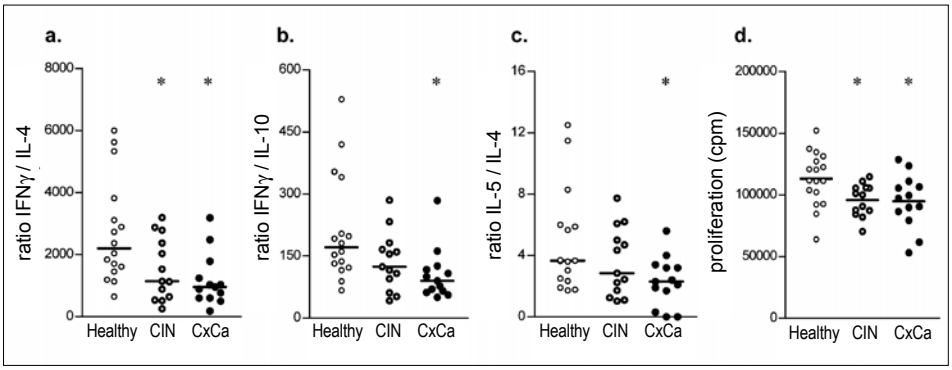
Even though the frequency at which HPV16 E2/E6-specific T-cell immunity was detected in patients and healthy females was roughly similar, it should be noted that all patients carried HPV16+ cervical lesions and therefore approximately half of the HPV16+ cervical carcinoma patients had failed to mount any detectable HPV16-specific Th response. Thus, failure of immune defense against cervical HPV16 infection can embody either HPV16 E2/E6-specific T-cell immunity with an impaired capacity to produce cytokines or a complete lack of such T-cell immunity.

Patients with HPV16+ cervical disease display altered cytokine profile of peripheral T-cells

The local cytokine environment in HPV-induced lesions differs from that observed in healthy tissue[14,16], and it has been suggested that this can influence the overall polarization of peripheral T-cells toward a Th2 type cytokine profile in patients bearing these lesions[17,30,31]. In our study, HPV16-specific CD4+ Th responses in patients with cervical cancer lack the clear Th1/Th2 cytokine profile that is exhibited by such responses in healthy subjects, whereas the cytokine profile of MRM-specific T-cell responses does not show significant differences between healthy and diseased individuals. We questioned whether the overall cytokine profiles of peripheral T-cells, as determined by mitogenic stimulation of PBMC, would reveal a difference between patients with HPV16-induced disease and healthy subjects. Supernatants from PHA-stimulated PBMC were analyzed for the levels of IFNγ, IL-4, IL-5, and IL-10. Per individual, the ratios of the different cytokines were calculated, which reflect the Th1/Th2 polarization of the T-cell repertoire. Cervical carcinoma patients revealed relatively

higher levels of IL-10 and IL-4 as compared with IFNγ, suggesting the loss of Th1 in favor of Th2 cytokines in the peripheral blood of the diseased population (Figure 5a,b; $p<0.01$, unpaired two-tailed t-test with Welch correction). However, the IFNγ/IL-5 ratios did not differ between the groups (data not shown), indicating that the cytokine profiles in cervical carcinoma patients do not represent a general Th2 bias. In line with this, the ratios of the Th2 type cytokines IL-5/IL-4 are also significantly lower in cancer patients (Figure 5c; $p<0.05$). The high-grade CIN patients revealed cytokine profiles similar to that observed in the cervical carcinoma patients, although the difference with the healthy subjects was less pronounced and was only significant for the IFNγ/IL-4 ratios (Figure 5a; $p<0.05$). Overall, the PHA-induced proliferation was slightly lower in both high-grade CIN and carcinoma patients compared with healthy individuals (Figure 5d). Taken together, the mitogen-induced cytokine profiles suggest the presence of an altered cytokine balance in HPV16-induced disease, which may have determined the lack in cytokine polarization of HPV16-specific Th responses found in patients and affected the proliferative capacity of these T-cells.

Figure 5. Patients with HPV16+ cervical disease display altered cytokine profile of peripheral T-cells.



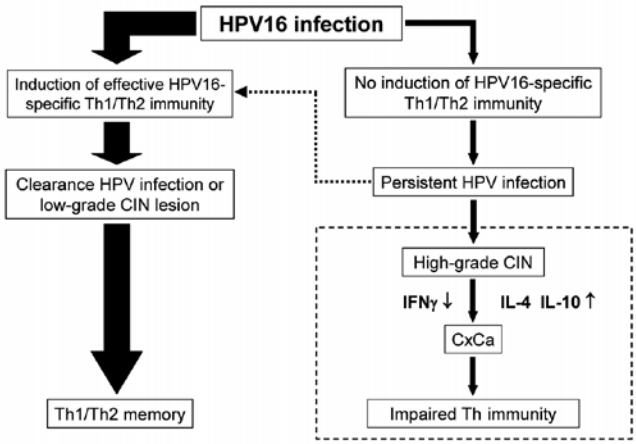
Thawed PBMC derived from healthy female individuals (n=17), CIN III patients (n=13), and cervical carcinoma patients (n=13) were stimulated with PHA (0,5 µg/ml) for 48 hours after which the proliferation was measured by [^{3}H]thymidine incorporation, and the supernatants were analyzed for the presence of IFNγ, IL-4, IL-5 and IL-10 by ELISA. The ranges of measured cytokines were 31-97 ng/ml, 5-109 pg/ml, 0-479 pg/ml, and 50-1390 pg/ml, respectively. No significant differences in the absolute cytokine concentrations were observed between the groups. The cytokine ratios per subject were plotted because these reflect the overall polarization of the T-cell repertoire, independent of the absolute amounts of cytokine secreted. Significant differences between patients and healthy subjects are indicated with an asterisk (*) ($p<0.05$, two-tailed t-test with Welch correction).

Discussion

Our analysis of CD4+ T-cell immunity against the HPV16 E2 and E6 antigens in healthy women and patients with HPV16+ cervical disease revealed that cervical cancer is associated with HPV16-specific immune failure. Of the HPV16+ carcinoma patients tested, approximately half lacked any detectable proliferative E2-, E6- and/or E7-specific T-cell responses. The other half of the carcinoma patients did show systemic proliferative responses, but this immunity was, in general, not associated with a strong inflammatory cytokine profile. Because specific CD4+ T-cell help is of crucial importance for the development of humoral and cellular effector mechanisms against viral infections, these impaired responses against the viral antigens can be considered unfavorable in view of protection against progressive HPV-induced neoplasia. In contrast to cervical cancer patients, healthy women frequently displayed strong CD4+ T-cell responses against HPV16 E2 and E6, and these responses were generally associated with the secretion of inflammatory cytokines, most predominantly IFNγ and IL-5. Our observation that the HPV16-specific memory response in healthy subjects displays a mixed Th1/Th2 cytokine profile is in accordance with the notion that both humoral and cellular immunity are required to clear viral infection and provide subsequent protection against reinfection (Figure 6, left panel). Others have previously observed a lack of E6- and E7-specific IL-2 secretion in PBMC of cervical carcinoma patients and have, on the basis of this observation, suggested that this loss of IL-2, a cytokine indicative of a Th1 type response, might be accompanied by an increased Th2 type response[20]. Our data indicate that the HPV-specific cytokine secretion in the diseased population indeed lacks IL-2 but is not skewed toward Th2 type. Instead, the HPV-specific T-cell response in patients features an overall defect in inflammatory cytokine production.

Compared with the frequency at which HPV16-specific proliferative responses are detected in cervical carcinoma patients, the frequency of such immunity in patients with high-grade CIN lesions is surprisingly low, and this does not correspond with the idea of a gradual loss of HPV16-specific T-cell reactivity during disease progression. A similarly low frequency of HPV16-specific T-cell responses in CIN patients was also described by Nakagawa *et al.*[32]. On the basis of these accumulated data, we hypothesize that failure of the HPV-specific CD4+ T-cell response allows persistent HPV infection and subsequent establishment of high-grade CIN (Figure 6, right panel). Indirect evidence supporting this hypothesis is given by reports showing that low CD4+ T-cell counts in HIV-infected individuals are associated with multiple

Figure 6. Proposed model for the association between HPV16-specific CD4+ T-cell immunity and the development of HPV16-induced disease.



Thick arrows represent the fate of the majority of HPV16-infected individuals; thin arrows represent the fate of the minority of HPV16-infected individuals. The dashed arrow indicates the option that persistently infected individuals with low-grade dysplasia can show spontaneous regression, presumably via the induction of effective Th1/Th2 immunity. The dashed box represents the altered cytokine balance observed in high-grade CIN and cervical carcinoma (CxCa) patients.

HPV infections, higher viral load, viral persistence, and cervical dysplasia[12]. Without surgical intervention, the majority of established high-grade CIN lesions will evolve toward cervical carcinoma. Our data suggest that in approximately half of the cervical carcinoma patients, the presence of the tumor will eventually trigger the induction of a CD4+ T-cell response. It is, however, conceivable that cervical cancers do not provide the appropriate proinflammatory environment for the induction of a potent and well-polarized T-cell response and that CD4+ T-cell priming at this stage of disease will most likely result in an ineffective HPV-specific antitumor immune response. The lack of responses in the remaining patients could reflect either a complete failure at the induction level or could be the result of silencing of preexisting impaired responses by tumor-induced or chronic infection-induced T-regulatory activity. We are currently performing longitudinal studies in cohorts of women to investigate the dynamics of the HPV16 E2/E6-specific CD4+ T-cell response in relation to clearance or persistence of HPV infection.

We frequently detected E2-specific CD4+ T-cell immunity in cervical carcinoma patients, despite the fact that integration of viral DNA into the cellular genome often results in the loss of functional E2 gene expression. This may be explained by the finding that in addition to integrated copies, episomal HPV16 DNA capable of encoding E2, can be found in cervical carcinoma[33,34].

Comparison of the cytokine profiles of mitogen-induced T-cell responses in PBMC from cervical cancer patients versus healthy subjects showed the loss of the Th1 type cytokine IFNγ in favor of the Th2 type cytokines IL-4 and IL-10 in patients. Others have previously described a similar finding in patients with extensive CIN lesions compared with healthy controls[17]. Although cross-sectional analysis does not allow definite conclusions with respect to the order of events, it is likely that this altered systemic cytokine balance is a consequence of HPV-induced disease because the local cytokine environment in HPV-induced lesions is directly and indirectly (via antigen-presenting cells, APC) capable of modifying cytokine profiles of T-cells[15,16,30,31]. Notably, we did not observe significant differences between patients and controls in the cytokine profile of recall (MRM)-specific T-cell responses. The discrepancy between the character of MRM-specific responses on one hand and mitogen-triggered and HPV-specific responses on the other can most readily be explained by the fact that MRM-specific T-cell memory was established and therefore properly polarized before the development of cervical neoplasia. These strongly polarized MRM-specific memory T-cells are, in contrast to naïve T-cells, relatively insensitive to modified APC function[35,36], such as that expected to be found in cervical cancer patients[37]. Mitogen-induced cytokine secretion, on the other hand, reflects the cytokine production of all peripheral T-cells, including that of naïve T-cells and those that have been primed against other pathogens during this period of HPV-induced disease.

In conclusion, our data demonstrate an association between HPV16+ cervical disease and partial or complete failure of CD4+ T-cell function in E2- and E6-specific immunity. The sophistically designed infection cycle of HPV16, which does not involve destruction of virus-infected keratinocytes and avoids proinflammatory signals that could stimulate recruitment and activation of APC, necessitates the role of CD4+ T-cell help in the process of anti-HPV immunity. In view of this consideration, our findings offer a possible explanation for the development of cervical disease.

Acknowledgments

We thank all healthy blood donors and patients participating in the CIRCLE study. We also thank Drs. D.L. Roelen and M. Griffioen for critical reading of the manuscript, W. Benckhuijsen for peptide synthesis, and Prof. J. Dillner for reanalysis of serum samples for anti-HPV16 IgG antibodies.

References

1. Ho GY, Bierman R, Beardsley L, et al. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998; 338: 423-428.
2. Schiffman M and Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. J Natl Cancer Inst Monogr 2003; 14-19.
3. Sellors JW, Karwalajtys TL, Kaczorowski J, et al. Incidence, clearance and predictors of human papillomavirus infection in women. CMAJ 2003; 168: 421-425.
4. Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis 1995; 171: 1026-1030.
5. Ronco LV, Karpova AY, Vidal M, et al. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev 1998; 12: 2061-2072.
6. Park JS, Kim EJ, Kwon HJ, et al. Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the E7-mediated immune evasion mechanism in cervical carcinogenesis. J Biol Chem 2000; 275: 6764-6769.
7. Chang YE and Laimins LA. Microarray analysis identifies interferon-inducible genes and Stat-1 as major transcriptional targets of human papillomavirus type 31. J Virol 2000; 74: 4174-4182.
8. Nees M, Geoghegan JM, Hyman T, et al. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. J Virol 2001; 75: 4283-4296.
9. Fausch SC, Da Silva DM, Rudolf MP, et al. Human papillomavirus virus-like particles do not activate Langerhans cells: a possible immune escape mechanism used by human papillomaviruses. J Immunol 2002; 169: 3242-3249.
10. Frazer IH, Thomas R, Zhou J, et al. Potential strategies utilised by papillomavirus to evade host immunity. Immunol Rev 1999; 168: 131-142.
11. Coleman N, Birley HD, Renton AM, et al. Immunological events in regressing genital warts. Am J Clin Pathol 1994; 102: 768-774.
12. Palefsky JM and Holly EA. Chapter 6: Immunosuppression and co-infection with HIV. J Natl Cancer Inst Monogr 2003; 41-46.
13. Bouwes Bavinck JN and Berkhout RJ. HPV infections and immunosuppression. Clin Dermatol 1997; 15: 427-437.
14. El-Sherif AM, Seth R, Tighe PJ, et al. Quantitative analysis of IL-10 and IFN-gamma mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. J Pathol 2001; 195: 179-185.
15. Mota F, Rayment N, Chong S, et al. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. Clin Exp Immunol 1999; 116: 33-40.
16. Giannini SL, Al-Saleh W, Piron H, et al. Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. Clin Exp Immunol 1998; 113: 183-189.
17. Clerici M, Merola M, Ferrario E, et al. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. J Natl Cancer Inst 1997; 89: 245-250.
18. de Gruijl TD, Bontkes HJ, Stukart MJ, et al. T cell proliferative responses against human papillomavirus type 16 E7 oncoprotein are most prominent in cervical intraepithelial neoplasia patients with a persistent viral infection. J Gen Virol 1996; 77: 2183-2191.
19. Luxton JC, Rowe AJ, Cridland JC, et al. Proliferative T cell responses to the human papillomavirus type 16 E7 protein in women with cervical dysplasia and cervical carcinoma and in healthy individuals. J Gen Virol 1996; 77: 1585-1593.
20. Tsukui T, Hildesheim A, Schiffman MH, et al. Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. Cancer Res 1996; 56: 3967-3974.
21. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: Identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 2001; 91: 612-618.
22. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.
23. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory T-helper cells in the healthy

population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.
24. Bosch FX and de Sanjose S. Chapter 1: Human papillomavirus and cervical cancer--burden and assessment of causality. J Natl Cancer Inst Monogr 2003; 3-13.
25. Claas EC, Melchers WJ, van der Linden HC, et al. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. Am J Pathol 1989; 135: 703-709.
26. van der Burg S, Kwappenberg KM, Geluk A, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. J Immunol 1999; 162: 152-160.
27. Kirnbauer R, Hubbert NL, Wheeler CM, et al. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J Natl Cancer Inst 1994; 86: 494-499.
28. Sasagawa T, Rose RC, Azar KK, et al. Mucosal immunoglobulin-A and -G responses to oncogenic human papilloma virus capsids. Int J Cancer 2003; 104: 328-335.
29. van der Burg SH, Menon AG, Redeker A, et al. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. Int J Cancer 2003; 107: 425-433.
30. Sheu BC, Lin RH, Lien HC, et al. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. J Immunol 2001; 167: 2972-2978.
31. Bonagura VR, Hatam L, DeVoti J, et al. Recurrent respiratory papillomatosis: altered CD8(+) T-cell subsets and T(H)1/T(H)2 cytokine imbalance. Clin Immunol 1999; 93: 302-311.
32. Nakagawa M, Stites DP, Farhat S, et al. T-cell proliferative response to human papillomavirus type 16 peptides: relationship to cervical intraepithelial neoplasia. Clin Diagn Lab Immunol 1996; 3: 205-210.
33. Maitland NJ, Conway S, Wilkinson NS, et al. Expression patterns of the human papillomavirus type 16 transcription factor E2 in low- and high-grade cervical intraepithelial neoplasia. J Pathol 1998; 186: 275-280.
34. Badaracco G, Venuti A, Sedati A, et al. HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. J Med Virol 2002; 67: 574-582.
35. Jonuleit H, Schmitt E, Steinbrink K, et al. Dendritic cells as a tool to induce anergic and regulatory T cells. Trends Immunol 2001; 22: 394-400.
36. Mahnke K, Schmitt E, Bonifaz L, et al. Immature, but not inactive: the tolerogenic function of immature dendritic cells. Immunol Cell Biol 2002; 80: 477-483.
37. Sombroek CC, Stam AG, Masterson AJ, et al. Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. J Immunol 2002; 168: 4333-4343.

CHAPTER 3

Distinct regulation and impact of type 1 T-cell immunity against HPV16 L1, E2 and E6 antigens during HPV16-induced cervical infection and neoplasia

Mariëtte I.E. van Poelgeest
Esther R. Nijhuis
Kitty M.C. Kwappenberg
Ineke E. Hamming
Jan Wouter Drijfhout
Gert Jan Fleuren
Ate G.J. van der Zee
Cornelis J.M. Melief
Gemma G. Kenter
Hans W. Nijman
Rienk Offringa
Sjoerd H. van der Burg

International Journal of Cancer 2006; 118: 675-683

Abstract

Cervical cancer is the possible outcome of a genital infection with high-risk human papillomavirus type 16 (HPV16) and is preceded by a phase of persistent HPV infection during which the host immune system fails to eliminate the virus. Our previous work showed that failure is reflected by the absence of type 1 T-cell immunity against HPV16 early antigens E2 and E6 in patients with HPV16+ cervical lesions. We now show that a majority of both patients with cervical lesions and healthy subjects display HPV16 L1 peptide-specific type 1 T-cell responses with similar magnitude. The T-cell response in patients was directed at a broad range of peptides within L1, suggesting that during persistent or repeated exposure to HPV16 L1, the immune system maximizes its efforts to counter the viral challenge. Unlike the type 1 T-cell responses against HPV16 early antigens E2 and E6, type 1 T-cell immunity against L1 does not correlate with health or disease. This argues that T-cell responses against early and late HPV16 antigens essentially differ in the manner in which they are induced and regulated, as well as in their impact on the subsequent stages of HPV16-induced cervical disease.

Introduction

Infection of both men and women with oncogenic human papillomavirus (HPV) types, such as HPV type 16 (HPV16), is quite common[1-3] and leads to progressive disease in only a minor fraction of infected subjects[4-6]. The majority of the infections and HPV-induced epithelial lesions spontaneously resolve, most likely through intervention by the adaptive immune response[7-9]. In a majority of healthy subjects (approximately 60%) strong type 1 T-cell reactivity directed at the nonstructural proteins HPV16 E2 and E6 can be detected[10-12], suggesting that these proteins are important target antigens for a protective immune response in humans, similar to what was found in animal models[13-15]. These animal models also revealed that L1-specific immunity can protect against infection with the cottontail rabbit papilloma virus[16] or the canine oral papilloma virus[17-19]. A large HPV16 L1-VLP vaccination trial in humans revealed that vaccine-induced immunity could prevent persistent HPV16 infections[20]. Although the immunologic evaluation of this trial was focused on the role of neutralizing antibodies in preventing viral infection, VLP vaccination must also have affected the underlying CD4+ Th responses and possibly Th-dependent cell-mediated effector functions.

Previous work has shown that patients diagnosed with HPV16+ CIN display proliferative responses against HPV16 L1[21,22] and that there was no difference in such proliferative responses (measured by IL-2 production) between patients who cleared their lesions or in whom lesions persisted[23]. Immunity in such subjects may differ not so much in the quantitative aspects of the immune response but more in the quality of the response, as is indicated by our studies on immunity against the HVP16 nonstructural antigens. Although we found patients with cervical cancer to display HPV16 E2 and/or E6-specific proliferation, this reactivity was not associated with the production of the type 1 cytokine IFNγ typical for the HPV16-specific response in healthy subjects[11]. To gain a better understanding of the role of HPV16 L1-specific T-cell response, we analyzed the L1 peptide-specific type 1 T-cell immunity in adult healthy subjects, patients with HPV16+ CIN II/III and patients with HPV16+ cervical cancer by IFNγ ELISPOT. In addition, we studied the HPV16 L1-specific T-cell response in 10-15-year-old females who have had no previous experience of penetrative sex, hence referred to as virgin female subjects. This sample group participated in an L1-VLP vaccination study, a setting that provided us with a unique opportunity to analyse L1-specific T-cell cross-reactivity between common HPV types (skin and low-risk) and HPV16, as well as to compare vaccine-induced T-cell reactivity with that induced upon infection.

Material and methods

Subjects

PBMCs were isolated from buffy coats of anonymous healthy blood bank donors after informed consent. Because these donors were anonymous, no data on medical history were available. There was no serum available to determine the HPV infection status by serological analysis. However, donors with a known recent history of infection, including abnormal pap smear were, as part of normal regulations, discouraged to donate blood.

Subjects with histologically proven cervical carcinoma or CIN were nested in the CIRCLE study, which investigates cellular immunity against HPV16- positive cervical lesions. The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center. Blood samples were collected on the day of treatment before surgery, and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on the same day. Cells were cryopreserved in liquid nitrogen until further use. Carcinoma subjects were staged FIGO IB-IIB and were treated by radical hysterectomy or chemoradiation. The patients with CIN were treated by loop electrosurgical excision procedure (LEEP) or by conization. The subjects were typed for HPV16 using HPV16-specific primers on DNA isolated from paraffin-embedded sections of biopsies or surgical resection specimens[24]. For the ELISPOT-analyses, only HPV16-positive subjects were included. The age of the cervical cancer patients (n=10) ranged from 27-57 years old (median 37 years), and the age of the CIN II/III patients (n=10) ranged from 25-70 years (median 36 years). For the clinical characteristics of the patients, see Table 1.

In addition, PBMC and serum samples from 15 healthy virgin female subjects (aged 10-15 years, median 12 years) were collected to determine vaccine-induced immunologic responses against HPV16 L1. These subjects participated in a phase III trial to examine the safety and immunogenicity of 3 injections of different doses of a L1-VLP vaccine consisting of HPV6, 11, 16 and 18 VLPs mixed with alumn adjuvant (Merck Research Laboratories, West Point, PA, USA). These subjects were enrolled at the Groningen University Medical Center (The Netherlands), and the protocol for this study was approved by the Central and Local Medical Ethical Committees. Subjects received vaccinations, injected i.m. into the deltoid muscle, at 0, 2 and 6 months. Blood specimens were collected for immunologic assays before the initial vaccination (i.e. at month 0) and at months 3 and 7 (1 month after the second and 1 month after the last vaccination, respectively) and PBMC and serum samples were stored in liquid nitrogen until further use.

Table 1. Patient characteristics

patient no	age (yrs)	diagnosis	grade/stage	treatment
1	35	CIN	II	LEEP
2	34	CIN	II	LEEP
3	33	CIN	II	LEEP
4	41	CIN	III	conization
5	25	CIN	II	LEEP
6	28	CIN	II	LEEP
7	29	CIN	III	LEEP
8	30	CIN	III	conization
9	70	CIN	III	hysterectomy[1]
10	31	CIN	III	conization
11	46	SCC	IB1	radical hysterectomy
12	32	SCC	IB1	radical hysterectomy
13	44	SCC	IB1	radical hysterectomy
14	43	AC	IB1	radical hysterectomy
15	45	SCC	IIB	chemoradiation
16	27	SCC	IB1	radical hysterectomy
17	36	SCC	IIB	chemoradiation
18	40	SCC	IB1	radical hysterectomy
19	45	SCC	IB1	radical hysterectomy
20	57	SCC	IB1	radical hysterectomy

CIN, cervical intraepithelial neoplasia; LEEP, loop electrosurgical excision procedure; SCC, squamous cell carcinoma; AC, adenocarcinoma. [1] hysterectomy performed because of patient's age and suspected malignancy.

Antigens

A set of peptides spanning the whole HPV16 L1, E2 and E6 protein were used for the ELISPOT assays. The L1 peptides consisted of 33 30-mer peptides with 15 amino acids overlap and the C-terminal peptide with 20 amino acids overlap. The length of the E2 and E6 peptides was 30 and 22 amino acids, respectively, with an overlap of 15 and 12 amino acids. The peptides were synthesized and dissolved as described previously[25].

Analysis of HPV16 L1, E2 and E6-specific T-cell reactivity by IFNγ ELISPOT

Interferon-γ (IFNγ) producing HPV-specific T-cells (CD4+ and/or CD8+) were quantified using ELISPOT that was performed as described previously[26,27]. Briefly, PBMC were thawed, washed and seeded at a density of 1-2 x 10^6 cells per well of a 24-well plate (Costar, Cambridge, MA) in 1 ml of IMDM (Bio Whittaker, Verviers, Belgium)

enriched with 10% human AB serum, in the presence or absence of indicated HPV 16 L1, E2, E6 and E7 peptide pools. As a positive control, PBMC were cultured in the presence of a memory recall mix (MRM), consisting of a mixture of tetanus toxoid (0.75 *limus flocculentius*/ml final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 µg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and *Candida albicans* (0.005%, HAL Allergenen Lab, Haarlem, The Netherlands). HPV16 peptides were used in pools of 4-5 peptides at a concentration of 5 µg/ml/peptide. The peptides, as indicated by their first and last amino acid in the protein, were used in the following pools: *E2-I*: 1-30, 16-45, 31-60, 46-75; *E2-II*: 61-90, 76-105, 91-120, 106-135; *E2-III*: 121-150, 136-165, 151-180, 166-195; *E2-IV*: 181-210, 196-225, 211-240, 226-255; *E2-V*: 241-270, 256-285, 271-300, 286-315; *E2-VI*: 286-315, 301-330, 316-345, 331-365; *E6-I*: 1-22, 11-32, 21-42, 31-52; *E6-II*: 41-62, 51-72, 61-82, 71-92; *E6-III*: 81-102, 91-112, 101-122, 111-132; *E6-IV*: 111-132, 121-142, 131-152, 137-158. For our first analysis of HPV16 L1-specific immunity in healthy donors, we used 17 pools of 2 peptides per pool. The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the 2 peptides (*e.g.,* $L1_{1\text{-}45}$, residues 1-30 + 16-45). For our analysis in healthy donors compared to HPV16-positive patients, we used 7 pools of 4 peptides, and the last pool consisted of 5 peptides. In addition, for analysis of the PBMC of the vaccinated virgin female subjects, 3 pools of 11 peptides covering the L1 protein were used to decrease the required number of cells. After 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in 4 replicate wells at a density of 10^5 cells per well in 100 µl IMDM enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ-catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT were performed according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (Bio Sys). Specific spots were calculated by subtracting the mean number of spots + 2 x SD of the medium-only control from the mean number of spots in experimental wells. Based on our previous reports, antigen-specific T-cell frequencies were considered to be increased compared to non-responders when specific T-cell frequencies were ≥ 1/10,000[27,28].

HPV16 VLP ELISA

For the detection of HPV16-specific antibodies in serum, we used an ELISA method previously described by Kirnbauer *et al*[29]. Each serum sample was diluted 30 times and

tested for reactivity against HPV16 virus-like particles (VLP, baculovirus-expressed capsids comprising the L1 protein) and against bovine papillomavirus (BPV) capsids, the latter disrupted by treatment with 0.1 M carbonate buffer to serve as a negative control. Both VLP and BPV were kindly provided by Prof. Dr. J. Dillner (LUNDS University, Sweden). The patients (all proven HPV16-positive by PCR) and vaccinated virgin female subjects were tested for both HPV16-specific IgG and IgA.
The induction of HPV16 L1-VLP-specific antibodies was analysed by ELISA in the sera of all 15 vaccinated virgin female subjects prevaccination and after all vaccinations. In addition, a set of sera of healthy children (n=8, mean age 7.3 years, range 4.3-14.1 years) was tested to determine background reactivity. All sera were diluted 30 times. For HPV16 L1-VLP IgG-type responses a cutoff OD value of 0.230 was used (mean OD=0.060; range –0.056 to 0.150; mean + 2 x standard deviation =0.230). For IgA-type responses, a cutoff of OD=0.178 was used (mean OD=0.043; range –0.062 to 0.128).

Homology search in protein database
The search for sequence homology of the overlapping HPV16 L1 peptides with other HPV types was performed in a protein database (SwissProt) using standard Basic Local Alignment Tool (BLAST)[30]. Included were those HPV types that are most prevalent in healthy women without HPV-associated cervical disease according to 2 large cohort studies[31,32] (high- risk types 16, 18, 26, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82, and low-risk types 6, 11, 40, 42, 43, 44, 54, 55, 57, 61, 70, 72, 81, and 83) as well as the common wart types (2, 3, 4, 7, 10, 27, 28, 29). To identify the major regions that could give rise to cross-reactivity, only reported matches with >70% amino acid homology with HPV16 L1 peptides were included.

Statistics
The strength of spontaneous and vaccine-induced L1-specific T-cell response was compared by entering all of the positive L1 responses found in healthy donors (Table 3) versus the responses found in CIN and cervical cancer patients (Table 3). The spread of the T-cell response in the healthy individuals versus the HPV16+ patients was calculated using the 2-sided Fisher's exact test. Statistical analyses were performed using Graphpad Instat Software.

Table 2. HPV16 L1 and E6-specific T-cell responses in healthy donors

Donor[a]	L1 peptides																	E6 peptides				MRM
	1[b] 45	31 75	61 105	91 135	121 165	151 195	181 225	211 255	241 285	271 315	301 345	331 375	361 405	391 435	421 465	451 495	466 505	1 52	41 92	81 132	111 158	
1	-[c]	-	-	-	-	-	**-**	**68**	-	-	**25**	-	-	**-**	-	**-**	-	-	-	-	-	**251**
2	-	-	-	-	-	-	-	*2*	-	*2*	**12**	**-**	**-**	*4*	-	**-**	**-**	-	**-**	-	-	**221**
3	-	-	-	**12**	**-**	**-**	-	**10**	*9*	-	**17**	**32**	*1*	**-**	-	**-**	**-**	**-**	**-**	-	**128**	**254**
4	**18**	-	-	-	-	**-**	-	-	-	-	**22**	*3*	-	**-**	-	-	-	**-**	-	-	-	**149**
5	*3*	**-**	-	*6*	-	*2*	-	-	-	-	**24**	-	*3*	*1*	*2*	*5*	-	*2*	*3*	*4*	**14**	**96**
6	-	**-**	-	-	-	-	**-**	-	-	**-**	-	**-**	-	-	-	**-**	**-**	-	-	-	-	**127**
7	*3*	*3*	*2*	*5*	-	-	**10**	-	*5*	**29**	*5*	*3*	*3*	*3*	*2*	-	-	-	-	-	-	**147**
8	-	*4*	*6*	*3*	-	-	*9*	-	-	**29**	**26**	*3*	*5*	**-**	-	*3*	-	-	-	*3*	**26**	**251**
9	-	-	-	-	-	-	-	-	-	-	-	-	*7*	*2*	-	-	**-**	-	**-**	-	-	**185**
10	-	*2*	*2*	*2*	-	*2*	-	-	*2*	-	-	-	-	**-**	*1*	-	*2*	*1*	-	*4*	**27**	**52**
Total[d]	**1**	**-**	**-**	**1**	**-**	**-**	**1**	**2**	**-**	**2**	**6**	**1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**4**	**10**

[a] PBMC from 10 healthy donors were tested for type 1 T-cell reactivity against HPV16 L1. PBMC were stimulated with different pools of HPV16 L1 and E6 peptides and tested for antigen-specific IFNγ production by ELISPOT. [b] Indicated are the first and last amino acid in the indicated protein of the peptide pool used. [c] Specific responses were calculated by subtracting the mean number of spots + 2 x SD of the medium control from the mean number of spots of experimental wells. The number of specific spots per 100,000 PBMC are given. Based on our previous reports, responses were considered positive if peptide pool-specific T-cell frequencies were ≥ 10/100,000 PBMC[27,28]. These values are indicated in bold. Values below this threshold are shown in italics. (–), no specific response to E6 or L1. MRM, memory recall mix was used as a positive control. [d] The total number of subjects responding to the indicated antigen are depicted.

Results

HPV16 L1-specific T-cell responses in healthy donors

So far, the HPV16 L1-specific T-cell response has been analysed by measuring the proliferative capacity of PBMC in the presence of either L1-VLP or limited sets of synthetic L1-derived peptides[21-23,33,34]. To obtain more detailed information about the magnitude, specificity and functionality of T-cell responses against HPV16 L1, we incubated PBMC isolated from the blood of a group of 20 healthy donors with an overlapping set of 30-mer peptides covering the entire HPV16 L1 sequence and tested T-cell reactivity by IFNγ ELISPOT assay. In addition, PBMC were incubated with HPV16 E6 peptides to relate the reactivity against L1 with our previous findings for early antigens. In a first group of 10 healthy individuals, HPV16 L1 peptide-specific T-cell responses against one or more of the HPV16 L1 peptides were detected in 7 of 10 healthy donors (Table 2). These data suggested that in healthy subjects T-cell reactivity against HPV16 L1 was observed even more commonly than reactivity against HPV16 E6 (4 of 10 subjects in this case). Interestingly, in 6 of 7 cases, a reaction was found to one particular region of HPV16 L1 (Table 2), indicating that these peptides must harbour one ore more particularly immunogenic epitopes.

We then analysed HPV16-specific T-cell immunity in a second group of 10 healthy individuals and compared the L1-peptide specific response with immunity against both HPV16 E2 and E6. To allow a full comparison with the reactivity found in the blood of patients with HPV16-associated cervical neoplasia, of which the number of isolated PBMC is limited, we used L1-peptide pools consisting of 4 peptides per pool. This analysis confirmed our results, in that again 7 of the 10 donors responded to one or more of the L1-peptide pools and 4 of 10 against E2 and/or E6 (Table 3). Taken together, T-cell reactivity against HPV16 L1 was detected in about 70% (14 of 20) of all healthy subjects. The most frequently recognized region of L1 was located between amino acids 301 and 435. The frequency of healthy subjects responding to E2 and/or E6 (8 of 20, Tables 2 and 3), which is comparable to what we have found in our previous studies[10-12], is somewhat lower than that against HPV16 L1, suggesting that not only E2 and E6 but also HPV16 L1 forms a target for the immune system during a transient infection.

HPV16 L1-specific T-cell immunity in patients with HPV16-positive cervical lesions

Our previous analyses of HPV16 E2- and E6-specific T-cell immunity in cervical cancer patients showed that, although antigen-specific proliferation could still be detected, the capacity of such T-cells to produce type 1 cytokines (IFNγ) was generally lost[11]. Others

Table 3. HPV16-specific T-cell responses in healthy individuals and HPV16+ patients

Donor[a]	E2 peptides						E6 peptides			
	1 75	61 135	121 195	181 255	241 315	286 365	1 52	41 92	81 132	111 158
11	-	-	**16**	-	*1*	**13**	*2*	**21**	*3*	*6*
12	-	*8*	**13**	-	-	-	-	**12**	*2*	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	*4*	*6*	*4*	*6*	-
15	-	-	**17**	-	-	-	*4*	*7*	**10**	-
16	*4*	-	*5*	-	*2*	-	*2*	-	*1*	-
17	**69**	**47**	-	-	-	*2*	-	-	-	-
18	-	-	*1*	-	-	*3*	-	*2*	-	-
19	-	-	-	*4*	-	-	-	-	-	-
20	-	*4*	-	-	-	*3*	*3*	-	-	-
Total	**1**	**1**	**3**	**-**	**-**	**1**	**-**	**2**	**1**	**-**
Patient										
CIN 1	-	-	-	-	-	-	-	-	-	-
CIN 2	-	-	-	-	*4*	-	-	-	-	-
CIN 3	*6*	*7*	-	-	*2*	*6*	-	-	**51**	**20**
CIN 4	-	**10**	*9*	*1*	**26**	**19**	**34**	-	*3*	-
CIN 5	-	*1*	*2*	-	-	-	-	-	*2*	-
CIN 6	*3*	*2*	-	-	*1*	*5*	-	*8*	-	-
CIN 7	-	-	-	-	-	-	*6*	**19**	*3*	-
CIN 8	-	-	-	-	-	*6*	*2*	*3*	-	-
CIN 9	*2*	-	-	-	-	*2*	-	**14**	*2*	**21**
CIN 10	-	-	*2*	-	-	-	-	-	*4*	*7*
Ca 11	-	-	-	-	*4*	-	-	-	-	-
Ca 12	-	*1*	*2*	-	*3*	*5*	-	*1*	-	*5*
Ca 13	-	-	-	-	**21**	**28**	*4*	-	*5*	*3*
Ca 14	-	-	-	-	*1*	**11**	-	-	*2*	-
Ca 15	-	-	-	-	-	-	-	-	-	-
Ca 16	-	-	-	-	-	-	-	-	-	-
Ca 17	-	-	-	-	-	-	-	-	-	-
Ca 18	*7*	-	*2*	-	*7*	*7*	-	-	-	-
Ca 19	-	-	-	-	-	-	*4*	-	-	-
Ca 20	-	*1*	*1*	-	-	-	-	-	*6*	-
Total	**-**	**1**	**-**	**-**	**2**	**3**	**1**	**2**	**1**	**2**

[a] For explanatory notes, see Table 2

[b] Sera of the HPV16+ patients were tested for the presence of HPV16 L1-VLP-specific IgG antibodies. Indicated is the presence (+) or absence (-) of antibodies. An empty box means not tested.

L1 peptides								MRM	VLP[b]
1 75	61 135	121 195	181 255	241 315	301 375	361 435	421 505		
6	*5*	*9*	*1*	**68**	**11**	**12**	*5*	**182**	
7	-	-	-	-	**29**	-	-	**57**	
62	-	-	-	-	*6*	**58**	-	**32**	
-	-	*4*	*4*	*4*	*4*	*7*	-	**185**	
-	-	-	-	-	*5*	-	-	**91**	
-	*4*	**15**	*9*	*2*	**18**	**11**	-	**148**	
-	-	**-**	-	*4*	**-**	**28**	-	**85**	
-	-	**-**	-	-	**18**	**-**	-	**43**	
-	-	**-**	-	-	**-**	**-**	-	**156**	
-	-	**13**	-	*3*	*2*	**-**	-	**34**	
1	**-**	**2**	**-**	**1**	**4**	**4**	**-**	**10**	
-	-	-	-	*8*	-	-	-	**89**	-
-	**17**	**54**	**17**	-	-	*2*	-	**231**	-
-	*3*	**-**	**14**	-	*5*	**21**	**35**	**156**	+
8	**10**	**10**	*1*	*5*	*4*	*3*	*9*	*5*	+
-	*9*	-	-	-	-	*1*	-	**42**	+
55	**57**	**76**	**17**	**20**	**45**	**108**	*9*	**144**	+
-	*4*	*3*	-	-	-	**48**	-	**186**	+
-	-	-	*1*	*1*	**29**	-	-	**51**	+
-	**42**	**23**	**59**	**59**	**33**	*1*	**61**	**237**	-
-	-	-	-	-	-	-	-	**32**	-
-	*2*	-	*8*	**159**	**14**	*1*	-	**33**	+
1	*2*	-	*7*	-	**26**	*5*	*3*	**114**	-
-	*6*	**12**	*2*	**13**	**29**	*5*	**10**	**202**	+
3	*4*	-	*2*	**10**	-	**26**	**18**	**207**	-
-	-	-	-	-	*6*	-	-	**90**	-
-	-	-	**12**	-	-	-	-	**28**	-
-	*1*	**23**	**10**	**17**	**15**	**38**	*2*	**10**	
2	**13**	*8*	*2*	**15**	**24**	**17**	*5*	**67**	+
-	1	-	*2*	-	*7*	**28**	*1*	**61**	-
1	-	-	-	**10**	**11**	-	*3*	**29**	-
1	**5**	**6**	**6**	**8**	**9**	**7**	**4**	**19**	

have reported that the majority of patients with high-grade CIN lesions displayed HPV16 L1-specific proliferative responses[22,23], but it is unclear whether these responses were associated with the production of IFNγ. We, thus, examined the type 1 (IFNγ)-associated HPV16 L1 peptide-specific T-cell response in HPV16 DNA-positive CIN III and cervical cancer patients and compared these responses with those found in healthy subjects. A group of 10 subjects with HPV16+ CIN II/III and 10 subjects diagnosed with HPV16+ cervical carcinoma (see Table 1 for patient characteristics) was analysed for reactivity against HPV16 L1, E2 and E6 by IFNγ ELISPOT assays. In accordance with our earlier studies, HPV16 E6-specific T-cell immunity associated with IFNγ production was absent in patients with HPV16+ cervical carcinoma, whereas only 2 patients exhibited a detectable HPV16 E2-specific IFNγ response (Table 3). In patients with high-grade CIN, HPV16 E2 and E6-specific IFNγ responses were found in, respectively, 1 and 4 of the 10 subjects, which was similar to what we found in the healthy control group. In contrast to the low number of IFNγ responders found against these non-structural antigens (6 of 20), HPV16 L1 peptide-specific IFNγ-producing T-cells were detected in the vast majority (16 of 20) of the patients diagnosed with HPV16+ cervical carcinoma (9 of 10) or CIN II/III (6 of 10) (Table 3; $p<0.004$, Fisher's exact test). In 9 of these 20 patients, an IFNγ-associated T-cell response was detected against the region HPV16 $L1_{300-375}$, corroborating previous results on L1 peptide-specific T-cell reactivity by proliferation[22,23]. In contrast to the healthy subjects, in which the T-cell reactivity was primarily focused on the region HPV16 $L1_{300-435}$, HPV16+ patients diagnosed with high-grade CIN or cervical carcinoma exhibited a significantly broader T-cell response against HPV16 L1. In 46 of the 160 different cultures, an L1 peptide-specific T-cell response could be detected, as compared to 12 of 80 L1-cultures in healthy donors (Table 3; $p=0.02$; 2-sided Fisher's exact test), suggesting that a broad T-cell repertoire is activated during a persistent HPV16 infection or repeated exposure to antigen by *de novo* infections.

CD4+ T-helper responses participate in the generation and maintenance of protective B-cell responses, and HPV16 L1 is a known target for antibodies[35]. We, therefore, analysed the sera of the CIN II/III and cervical carcinoma patients for the presence of HPV16 L1-VLP-specific antibodies. Compared to cervical carcinoma patients, more subjects with high-grade CIN had detectable IgG antibodies against HPV16 L1 (6 of 10 and 3 of 9, respectively, Table 3), which is in accordance with the fact that progression of cervical neoplasia is associated with a shutdown of L1 expression, while maintenance of high levels of antibodies benefit from sustained virus production. No circulating IgA antibodies against HPV16 L1 were found in these groups of HPV16+ subjects (data not shown). As expected, patients with detectable HPV16 L1 IgG levels

(8 of 9) generally displayed a concurrent HPV16 L1 peptide-specific T-cell response. Nevertheless, in only half of the patients (8 of 16), L1-specific T-cell reactivity was accompanied by detection of L1-specific IgG, indicating that in half of the patients the presence of antigen-specific T-cell immunity does not suffice to induce and/or sustain systemic HPV16 L1-specific IgG levels.

Spontaneous HPV16 L1 peptide-specific type 1 T-cell immunity is not detected in virgins

Due to the common nature of HPV infections, a majority of the human population is likely to encounter multiple HPV types[2,36]. It is therefore possible that at least a fraction of the T-cell repertoire induced by a previous encounter with HPV types other than HPV16 could cross-react with the HPV16 L1 peptides (Table 4). We had the unique opportunity to analyse the HPV16 L1-specific T-cell response in the PBMC isolated from the blood of virgin female subjects aged 10-15 years who were recruited for a phase III VLP vaccination study. Most (50-70%) of the preadolescents at this age will have been infected with the skin types of HPV[37,38], and a considerable number of them will have encountered common (approximately 40%) and low-risk (approximately 20%) HPV types[38-43]. The small amounts of blood available from these children necessitated the use of 3 large pools of HPV16 L1 peptides, covering amino acids 1-180, 166-345 and 331-505. The use of pools of 11 peptides will result in some loss of specificity with respect to the exact sequences recognized. However, the sensitivity of the assay will be increased because the number of detected spots is the result of multiple L1 peptide-specific T-cells responses. In none of these preadolescents, HPV16 L1 peptide-specific type 1 T-cell reactivity was detected before vaccination by IFNγ ELISPOT (Table 5; T=0), suggesting that T-cell priming against the common and skin types of HPV is not likely to result in a T-cell response with strong cross-reactivity to HPV16. This also suggests that in healthy subjects, detected HPV16 $L1_{301-435}$-specific T-cell responses, a region which shows only limited homology with the skin and low-risk HPV types (Table 4), are not likely to represent T-cell immunity against these skin and low-risk HPV types but are more likely to be specific for HPV16 and/or closely related types.

In addition, the HPV16 L1 peptide-specific T-cell type 1 response was measured in the peripheral blood of these preadolescents following 1 month after the second vaccination (T=3) and 1 month after the last vaccination (T=7) (3 injections; prime-boost-boost). Although in some cases not enough material was available at all time points, all 14 female subjects displayed HPV16 L1 peptide- specific T-cell immunity

Table 4. HPV16 L1 30-mer peptides sharing >70% homology with those of several other HPV types that are prevalent ≥0.1% in women with normal cytological Pap smears[1]

L1 peptides	common/flat warts								low-risk anogenital HPV types[a]							
	2	3	4	7	10	27	28	29	6	11	40	42	43	54	70	72
1[b]	83				93	83	93	93				83[c]		82		
16		93			93		93	93						93		
31	85	86			90	86	86									
46					83	83	76									
61														82		
76						100						100			89	
91																
106												93	86	93		
121												73		76		
136												88		88		
151									83	90						
166									76	83						
181														92		
196					93		93				96					93
211	93					96										
226	90					93								93		
241										89				96		
256																
271																
286												93				
301												100				
316									100							
331						80										
346																
361	93				93		93									
376																
391										90						
406																
421																
436																
451												90		96		
466												83				
476																

[1]The prevalence of mucosotropic HPV types were calculated in healthy subjects using 2 published large cohort studies in which HPV was typed on a cytologic normal Pap smear taken from control women in the Netherlands[31]; n=3305 and from control women in Africa, South America, Asia and Spain[32]; n=1928.
The amino acid sequences of the HPV16 L1 peptides were aligned with the sequences of all HPV types that displayed a prevalence ≥0.1% in at least one of the two studies.

high-risk anogenital HPV types											
18	31	33	34	35	39	45	51	52	58	59	66
	93	96	83	86				96			
	93	100		93				96			
		86	86	93							
		83	80	90				75			
	96	96	93	96				96			82
86	100	100	89	89				96			
	100	100			100						
	93			93				90			
	80			78				80			
	86	96		93				90			
	96	86		100				96			
	90							90			
	96			100		96	100	96			90
		93		100				93			
	83	89		100				96			
	100	93		96				96			
	96	96	100	96				96			
	93	93	93	85							
	90	93	86	80				81			
	96	93		96				96			
	100			96				100			
	100							100			
	96	86		96				83			
	96			96				95			
	100		90	100				100			
96	93			96				93			
				90							
								82			
				83							
	90			96							
92	96	96		96				96			
76	80	76		76							

a. Low-risk and high-risk HPV types that are prevalent in ≥0.1% of women with normal cytological Pap smears[31,32]

b. Indicated is the first amino acid of the 30-mer peptide of the HPV16 L1 protein,

c. Percentage of the HPV16 L1 peptides sharing >70% homology with indicated HPV types. The HPV16 $L1_{301-435}$ region (in border) is most frequently recognized in healthy subjects (Tables 2 and 3).

Table 5. HPV16 L1-VLP-induced T-cell responses in female subjects 10-15 years of age

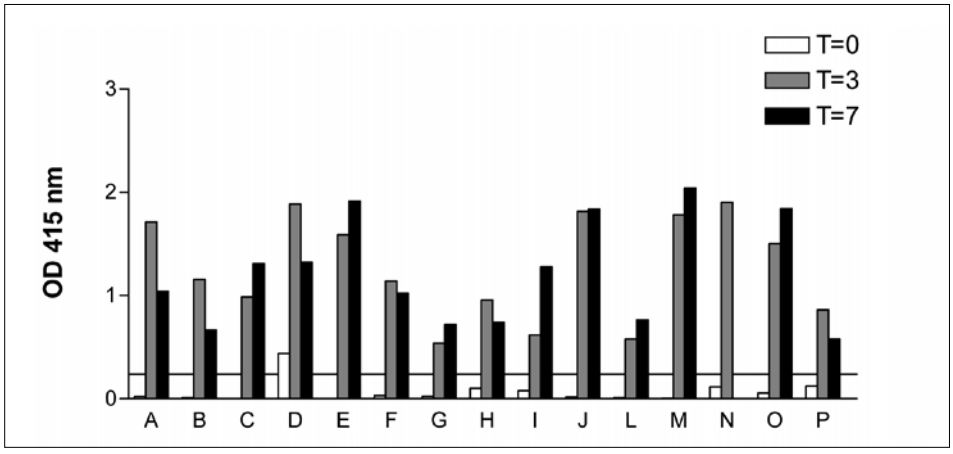
	T=0[b]				T=3				T=7				
Subject[a]	L1 1-180[c]	L1 166-345	L1 331-505	MRM	L1 1-180	L1 166-345	L1 331-505	MRM	L1 1-180	L1 166-345	L1 331-505	MRM	Vaccine induced[d]
A									**16**[c]	*4*	**20**	*1*	?
B	-	-	*3*	**24**	**95**	**147**	**57**	**61**	*7*	-	-	**58**	+
C	-	-	-	**103**					**25**	**77**	*7*	**108**	+
D	-	-	-	**182**	*4*	-	-	**219**	**140**	**94**	**15**	**330**	+
E					**31**	**18**	**53**	**29**	**90**	**97**	**122**	**47**	?
F					**26**	-	-	**103**	**38**	**10**	*5*	**26**	?
G	-	-	-	**61**	**51**	**27**	**20**	**42**	**145**	**33**	**40**	**22**	+
H	-	-	-	**152**	**37**	**23**	**17**	**316**	**138**	**108**	**82**	**347**	+
I	-	-	-	**11**	-	**16**	*5*	**106**	*4*	**88**	**55**	**46**	+
J	*6*	-	-	**173**	**61**	*3*	-	**150**					+
M	-	-	-	**13**	**61**	**11**	-	**36**	-	-	-	**128**	+
N	-	-	-	**329**	**17**	**38**	*7*	**148**					+
O	-	*4*	-	**225**	**93**	**108**	**32**	**139**					+
P					**11**	**124**	**23**	**194**	**25**	**159**	**62**	**108**	?
Total[e]	**-**	**-**	**-**	**10**	**10**	**9**	**6**	**12**	**8**	**8**	**7**	**10**	

a. Fifteen 10-15-year-old female subjects were vaccinated 3 times with a L1-VLP combination vaccine consisting of HPV6, 11, 16 and 18 VLPs.
b. PBMC were isolated before (T=0) and 1 month following the second (T=3) and 1 month following the last vaccination (T=7).
c. For explanatory notes, see Table 2. A blank field indicates that not enough material was available to perform this analysis.
d. A T-cell response after vaccination of at least 3-fold the prevaccination response was considered to be vaccine enhanced. (+) A definitive vaccine-induced response, (?) A potential vaccine-induced response.
e. The total number of subjects responding to the indicated antigen are depicted.

after vaccination. In 10 cases, we could establish that these responses were vaccine-induced (B, C, D, G, H, I, J, M, N, and O, Table 5). It is likely that the HPV16 L1 peptide-specific T-cell responses detected in the other 4 subjects (A, E, F, P) were also vaccine-induced, but the absence of prevaccination samples in these cases precludes a definite conclusion. Except for one case, all subjects responded to at least 2 of the 3 L1-peptide pools and half of the subjects tested responded to all 3 L1-peptide pools. This shows that a repeated exposure to sufficient quantities of virus-like particles results in a strong and broad HPV16 L1 peptide-specific T-cell response, a feature reminiscent of that found for L1-specific immunity in HPV16+ patients (Table 3). In 7 of 9 subjects from whom both postvaccination samples were available, systemic immunity against HPV16 L1 peaked after completion of all vaccinations (T=7) rather than after 2 vaccinations (T=3) (Table 5). In the 2 other subjects, the induced L1-specific type 1 immune response was apparently transient since L1-peptide specific IFNγ production could only be detected after 2 vaccinations.

In all cases, vaccination resulted in induction of clear-cut anti-HPV16 L1 VLP IgG levels in the serum (Figure 1). A low preexisting HPV16 L1-VLP-specific IgG response was observed in 1 subject (D), all the other subjects were negative. The serologic responses remained stable over at least the study period (Figure 1). No serum HPV16 L1-VLP-specific IgA was detected (data not shown).

Figure 1. Strong serologic responses after vaccination with VLP vaccine.



HPV16 L1 IgG antibodies in serum from 15 vaccinated female subjects prior to vaccination (T=0) and at months 3 and 7 (T=3 and T=7, 1 month after the second and 1 month after the last vaccination, respectively). Serum antibody responses were measured by ELISA. Depicted are the OD values at 415 nm of 30x diluted sera. The cutoff point for the antibody levels (solid line) was based on the OD values from 8 healthy children aged 4-14 years and was calculated as the mean of the OD values + 2 x SD.

Discussion

We have analysed the HPV16 L1 peptide-specific type 1 (IFNγ) T-cell responses in healthy individuals, in patients with HPV16+ cervical neoplasia as well as in a unique group of virgin female subjects (10-15 years) before and after vaccination with a combined L1-virus-like particle (L1-VLP) vaccine of types 6, 11, 16 and 18. The response rate against HPV16 L1 was compared to immunity against 2 other frequently recognized antigens HPV16 E2 and E6. Our data show that HPV16 L1 peptide-specific T-cell immunity occurs in a high frequency in healthy individuals but is absent in virgin female subjects before vaccination, indicating that the observed immunity in healthy control subjects has resulted from exposure to HPV16 or closely related types, most likely through sexual transmission. Accordingly, strong HPV16 L1 peptide-specific T-cell responses were observed in all subjects after VLP vaccination. Analysis of HPV16 L1 peptide-specific immunity in patients with HPV16+ CIN II/III also revealed that the vast majority of these subjects displayed strong type 1 T-cell immunity against this antigen, corroborating the data of others[21-23,34]. In addition, this study shows that strong HPV16 L1 peptide-specific type 1 T-cell immunity is also present in the majority of patients with an HPV16+ cervical carcinoma. These responses were covering a broad range of peptides within L1, suggesting that during persistent or repeated exposure to HPV16 L1, the immune system maximizes its efforts to counter the viral challenge. This broad T-cell reactivity is reminiscent of what we observed for the influenza virus matrix 1-specific T-cell response[12,27].

The frequent detection of type 1 anti-L1 responses in patients contrasts with our previous observation that such patients generally lack such IFNγ-associated T-cell immunity against the HPV16 early antigens E2 and E6 ([11], this study). Thus, although failure of the immune defense does extend to the L1 peptide-specific response in a minority of the patients tested, the presence and character of these IFNγ-associated L1 peptide-specific responses is not a good indicator for efficient protection against HPV16-induced cervical infection. A lack of protective capacity of such L1 peptide-specific T-cell responses at the stage of CIN and cervical carcinoma may very well be attributed to a loss in the active production of L1 in these lesions[44]. Nevertheless, the question remains why the majority of cancer patients have been able to mount a systemic type 1 T-cell response against L1 (7 of 10, Table 3), but failing to do so against the early antigens E2 and E6 (2 of 10, Table 3 and [11]). A possible explanation for this disparity may lie in the fact that the APC required for cross priming of HPV-specific T-cells acquire the early and late HPV antigens from distinct sources. During the productive phase of cervical HPV16 infection, the L1 protein is primarily

contained within the progeny virus particles that are released from the apical layers of keratinocytes. In contrast, E2 and E6 are primarily found in the keratinocytes positioned in the lower half of the epithelium. HPV infection, at least in the beginning, is a subtle process that does not induce keratinocyte death and that does not result in major proinflammatory signals. Consequently, one could envision that this setting does not trigger efficient transfer of antigen from HPV-infected keratinocytes to the APC, leaving free viral particles as the only truly accessible antigen source at this stage. Early antigens, such as E2 and E6, will only become available for cross-presentation at later stages in infection. This scenario implies that presentation of L1 takes place in a setting that is more favorable to induction of systemic type 1 T-cell immunity. This notion is supported by data suggesting that the uptake of HPV16 L1-VLP directly activates dendritic cells[45-47]. Conversely, cross-presentation of the early antigens lack such activation signals and may under the wrong circumstances result in an improperly primed T-cell response[48]. Taken together, our data argue that systemic T-cell responses against HPV16 early (E2, E6) and late (L1) antigens essentially differ in the manner in which they are induced and regulated, as well as in their impact on the subsequent stages of HPV16-induced cervical disease. Furthermore, the presence of L1-specific type 1 immune responses suggests that patients diagnosed with HPV-induced cervical lesions are not profoundly predisposed to an overall type 2 immune response.

A recent analysis of HPV type 11 L1-specific T-cell immunity suggested that a significant fraction of HPV16 L1-specific T-cell responses as detected in our study may be caused by cross-reactivity between skin and anogenital types of HPV, in that T-cell cultures stimulated with HPV11 L1 peptides simultaneously responded to corresponding peptides of HPV 1-4 and HPV16[49]. The cross-reactive peptides are predominantly located at the N-terminal side of HPV16 L1, which indeed shows homology with several low-risk anogenital types (Table 4) as well as skin types (data not shown). Interestingly, the most immunodominant region of HPV16 L1 that is recognized by healthy subjects in our study is located outside this area and shows predominantly homology with HPV types that are members of the HPV-clade A9 (types 16, 31, 33, 35, 52, 58; Table 4) . The total absence of detectable HPV16 L1 peptide-specific T-cell immunity in the group of young virgin female subjects 10-15 years of age, who will have encountered most of the low-risk and skin types of HPV[37-43], further indicates that cross-reactivity at the T-cell level does not readily occur between these common HPV types and the high-risk HPV types. The high conservation of HPV16 L1 within the HPV types of clade A9 suggests that L1-specific T-cell cross-reactivity against

these group members could occur.

Vaccination of young virgin female subjects (10-15 years of age) with a vaccine containing HPV16 L1-VLP resulted in the induction of a strong L1 peptide-specific IFNγ-associated T-cell response and a concomitant B-cell response producing L1-VLP-specific IgG type antibodies. A similar T-cell response was detected in adult female volunteers 18-25 years of age of whom PBMC were stimulated *in vitro* with L1-VLP[50]. Studies should be conducted to understand whether the presence of these preexistent vaccine-induced L1-specific T-cells may assist in establishing a conducive environment that allows a rapid induction and deployment of strong type 1 T-cell responses against the other HPV16 antigens at the time that the vaccinated virgin female subjects become sexually active and encounter a genuine HPV16 infection.

In conclusion, HPV16 L1 peptide-specific type 1 T-cell immunity is induced in human subjects by either natural exposure to transient HPV16 infections, the persistence of this virus in low- or high-grade cervical lesions[44] or by L1-VLP vaccination. Although the presence of L1-specific type 1 T-cell responses may assist in protective immunity, the detection of these T-cells, unlike that of type 1 T-cell immunity against E2 and/or E6, is not indicative of successful control of this virus.

Acknowledgments

We thank all patients who have participated in this study, Prof. J. Dillner for helpful advice on HPV serology and the Merck Research Laboratories for funding the trial that recruited 10-15- year-old virgins evaluated in this study.

References

1. Burk RD, Kelly P, Feldman J, et al. Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. Sex Transm Dis 1996; 23: 333-341.
2. Koutsky L. Epidemiology of genital human papillomavirus infection. Am J Med 1997; 102: 3-8.
3. Schiffman M, Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. J Natl Cancer Inst Monogr 2003; 14-19.
4. Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis 1995; 171: 1026-1030.
5. Ho GY, Bierman R, Beardsley L, et al. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998; 338: 423-428.
6. Woodman CB, Collins S, Winter H, et al. Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. Lancet 2001; 357: 1831-1836.
7. Sun XW, Kuhn L, Ellerbrock TV, et al. Human papillomavirus infection in women infected with the human immunodeficiency virus. N Engl J Med 1997; 337: 1343-1349.
8. Palefsky JM, Minkoff H, Kalish LA, et al. Cervicovaginal human papillomavirus infection in human immunodeficiency virus-1 (HIV)-positive and high-risk HIV-negative women. J Natl Cancer Inst 1999; 91: 226-236.
9. Serraino D, Carrieri P, Pradier C, et al. Risk of invasive cervical cancer among women with, or at risk for, HIV infection. Int J Cancer 1999; 82: 334-337.
10. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.
11. de Jong A, van Poelgeest MI, van der Hulst JM, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004; 64: 5449-5455.
12. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.
13. Han R, Cladel NM, Reed CA, et al. Protection of rabbits from viral challenge by gene gun-based intracutaneous vaccination with a combination of cottontail rabbit papillomavirus E1, E2, E6, and E7 genes. J Virol 1999; 73: 7039-7043.
14. Selvakumar R, Ahmed R, Wettstein FO. Tumor regression is associated with a specific immune response to the E2 protein of cottontail rabbit papillomavirus. Virology 1995; 208: 298-302.
15. Selvakumar R, Borenstein LA, Lin YL, et al. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. J Virol 1995; 69: 602-605.
16. Lin YL, Borenstein LA, Selvakumar R, et al. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. Virology 1992; 187: 612-619.
17. Suzich JA, Ghim SJ, Palmer-Hill FJ, et al. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci USA 1995; 92: 11553-11557.
18. Bell JA, Sundberg JP, Ghim SJ, et al. A formalin-inactivated vaccine protects against mucosal papillomavirus infection: a canine model. Pathobiology 1994; 62: 194-198.
19. Yuan H, Estes PA, Chen Y, et al. Immunization with a pentameric L1 fusion protein protects against papillomavirus infection. J Virol 2001; 75: 7848-7853.
20. Koutsky LA, Ault KA, Wheeler CM, et al. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 2002; 347: 1645-1651.
21. Luxton JC, Nath R, Derias N, et al. Human papillomavirus type 16-specific T cell responses and their association with recurrence of cervical disease following treatment. J Gen Virol 2003; 84: 1063-1070.
22. Shepherd PS, Rowe AJ, Cridland JC, et al. Proliferative T cell responses to human papillomavirus type 16 L1 peptides in patients with cervical dysplasia. J Gen Virol 1996; 77 (Pt 4): 593-602.
23. de Gruijl TD, Bontkes HJ, Walboomers JM, et al. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. I. Differential T-helper and IgG responses in relation to HPV infection and disease outcome. J Gen Virol 1999; 80 (Pt 2): 399-408.
24. Claas EC, Melchers WJ, van der Linden HC, et

al. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. Am J Pathol 1989; 135: 703-709.
25. van der Burg SH, Kwappenberg KM, Geluk A, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. J Immunol 1999; 162: 152-160.
26. de Jong A, O'Neill T, Khan AY, et al. Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T-cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. Vaccine 2002; 20: 3456-3464.
27. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II- restricted epitopes. Int J Cancer 2001; 91: 612-618.
28. Smyth LJ, van Poelgeest MI, Davidson EJ, et al. Immunological responses in women with human papillomavirus type 16 (HPV-16)-associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV-16 oncogene vaccination. Clin Cancer Res 2004; 10: 2954-2961.
29. Kirnbauer R, Hubbert NL, Wheeler CM, et al. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J Natl Cancer Inst 1994; 86: 494-499.
30. Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25: 3389-3402.
31. Jacobs MV, Walboomers JM, Snijders PJ, et al. Distribution of 37 mucosotropic HPV types in women with cytologically normal cervical smears: the age-related patterns for high-risk and low- risk types. Int J Cancer 2000; 87: 221-227.
32. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348: 518-527.
33. Nakagawa M, Stites DP, Farhat S, et al. T-cell proliferative response to human papillomavirus type 16 peptides: relationship to cervical intraepithelial neoplasia. Clin Diagn Lab Immunol 1996; 3: 205-210.
34. Davidson EJ, Sehr P, Faulkner RL, et al. Human papillomavirus type 16 E2- and L1-specific serological and T-cell responses in women with vulval intraepithelial neoplasia. J Gen Virol 2003; 84: 2089-2097.
35. Lowy DR, Frazer IH. Chapter 16: Prophylactic human papillomavirus vaccines. J Natl Cancer Inst Monogr 2003; 111-116.
36. Thomas KK, Hughes JP, Kuypers JM, et al. Concurrent and sequential acquisition of different genital human papillomavirus types. J Infect Dis 2000; 182: 1097-1102.
37. Antonsson A, Karanfilovska S, Lindqvist PG, et al. General acquisition of human papillomavirus infections of skin occurs in early infancy. J Clin Microbiol 2003; 41: 2509-2514.
38. Cubie HA, Plumstead M, Zhang W, et al. Presence of antibodies to human papillomavirus virus-like particles (VLPs) in 11-13-year-old schoolgirls. J Med Virol 1998; 56: 210-216.
39. Syrjanen S, Puranen M. Human papillomavirus infections in children: the potential role of maternal transmission. Crit Rev Oral Biol Med 2000; 11: 259-274.
40. Handley J, Hanks E, Armstrong K, et al. Common association of HPV 2 with anogenital warts in prepubertal children. Pediatr Dermatol 1997; 14(5): 339-343.
41. Powell J, Strauss S, Gray J, Wojnarowska F. Genital carriage of human papilloma virus (HPV) DNA in prepubertal girls with and without vulval disease. Pediatr Dermatol 2003; 20: 191-194.
42. af Geijersstam V, Eklund C, Wang Z, et al. A survey of seroprevalence of human papillomavirus types 16, 18 and 33 among children. Int J Cancer 1999; 80: 489-493.
43. Stevens-Simon C, Nelligan D, Breese P, et al. The prevalence of genital human papillomavirus infections in abused and nonabused preadolescent girls. Pediatrics 2000; 106: 645-649.
44. Melsheimer P, Kaul S, Dobeck S, et al. Immunocytochemical detection of HPV high-risk type L1 capsid proteins in LSIL and HSIL as compared with detection of HPV L1 DNA. Acta Cytol 2003; 47: 124-128.
45. Lenz P, Day PM, Pang YY, et al. Papillomavirus-like particles induce acute activation of dendritic cells. J Immunol 2001; 166: 5346-5355.
46. Fausch SC, Da Silva DM, Kast WM. Differential uptake and cross-presentation of human papillomavirus virus-like particles by dendritic cells and Langerhans cells. Cancer Res 2003; 63: 3478-3482.
47. Yan M, Peng J, Jabbar IA, et al. Despite differences between dendritic cells and Langerhans cells in the mechanism of papillomavirus-like

particle antigen uptake, both cells cross-prime T cells. Virology 2004; 324: 297-310.
48. Offringa R, de Jong A, Toes RE, et al. Interplay between human papillomaviruses and dendritic cells. Curr Top Microbiol Immunol 2003; 276: 215-240.
49. Williams OM, Hart KW, Wang EC, et al. Analysis of CD4(+) T-cell responses to human papillomavirus (HPV) type 11 L1 in healthy adults reveals a high degree of responsiveness and cross-reactivity with other HPV types. J Virol 2002; 76: 7418-7429.
50. Pinto LA, Edwards J, Castle PE, et al. Cellular immune responses to human papillomavirus (HPV)-16 L1 in healthy volunteers immunized with recombinant HPV-16 L1 virus-like particles. J Infect Dis 2003; 188: 327-338.

CHAPTER 4

Human papillomavirus type 16 E2, E6 and E7 peptide-specific skin reactions in health and disease, results of a pilot study

Mariëtte I.E. van Poelgeest
Muriel van den Hende
Jeanette M. van der Hulst
Jan Wouter Drijfhout
Gert Jan Fleuren
Amon Wafelman
Cornelis J.M. Melief
Rienk Offringa
Sjoerd H. van der Burg
Gemma G. Kenter

Submitted

Abstract

Purpose: To validate the use of skin tests as a safe and simple method to detect HPV16 E2, E6 and E7-specific type 1 T-cell responses in humans.
Experimental design: A group of eleven women with cervical neoplasia and 9 healthy individuals were challenged with intracutaneous injections of 8 different pools of HPV16 E2, E6 and E7 peptides. In addition, peripheral blood was isolated to determine the number of circulating HPV16-specific type 1 T-cells by IFNγ ELISPOT analysis.
Results: In our study population, only two classical (<72 hours) delayed-type hypersensitivity (DTH) reactions were detected. However, we found positive skin reactions in the majority of healthy subjects and in a part of the patients 1 to 3 weeks after injection. In healthy subjects, early skin reactions (within 11 days) developed to peptides of which the T-cell frequency exceeded 1/20,000 in our IFNγ ELISPOT analysis while late skin reactions developed in response to peptides for which we were not able to detect Th1 immunity (p=0.001). In vitro expansion of the T-cells present in the positive skin test sites revealed that this population comprised HPV16-specific CD4+ and CD8+ T-cells and that both IFNγ and IL-10 were produced. This suggests that both Th1 and Th2 cells infiltrated the skin test site. In the patients, positive skin reactions were not associated with systemic type 1 T-cell reactivity, suggesting that HPV16-specific T-cells producing cytokines other than IFNγ infiltrated the skin.
Conclusions: Our pilot study did not unequivocally show that HPV16 peptide-induced skin reactions were associated with an HPV16-specific type 1 T-cell response. Further analyses of the type and character of T-cells infiltrating the skin of patients with cervical cancer are needed before larger field trials are initiated.

Introduction

Genital infection with human papillomavirus (HPV) is one of the most common sexually transmitted diseases[1-4]. Persistent infections with one of the high-risk types of HPV, in particular type 16 (HPV16), promote the development of cervical cancer[5]. This is the second leading cause of death in women worldwide, and the most common malignancy among women in developing countries[6,7].

Immunocompromised subjects, such as renal transplant patients and HIV-infected subjects display an increased incidence of HPV infections and cervical carcinoma[8,9] and this reveals the important role of the immune system in controlling HPV infections. In accordance, strong T-cell responses against the early antigens of the high risk HPV type 16 are detected in the peripheral blood of most healthy women[10-17]. However, in a minority of immunocompetent individuals the immune system fails to mount an effective HPV16-specific immune response and this results in viral persistence and the development of malignancies.

Our recent studies suggested an important role for the HPV16 E2, E6 and E7 early antigen-specific type 1 T-cell response in the control of HPV16-induced progressive disease[13,16]. Based on these results we hypothesized that subjects who live in HPV endemic geographic regions and who failed to mount such an HPV16-specific type 1 response may be at risk for developing HPV16-induced malignancies and would directly benefit from therapeutic vaccination. In general, these endemic regions are located in the developing countries, which by definition have low resources. Therefore, an immunological screening strategy to identify individuals at risk should be simple.

Intracutaneous antigen challenges (skin tests) have been used to monitor the development of delayed type IV hypersensitivity reactions, detected as flat red papules that arise 24-72 hours after injections. These skin reactions are the result of circulating antigen-experienced CD4+ Th1 and/or Th2 cells, which upon recognition of their cognate antigen migrate to the antigen site to attract macrophages or eosinophils, respectively[18,19]. The use of a skin test to detect HPV16-specific type 1 T-cell immunity would have the advantage that there is no need for laboratory facilities and highly skilled persons. The first demonstration of a skin test to detect HPV16 L1-specific T-cell mediated immunity was in individuals with HPV16-associated CIN who exhibited a delayed type hypersensitivity (DTH) reaction to a subcutaneous challenge with HPV16 capsid protein L1[20]. Subsequently, it was shown that an HPV16 E7-specific DTH response could be detected in subjects with spontaneously regressing CIN lesions[21].

The aim of the current pilot study was to validate the use of skin tests as a safe and simple method to detect HPV16 E2, E6 and E7-specific type 1 T-cell responses. We, therefore, injected different pools of overlapping clinical grade E2, E6 and E7 peptides in both healthy subjects and patients with HPV-associated cervical cancer and in parallel determined the presence of circulating HPV16-specific type 1 T-cells in the peripheral blood by IFNγ ELISPOT.

Materials and Methods

Subjects

Eleven non-pregnant women (age 29-72 years, median 49 years) with histories of cervical intraepithelial neoplasia (CIN) (n=3) or cervical carcinoma (n=8) were recruited for this study at the Department of Gynecology of the Leiden University Medical Center, The Netherlands, from October to December 2004. The clinical characteristics of the patients are summarized in Table 1. A group of nine healthy subjects (4 males, 5 females) within the age of 29-51 years (median 38 years) participated in this study after informed consent. Blood samples for the analysis of HPV16-specific T-cell reactivity were obtained from all subjects immediately before skin testing. The study design was approved by the Medical Ethical Committee of the LUMC and all subjects gave written informed consent.

Table 1. Patient characteristics

patient	age (yrs)	diagnosis	grade/ stage	treatment	time (months)[a]	HPV
1	72	CxCa	IA[b]	radical hysterectomy	9	16
2	57	CxCa	IA	radical hysterectomy	18	16
3	57	CxCa	IB	radical hysterectomy	36	?
4	50	CxCa	IB	radical hysterectomy	6	16
5	44	CxCa	IA	hysterectomy	36	16
6	53	CxCa	IIB	chemoradiation	36	16neg
7	34	CxCa	IA	radical hysterectomy	48	?
8	44	CxCa	IB	radical hysterectomy	7	16
9	29	CIN	III	LEEP[c]	2	?
10	42	CIN	II	LEEP	3	?
11	49	CIN	III	LEEP	60	18

[a] Time of treatment before skin tests were performed [b] cervical cancer stage according to FIGO [c] Loop electrosurgical excision procedure

Antigens

A set of peptides spanning the whole HPV16 E6 and E7 protein, and the most immunogenic regions of the E2 30-mer peptides[12,16] were used for the IFNγ ELISPOT assays and skin tests. For the skin tests, 8 pools of 2 or 3 long synthetic peptides were used (Table 2). The peptides were produced under GMP conditions in our own production facility (Interdivisional GMP Facility LUMC, IGFL) and were synthesized and dissolved as decribed previously[22]. The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (*e.g.*, $E2_{31-75}$, residues 31-60 and 46-75). IFNγ ELISPOT assays were performed with the same peptide pools, only peptide pools 4 and 5 differed slightly from the peptides used for the skin tests (peptide pool 4 used for IFNγ ELISPOT: $E6_{37-68}$, $E6_{55-86}$, and $E6_{73-104}$; peptide pool 5: $E6_{91-122}$ and $E6_{73-104}$) (Table 2). Memory response mix (MRM), consisting of a mixture of tetanus toxoid (0.75 *limus flocculentius*/ml final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 µg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and *Candida albicans* (0.005%, HAL Allergenen Lab, Haarlem, The Netherlands), was used as a positive control in the ELISPOT assays.

Table 2. HPV16 peptide pools used for skin tests and ELISPOT assays

pool	antigen	amino acid sequence
1	E2 31-60	DYWKHMRLECAIYYKAREMGFKHINHQVVP
	E2 46-75	AREMGFKHINHQVVPTLAVSKNKALQAIEL
2	E2 301-330	LRYRFKKHCTLYTAVSSTWHWTGHNVKHKS
	E2 316-345	SSTWHWTGHNVKHKSAIVTLTYDSEWQRDQ
3	E6 1-32	MHQKRTAMFQDPQERPRKLPQLCTELQTTIHD
	E6 19-50	LPQLCTELQTTIHDIILECVYCKQQLLRREVY
4[a]	E6 41-65	KQQLLRREVYDFAFRDLCIVYRDGN
	E6 55-80	RDLCIVYRDGNPYAVCDKCLKFYSKI
	E6 71-95	DKCLKFYSKISEYRHYCYSLYGTTL
5[b]	E6 85-109	HYCYSLYGTTLEQQYNKPLCDLLIR
	E6 91-122	LYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEK
6	E6 109-140	RCINCQKPLCPEEKQRHLDKKQRFHNIRGRWT
	E6 127-158	DKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL
7	E7 1-35	MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEE
	E7 22-56	LYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVT
8	E7 43-77	GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR
	E7 64-98	TLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP

[a]For the IFNγ ELISPOT assays pool 4 consisted of HPV16 $E6_{37-68}$, $E6_{55-86}$, and $E6_{73-104}$ peptides
[b]For the IFNγ ELISPOT assays pool 5 consisted of HPV16 $E6_{91-122}$ and $E6_{73-104}$ peptides

Skin tests

Skin tests, using HPV16 E2, E6 and E7 antigens, were performed similar to previous studies[20,21]. The skin test preparations consisted of 0.2 mg/ml peptides in 16% DMSO in 20 mM isotonic phosphate buffer. Skin tests included intracutaneous injection of 0.05 ml (10 µg/peptide) of the 8 peptide pools and the negative control (dissolvent only), at individual skin test sites of the upper arm. Skin test sites were inspected minimally two times, at 3 and 10 days after injection of the peptides, and after contact by telephone in case of positive skin reactions that appeared after this time. Reactions were considered positive when papules greater than 2 mm in diameter arose no less than 2 days after testing. All positive skin reactions were documented by photography.

Analysis of HPV16-specific T-cell reactivity by IFNγ ELISPOT

The HPV-specific T-cell reactivity in vitro was analyzed using ELISPOT that was performed as described previously[15,23]. Briefly, fresh PBMC were washed and seeded at a density of 2 x 10^6 cells per well of a 24-well plate (Costar, Cambridge, MA) in 1ml of IMDM (Bio Whittaker, Verviers, Belgium) enriched with 10% human AB serum, in the presence or absence of indicated HPV 16 E2, E6 and E7 peptide pools. Peptides were used at a concentration of 5 µg/ml/peptide. Following 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells per well in 100 µl IMDM enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT were performed according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (Bio Sys). Specific spots were calculated by subtracting the mean number of spots + 2 x SD of the medium control from the mean number of spots in experimental wells[15].

Statistics

Statistical analysis of the number of positive skin reactions in healthy subjects versus patients with cervical neoplasia and the association between the time of development of positive skin reactions in healthy subjects and the number of specific spots in the ELISPOT analyses was performed using 2-tailed Fisher's exact test. Statistical analyzes were performed using Graphpad Instat Software (version 3.0).

Results

Skin reactions to intracutaneous injection with HPV16 E2, E6 and E7 peptides

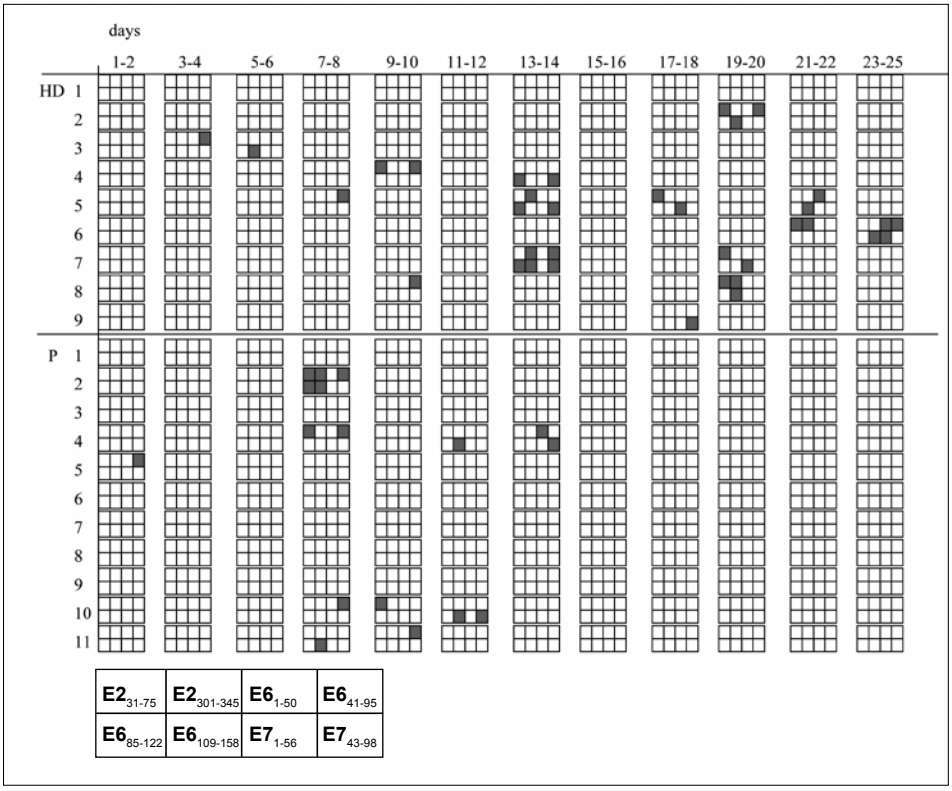
Skin tests are used as a simple assay to test the presence and type of immunity against recently encountered pathogens. In general, red papules that arise within 24-72 hours after an intracutaneous challenge with antigen are the result of the influx of mononuclear cells indicating the presence of a Th1 and/or Th2 response. This type of response is known as the classical delayed-type hypersensitivity (DTH) reaction[18]. We have studied skin reactions in both healthy subjects and patients with HPV-induced high-grade disease after an intracutaneous injection with HPV16 E2, E6- and E7 peptides. In our study population, positive skin reactions appeared as flat reddish papules of 2-20 mm in diameter arising within 2 to 25 days after skin testing. In total, 35 out of 72 skin tests were found positive in the healthy controls, whereas in the patient group a significantly lower number of skin reactions were detected (17 out of 88; $p<0.0001$, 2-sided Fisher's exact test). The time for skin reactions to appear differed considerably between healthy controls and patients. A classical DTH reaction could be detected only in 1 female healthy control and in one of the patients (Figure 1). However, the majority of reactions in the patient group developed around 7 days (range 2-14 days). In the healthy controls, the number of skin reactions peaked around 13 days and again 20 days after skin testing, revealing a bi-phasic response pattern (Figure 2A and 2B). This late reactivity suggests that the frequency of the responding HPV16 peptide-specific T-cells capable of migrating to the antigen depot in the skin is lower in the healthy population than in patients with HPV16-associated cervical neoplasia.

In alignment with our previous work[13,16], we found frequent skin reactions against the $E2_{31-75}$ (6/9 subjects) and $E2_{301-345}$ region (4/9 subjects), and E6 protein (7/9 subjects) in a substantial part of the healthy subjects. Furthermore, we detected positive skin reactions against the E7 protein (5/9 subjects), most of which appeared after day 13 (Figure 1). A similar reaction pattern was found in the patient group. The mean size of the skin reactions did not differ between patients and healthy subjects (data not shown).

A positive skin test is associated with the infiltration of HPV16-specific CD4+ and CD8+ T-cells

In order to confirm that positive skin test sites were infiltrated with HPV16-specific T-cells a small biopsy from one E6-peptide positive skin test site as well as a negative control site at the other arm were taken from one healthy subject (HD2) at day 21 after antigen injection (Figure 3). After 25 days of culture in medium supplemented

Figure 1. Skin reactions to intracutaneous injection with HPV16 E2, E6 and E7 peptides in healthy individuals and in patients with cervical neoplasia



An overview of the number, day of appearance and injected antigen that induced a positive skin reaction in the group of 9 healthy subjects (HD) and 11 patients (P) with history of cervical neoplasia.
Eight pools of peptides spanning the whole HPV16 E6 and E7 protein and the most immunogenic regions of the HPV16 E2 protein were injected separately at individual skin test sites of the upper arm. As a negative control the dissolvent only was used. Skin reactions were considered positive when papules greater than 2 mm in diameter arose no less than 2 days after testing. The indicated layout is used for the 8 peptide pools; a filled square represents a new developed, positive skin reaction to the indicated peptide pool.

with TCGF, IL-15, and FCS, the infiltrating immune cells were expanded to sufficient numbers for further analysis. The expanded infiltrating T-cells recognized their cognate epitope on monocytes pulsed with either peptide or protein when tested in a standard short-term proliferation assay (Figure 3). Analysis of the supernatants of these cultures revealed a predominant production of the Th1 cytokine IFNγ but also low amounts of the Th2 cytokine IL-10 (Figure 3). Flowcytometric analyses of

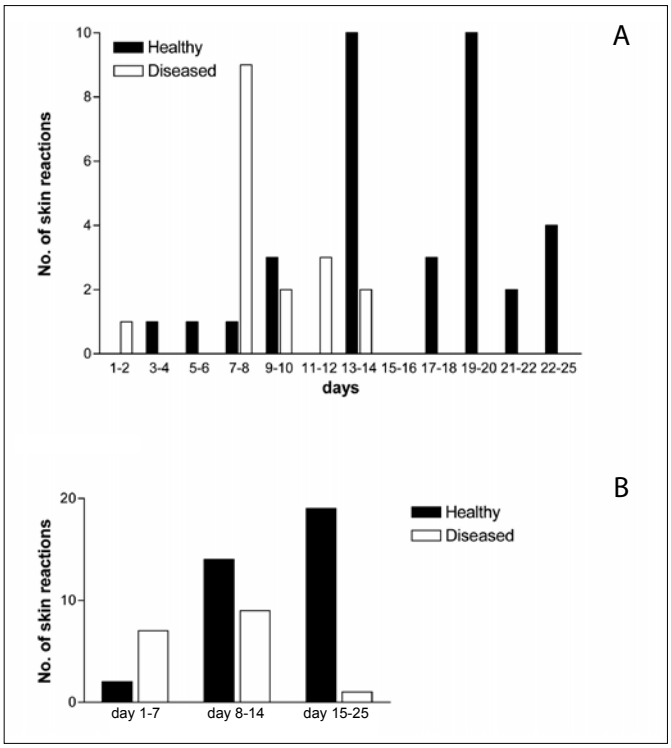


Figure 2. The number of skin reactions to HPV16 E2, E6 and E7 peptides in relation to the time of appearance in healthy individuals and in patients with HPV-induced cervical disease (A,B). The total number of skin reactions are depicted. The majority of skin reactions in the patient group developed around day 7 (A), whereas in the healthy controls the number of skin reactions showed two peaks at day 13 and day 20.

antigen-specific cytokine production by the IFNγ and IL-10 capture assay[17] revealed that the expanded T-cell population comprised both HPV16-specific CD4+ and CD8+ T-cells (data not shown). These preliminary results suggest that the skin-infiltrating immune cells after an intradermal challenge with HPV16 peptides comprises HPV16-specific Th1, Th2, and CD8+ T-cells. We are currently addressing this in a larger patient group.

Early skin reactions are associated with higher frequencies of HPV16-specific T-cells in the peripheral blood

In order to examine how the results of the skin test correspond to the detection of HPV16-specific Th1 immunity by IFNγ ELISPOT, PBMC were collected from 8/9 healthy subjects and all patients immediately before the intracutaneous injection of the HPV16 peptide antigens.

In 4 of 8 healthy subjects we were able to detect HPV16-specific IFNγ ELISPOT reactivity. When we compared the number of HPV16 peptide-specific spots detected by ELISPOT to the day that a skin reaction to this particular peptide appeared we found

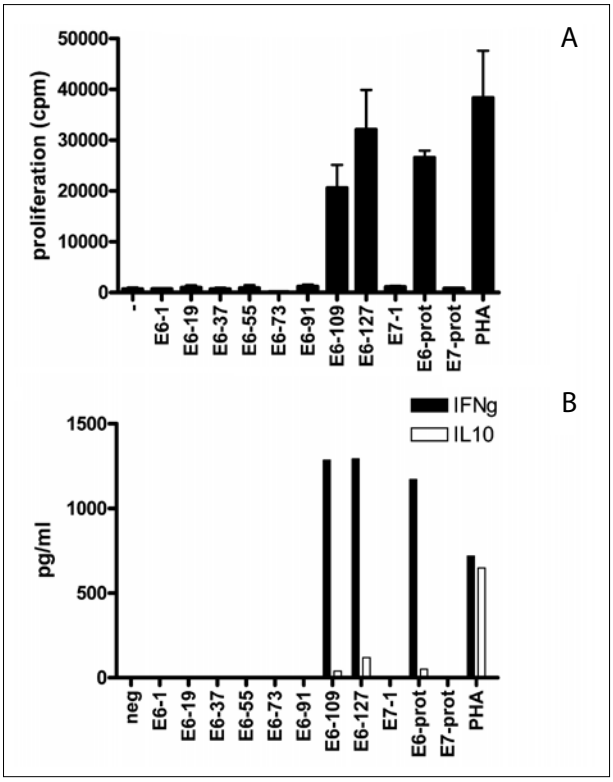
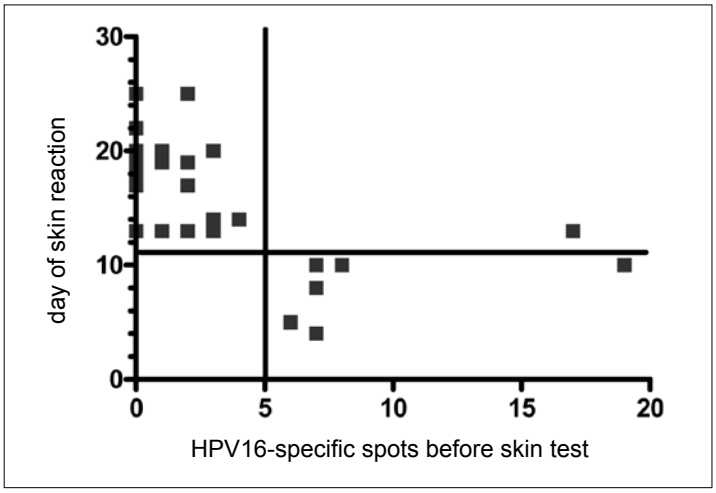


Figure 3. T-cell cultures of healthy donor 2 derived from a E6-peptide positive skin test site that recognize HPV16 E6-specific peptide pools.
A. The responses are shown of a standard short-term proliferation assay, indicating that skin-site T-cells of healthy donor 2 recognized $E6_{109-140}$ and $E6_{127-158}$ peptides corresponding to the E6 peptides injected at the skin test site.
B. Supernatants were tested for IFNγ and IL-10 production in a standard ELISA.

that ELISPOT response of ≥ 5 spots per 100,000 PBMC was associated with the presence of an early skin reaction (within 11 days) to that particular peptide (p=0.001, two-tailed Fisher's exact test) (Figure 4). Late skin reactions were found to peptides for which we were not able to detect a response by ELISPOT, suggesting that the frequency of circulating antigen-specific cells determines the delay time for skin reactions to appear.
We were able to isolate blood from 5 healthy subjects at the time that skin reactions were present. In these subjects, 28 skin reactions were found and in 22 cases a positive (≥ 5 spots/100,000 PBMC) Th1 response was detected by IFNγ ELISPOT (data not shown) indicating that the frequency of HPV16-specific T-cells in the blood of healthy subjects was higher at the time a skin reaction was present. These data suggest that the intracutaneous injection of peptide antigens enhances the number of HPV16-specific T-cells in the blood of healthy individuals.
In contrast to the healthy subjects, an occasional positive IFNγ ELISPOT response was found in the HPV16-positive patients and within this small group the appearance of early skin reactions was not associated with HPV16-specific Th1 immunity in the peripheral blood, suggesting that other types of HPV16-specific cells migrate into the skin.

Figure 4. Early skin reactions are associated with higher frequencies of circulating HPV16-specific T-cells in healthy individuals



Immediately before injection of the HPV16 peptide antigens, PBMC of 8 healthy subjects were tested with IFNγ ELISPOT. Detection of HPV16-specific T-cells by INFγ ELISPOT in the prechallenge blood sample of healthy subjects is significantly correlated with the appearance of an early (<11 days) positive skin reaction to the recognized peptide pool (p=0.001, two-tailed Fisher's exact test). Specific responses were calculated by subtracting the mean number of spots +2 x SD of the medium control from the mean number of spots in experimental wells. The number of specific spots per 100.000 PBMC is given. Responses were considered positive if peptide pool-specific T-cell frequencies were ≥ 5 in 100.000 PBMC.

Discussion

In this pilot study we have analyzed the HPV16-specific T-cell immunity *in vivo* in a group of nine healthy subjects and eleven patients with HPV16-associated cervical neoplasia by a peptide-based skin test using HPV16 E2, E6 and E7 peptides. One main observation of the present study is that the time for skin reactions to appear is correlated with the number of circulating HPV16-specific CD4+ T-cells in the peripheral blood. In healthy subjects, early skin reactions (within 11 days) developed to peptides of which the T-cell frequency exceeded 1/20,000 in our IFNγ ELISPOT analysis while late skin reactions developed in response to peptides for which we were not able to detect Th1 immunity (p=0.001). In line with this is the observation that early HPV16 E7 peptide-specific skin reactions (2-6 days after skin testing) can be detected in patients with immune driven regression of CIN lesions[21]. These patients who suc-

cessfully fight an active HPV infection have high numbers of HPV16-specific T-cells in their blood (van der Burg & Hopfl, unpublished data). It is highly likely that the HPV16-specific T-cell frequencies gradually decline in individuals who have successfully dealt with an HPV16 infection, such as will be the case with our healthy subject group. Similarly, a decline in responsiveness was observed in individuals vaccinated with BCG[24]. Upon intracutaenous challenge with HPV16 peptide-antigen such specific T-cells will be reactivated, start to divide and migrate to site of antigen. Indeed, at the time that skin reactions appeared, strong HPV16-specific Th1 cells were also detected in the peripheral blood of these healthy subjects by IFNγ ELISPOT.

Similar to the earlier observations[21], we found in the group of patients that the majority of the skin reactions appeared in about a week. These skin reactions were not associated with circulating HPV16-specific Th1 cells as measured by IFNγ ELISPOT, suggesting that HPV16-specific T-cells producing cytokines other than IFNγ infiltrated the skin. Although delayed-type skin reactions to soluble antigens are usually regarded as type 1 T-helper cell responses, Th2 cells can also infiltrate DTH sites for instance in chronic asthma[25-27]. We recently showed that HPV16-specific IFNγ-associated T-cell responses were lacking in the great majority of cervical cancer patients, whereas the secretion of the Th2 cytokine IL-10 was less affected[13]. Therefore, the skin reactions in cancer patients could well be the result of infiltrating HPV16-specific Th2 cells. Indeed, recent data of our group have shown that skin biopsies of positive DTH sites of healthy volunteers contain both Th1 and Th2 cells (van den Hende, unpublished results).

Unexpectedly, we observed a bi-phasic pattern in the number of skin reactions in healthy subjects. Skin reactions peaked 2 weeks and again 3 weeks after intracutaneous injection of peptide (Figure 2A). Low numbers of memory T-cells that circulate and that need to be activated and divide before they migrate to the antigen site can explain the first peak. It is, however, more difficult to explain the second peak in a similar way. If to some of the peptides even lower numbers of memory T-cells were present, one would expect a more evenly distributed pattern of skin reactions from day 13 to day 25. It may well be that the very late skin reactions (~day 20) are the result of T-cell priming, in which the T-cell response develops more slowly. Skin-test induced priming of T-cells has also been noted in 29% of patients whom underwent a 2-step tuberculin skin testing protocol[28]. Interestingly, in cancer patients, who are known to respond weakly to HPV16 antigens, we did not observe such very late skin reactions.

The main objective of this pilot study was to examine if an HPV16 peptide-based skin test could be used as a tools for measuring HPV-specific T-cell immunity in vivo. It is clear that at least two items need to be clarified before larger field tests are initiated. First, we need to know the Th1/Th2 character of the HPV-specific T-cells present in skin test sites of patients with HPV16-induced high-grade neoplasia. Secondly, we should firmly establish the maximal timeframe in which memory T-cell mediated skin reactions appear.

References

1 . Burk RD, Kelly P, Feldman J, et al. Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. Sex Transm Dis 1996; 23: 333-341.

2 . Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis 1995; 171: 1026-1030.

3 . Ho GY, Bierman R, Beardsley L, et al. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med1998; 338: 423-428.

4 . Koutsky L. Epidemiology of genital human papillomavirus infection. Am J Med 1997; 102: 3-8.

5 . Bosch FX, Lorincz A, Munoz N, et al. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002; 55: 244-265.

6 . Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993; 54: 594-606.

7 . Bosch FX, de Sanjose S. Chapter 1: Human papillomavirus and cervical cancer--burden and assessment of causality. J Natl Cancer Inst Monogr 2003; 3-13.

8 . Palefsky JM, Minkoff H, Kalish LA, et al. Cervicovaginal human papillomavirus infection in human immunodeficiency virus-1 (HIV)-positive and high-risk HIV-negative women. J Natl Cancer Inst 1999; 91: 226-236.

9 . Serraino D, Carrieri P, Pradier C, et al. Risk of invasive cervical cancer among women with, or at risk for, HIV infection. Int J Cancer 1999; 82: 334-337.

10 . Bontkes HJ, de Gruijl TD, Bijl A, et al. Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia. J Gen Virol 1999; 80 (Pt 9): 2453-2459.

11 . de Gruijl TD, Bontkes HJ, Walboomers JM, et al. Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study. Cancer Res 1998; 58: 1700-1706.

12 . de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.

13 . de Jong A, van Poelgeest MI, van der Hulst JM, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004; 64: 5449-5455.

14 . Luxton JC, Rowe AJ, Cridland JC, et al. Proliferative T cell responses to the human papillomavirus type 16 E7 protein in women with cervical dysplasia and cervical carcinoma and in healthy individuals. J Gen Virol 1996; 77 (Pt 7): 1585-1593.

15 . van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II- restricted epitopes. Int J Cancer 2001; 91: 612-618.

16 . Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.

17 . de Jong A, van der Hulst JM, Kenter GG, et al. Rapid enrichment of human papillomavirus (HPV)-specific polyclonal T cell populations for adoptive immunotherapy of cervical cancer. Int J Cancer 2005; 114: 274-282.

18 . Black CA. Delayed type hypersensitivity: current theories with an historic perspective. Dermatol. Online J 1999; 5: 7.

19 . Kobayashi K, Kaneda K, Kasama T. Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech 2001; 53: 241-245.

20 . Hopfl R, Sandbichler M, Sepp N, et al. Skin test for HPV type 16 proteins in cervical intraepithelial neoplasia. Lancet 1991; 337: 373-374.

21 . Hopfl R, Heim K, Christensen N, et al. Spontaneous regression of CIN and delayed-type hypersensitivity to HPV- 16 oncoprotein E7. Lancet 2000; 356: 1985-1986.

22 . van der Burg SH, Kwappenberg KM, Geluk A, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. J Immunol 1999; 162: 152-160.

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

24 . Floyd S, Ponnighaus JM, Bliss L, et al. Kinetics of delayed-type hypersensitivity to tuberculin induced by bacille Calmette-Guerin vaccination in northern Malawi. J Infect Dis 2002; 186: 807-814.
25 . Woodfolk JA, Platts-Mills TA. Diversity of the human allergen-specific T cell repertoire associated with distinct skin test reactions: delayed-type hypersensitivity-associated major epitopes induce Th1- and Th2-dominated responses. J Immunol 2001; 167: 5412-5419.
26 . Wang S, Fan Y, Brunham RC, et al. IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. Eur J Immunol 1999; 29: 3782-3792.
27 . Tsicopoulos A, Hamid Q, Haczku A, et al. Kinetics of cell infiltration and cytokine messenger RNA expression after intradermal challenge with allergen and tuberculin in the same atopic individuals. J Allergy Clin Immunol 1994; 94: 764-772.
28 . Akcay A, Erdem Y, Altun B, et al. The booster phenomenon in 2-step tuberculin skin testing of patients receiving long-term hemodialysis. Am J Infect Control 2003; 31: 3

CHAPTER 5

Immunological responses in women with HPV16-associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV16 oncogene vaccination

Lucy J.C. Smyth
Mariëtte I.E. van Poelgeest
Emma J. Davidson
Kitty M.C. Kwappenberg
Debbie Burt
Peter Sehr
Michael Pawlita
Stephen Man
Julian K. Hickling
Alison N. Fiander
Amanda Tristram
Henry C. Kitchener
Rienk Offringa
Peter L. Stern
Sjoerd H. van der Burg

Clinical Cancer Research 2004; 10: 2954-2961

Abstract

Purpose: To study the immunogenicity of heterologous prime-boost human papillomavirus (HPV) oncogene vaccination in patients with anogenital intraepithelial neoplasia (AGIN).

Experimental Design: Twenty-nine women with high-grade AGIN received three i.m. doses of TA-CIN (HPV16 L2/E6/E7 protein) at four weekly intervals followed by a single dermal scarification of vaccinia HPV16/18 E6/E7 and were followed up for 12 weeks. Immunity to HPV16 was assessed by lymphoproliferation, IFNγ enzyme-linked immunospot (ELISPOT), and ELISA.

Results: The patient group significantly responded to TA-CIN and not to the control antigen HPV6 L2/E7 at all postvaccination time points when compared with baseline responses ($p \leq 0.05$). Ten of the patients showed at least a 3-fold increase in TA-CIN-specific proliferation at one or more time points after vaccination. Comparison of stimulation with HPV16 E6- or E7-GST fusion proteins showed that proliferative responses were biased to HPV16 E6. This bias was also seen by IFNγ ELISPOT using overlapping peptides, with HPV16 E6- or E7- specific T-cells being detected in 9 and 2 patients, respectively. In addition, vaccination resulted in the induction of antibodies against the HPV16 oncoproteins. Of the 6 clinical responders, 2 patients showed both a proliferative TA-CIN-specific response and an E6-specific IFNγ response, whereas 3 other patients displayed E6-specific reactivity only. Stable disease was recorded in 19 patients, 8 of whom showed a concomitant TA-CIN-specific proliferative and/or E6-specific T-cell response. Of the 4 progressors, 2 failed to make a T-cell response and 2 responded by either proliferation or E6 ELISPOT alone.

Conclusions: The prime-boost regimen is immunogenic in AGIN patients (humoral and cellular immunity), but there is no simple relationship between induction of systemic HPV16-specific immunity and clinical outcome. Other factors that may play a role in the eradication of long-term established AGIN lesions need to be determined to identify the patient group that would benefit from immunotherapy with the vaccines used in this study.

Introduction

Human papillomavirus (HPV) oncogenes E6 and E7 are expressed throughout the spectrum of HPV-associated anogenital intraepithelial neoplasia (AGIN) and therefore are potential targets for immunotherapy[1]. A number of vaccine strategies are being evaluated in clinical trials[2]. In the pioneering studies, vaccinia HPV16/18 E6/E7 (TA-HPV), a live recombinant vaccinia virus encoding modified versions of HPV16 and 18 E6 and E7, was used in patients with advanced cervical carcinoma[3] and subsequently in early-stage cervical carcinoma patients [4]. These studies established safety and immunogenicity in that modest T-cell and antibody responses were induced. These studies in cancer patients, however, did not allow the evaluation of any clinical effects in the short term. Two recent studies[5,6] have tested a single dose of TA-HPV in patients with high-grade vulval intraepithelial neoplasia (VIN), a chronic, premalignant, frequently symptomatic disease. In the first study[6], a single dose of TA-HPV was shown to be immunogenic in 13 of 18 women as demonstrated by lymphocyte proliferation, enzyme-linked immunospot (ELISPOT), and/or antibody responses. Eight patients demonstrated a reduction in lesion diameter of at least 50%, and an additional 4 patients showed significant symptom relief. The lesions that shrank showed significantly higher levels of infiltrating CD4+, CD8+, and CD1a+ immune cells before vaccination as demonstrated by immunohistochemistry. In a second study in VIN patients[5], 6 of 10 women demonstrated vaccine-induced HPV16-specific T-cells by IFNγ ELISPOT, 5 with a concomitant significant reduction in lesion diameters. Delivery of cure in patients with this and other HPV-associated lesions may depend on viral clearance or at least sustained anti-HPV immunity, and a single vaccination may, therefore, not be enough.

A second vaccine formulation that has been tested in humans consists of a HPV16 L2E6E7 fusion protein (TA-CIN). It was well tolerated when administered to healthy volunteers and induced antibody and proliferative responses against TA-CIN, as well as IFNγ ELISPOT responses to the HPV16 oncoproteins[7]. A small study of VIN patients who received three booster vaccinations with TA-CIN between 7 and 15 months after the TA-HPV vaccination demonstrated HPV16-specific proliferative T-cell and/or serological activity, but there was no direct correlation between immunological and clinical responses[8].

In preclinical studies, a heterologous prime-boost immunization strategy using a HPV16 L2E6E7 fusion protein (TA-CIN), in combination with TA-HPV, showed enhanced immunogenicity compared with the use of either agent alone[9]. The protocol of TA-CIN followed by TA-HPV was superior to the reciprocal as defined by the

induction of T-cell reactivity against the oncoproteins. This may be related to the fact that TA-HPV comprises a multitude of vaccinia-specific T-cell epitopes, which can efficiently compete with the two HPV16 oncogenes for the attention of the T-cell-mediated immune response. In the case that the immune response is naïve to either of these epitopes, immunity may very well be skewed toward vaccinia-derived epitopes. Immunization with TA-CIN is thus likely to focus the immune response to the oncoproteins, whereas boosting with TA-HPV will increase the magnitude of this oncoprotein-specific T-cell response. Here, we describe the immunogenicity of a similar heterologous prime-boost strategy of three monthly immunizations with TA-CIN, followed by a single boost with TA-HPV in patients with HPV16-associated AGIN.

Patients and methods

The United Kingdom Medicines Control Agency, the Gene Advisory Committee, and the appropriate Local Ethics Committees approved the study, and all patients gave written informed consent. Twenty-nine women with stable, noncervical AGIN (27 VIN 3 and 2 VAIN 3) were recruited in three centers: St Mary's Hospital (Manchester, United Kingdom); University Hospital of Wales, Llandough Hospital (Cardiff, United Kingdom); and Addenbrooke's Hospital (Cambridge, United Kingdom). Three prime vaccinations of TA-CIN (533 μg) were given i.m. at four weekly intervals, followed 4 weeks later by a boost vaccination comprising a single dose (2.5×10^5 plaque-forming units) of TA-HPV by dermal scarification. Women were followed up for 12 weeks after completion of the vaccination schedule. Blood was taken before vaccination (week 0) and at 12, 16, 20, and 24 weeks for assessment of cell-mediated and humoral immunity. Prevaccination samples were limited for 2 patients (404 and 405), and patient 416 baseline sample was taken at week 4. Biopsies were taken at weeks 12 and 24 for histology and HPV status as described previously[6]. At entry, 26 patients had lesions with HPV16, 1 had HPV33 and HPV58, 1 was HPV high-risk but could not be typed, and 1 was HPV negative. Clinical responses were defined as partial if lesion area was reduced by ≥ 50% and a complete response by complete reduction of the lesion with no evidence of disease in the biopsy. Progression was defined as an increase in lesion area by ≥ 50%. The clinical findings will be reported elsewhere*.

*Kitchener H, Tristram A, Davidson EJ, Tomlinson A, Dobson J, Baldwin P, Sterling J, and Fiander A. The clinical effects of a multicentre trial of a prime boost vaccination strategy in women with high grade anogenital intraepithelial neoplasia. Manuscript submitted.

Analysis of HPV16-specific T-cell proliferation

Blood samples were collected in monovettes containing citrate and peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation. Cells were cryopreserved in liquid nitrogen until additional use. PBMC from all time points for each patient were assessed in a slightly modified assay previously described[6] and as follows. Briefly, cells were thawed and placed into 24-well suspension cell plates (Sarstedt) for a recovery period of 2 h before being seeded in triplicate wells of a 96-well round bottomed microtitre plate (Alpha Laboratories Ltd.) at 2 x 10^5 cells/well in RPMI 1640 supplemented with 10% human AB serum (Quest Biomedical), 100 µg/ml streptomycin, and 100 IU/ml penicillin (Life Technologies, Inc.). PBMC alone (medium control) or PBMC with 50 µg/ml recombinant HPV16 L2E6E7 protein (TA-CIN; Xenova Research Ltd.), 50 µg/ml recombinant HPV6 L2E7 protein (TA-GW, Xenova Research Ltd.), 25 µg/ml HPV16 GST-E6, 25 µg/ml HPV16 GST-E7, 25 µg/ml glutathione S-transferase (GST) tag protein alone (all proteins purified on glutathione-Sepharose; ref.[10]), or 7000 units/ml tuberculin purified protein derivative (PPD, Evans Vaccines Ltd., Liverpool, United Kingdom) were incubated for 6 days at 37°C. During the final 18 h of culture, 1 µCi/well of [^{3}H]thymidine (NEN Life Science Products) was added. The cells were harvested using a Packard 96-well vacuum cell harvester onto Unifilter plates (Packard BioSciences), left to dry overnight, and 30 µl/well of Microscint 20 scintillation fluid (Packard) added. [^{3}H]Thymidine incorporation was measured using a Topcount scintillation counter (Packard). Replicates were within 10%. Results are presented as stimulation index (SI) = the mean number of counts incorporated by antigen-stimulated PBMC divided by the mean number of counts for PBMC in medium alone (negative control); SIs for GST-E6 and GST-E7 were calculated using the response to GST as control. A preexisting proliferative T-cell response to HPV16 L2E6E7 was defined as SI $\geq$ 2. A postvaccination proliferative T-cell response to HPV16 L2E6E7 was defined as a 3-fold increase in the SI compared with the prevaccination value. Paired Wilcoxon's signed ranks tests were used to test for population differences in the responsiveness to each antigen before and after vaccination with $p \leq 0.05$ considered statistically significant. The response to TA-HPV boost vaccination (week 12 *versus* week 16 and so on) was also compared by Wilcoxon's signed rank tests.

Analysis of HPV16 E6- and E7-specific T-cell reactivity by IFNγ ELISPOT

IFNγ-producing HPV-specific T-cells were quantified using ELISPOT that was performed as described previously[7,11]. Briefly, PBMC were thawed, washed, and seeded at a density of 2 x 10^6 cells/well of a 24-well plate (Costar, Cambridge, MA) in 1 ml of Iscove's modified Dulbecco's medium (BioWhittaker, Verviers, Belgium) enriched with

10% human AB serum in the presence or absence of indicated HPV16 and HPV18 E6 and E7 peptide pools. As a positive control, PBMC were cultured in the presence of a memory recall mix, consisting of a mixture of tetanus toxoid (0.75 *limus floccolentius*/ml final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 µg/ml; generously donated by Dr. Paul Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and *Candida albicans* (0.005%, HAL Allergenen Laboratory, Haarlem, The Netherlands). The peptides used spanned the HPV16 and HPV18 E6 and E7 protein and consisted of 15 E6 and 9 E7 overlapping 22-mer peptides. Peptides were used in pools of 4-5 peptides at a concentration of 5 µg/ml/peptide. The peptides, as indicated by their first and last amino acid in the protein, were used in the following pools: *E6-I*: 1-22, 11-32, 21-42, 31-52; *E6-II*: 41-62, 51-72, 61-82, 71-92; *E6-III*: 81-102, 91-112, 101-122, 111-132; *E6-IV*: 111-132, 121-142, 131-152, 137-158; *E7-I*: 1-22, 11-32, 21-42, 31-52; *E7-II*: 41-62, 51-72, 61-82, 71-92, 77-98 (HPV18: last peptide 81-105). After 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells/well in 100 µl Iscove's modified Dulbecco's medium enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ catching antibody (Mabtech AB, Nacka, Sweden). Additional antibody incubations and development of the ELISPOT were performed according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted video imaging analysis system (Bio Sys). Specific spots were calculated by subtracting the mean number of spots + 2 x SD of the medium only control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be increased compared with nonresponders when specific T-cell frequencies were ≥ 1/10,000[11]. T-cell frequencies were considered to be boosted by the vaccine when they were at least 3-fold higher than before vaccination[7].

Analysis of HPV-specific IgG antibodies by ELISA

HPV16/18 E6/E7 and HPV16 L1 IgG levels were measured by ELISA using recombinant proteins fused to GST; GST without HPV protein sequences was used as specificity control, and any background response to the latter was subtracted to give the HPV-specific absorbance as described previously[10,12]. Antibody levels were measured in sera from a group of normal blood donors (n=15). Cutoff values for sera positivity were HPV16 E6, 119; HPV16 E7, 31; HPV18 E6, 356; HPV18 E7, 500; and HPV16 L1 101. In addition to a 3-fold increase over week 0 baseline absorbance values, the mean specific absorbance + 3 x SD of this group of normals for each assay was used as the cutoff value to define seropositivity in the AGIN patients. The normal and

baseline AGIN patient serological responses were compared using Mann-Whitney U (nonparametric) tests. Pre- and postvaccination AGIN patient group serological responses were compared by paired Wilcoxon's signed rank tests with $p \leq 0.05$ considered statistically significant.

Results

HPV16-specific proliferative T-cell responses

The capacity of the heterologous prime-boost protocol to induce HPV16-specific proliferative T-cell responses was examined by analysis of HPV-specific immunity before vaccination, after 3 vaccinations with TA-CIN (week 12), and after booster vaccination with TA-HPV (weeks 16, 20 and 24). Figure 1A shows an example of such proliferative responses detected in an individual patient. A 3-4-fold increase in TA-CIN-specific proliferation was detected after the three prime vaccinations with TA-CIN. This is not boosted by TA-HPV at week 16 but continues above baseline over the course of the study. The group responses to the HPV antigens are shown as box whisker plots in Figure 1B. The patient group as a whole showed significantly increased TA-CIN-specific responses after the three TA-CIN vaccinations, which was sustained on weeks 16-24. There was no significant change in response to the control antigen HPV6 L2/E7 at any time point (Table 1).

When examining individual patient responses, 10 of 27 patients tested had evidence of a preexisting proliferative T-cell response to TA-CIN, and 7 of these also showed

Table 1. P-values from Wilcoxon's signed rank tests showing statistical evaluation of patient group proliferative responses to human papillomavirus (HPV)16 L2/E6/E7 protein (TA-CIN), HPV6 L2/E7 (TA-GW), HPV16 GST-E6, and HPV16 GST-E7[a].

Antigen	Week 12	Week 16	Week 20	Week 24	Wk 12 vs Wk 16
TA-CIN	**0.0001**	**0.0003**	**0.0002**	**0.0017**	0.1613
TA-GW	0.2135	0.1979	0.205	0.2505	0.273
HPV16 E6	0.1681	**0.0452**	0.1146	0.0537	**0.0453**
HPV16 E7	0.533	0.6043	0.2902	0.6265	0.8758

[a] Vaccine-induced proliferation (stimulation index) was compared at time 0 *versus* postvaccination time points in a Wilcoxon's signed rank test. Data were considered significant when $p \leq 0.05$. Group responses to TA-CIN, TA-GW, HPV16 GST-E6, and HPV16 GST-E7 were compared after vaccinations with TA-CIN (week 12), after boosting with HPV16/18 E6/E7 (week 16) and in the follow-up. Specific booster activity of HPV16/18 E6/E7 was tested by comparison of group responses in week 12 *versus* week 16.

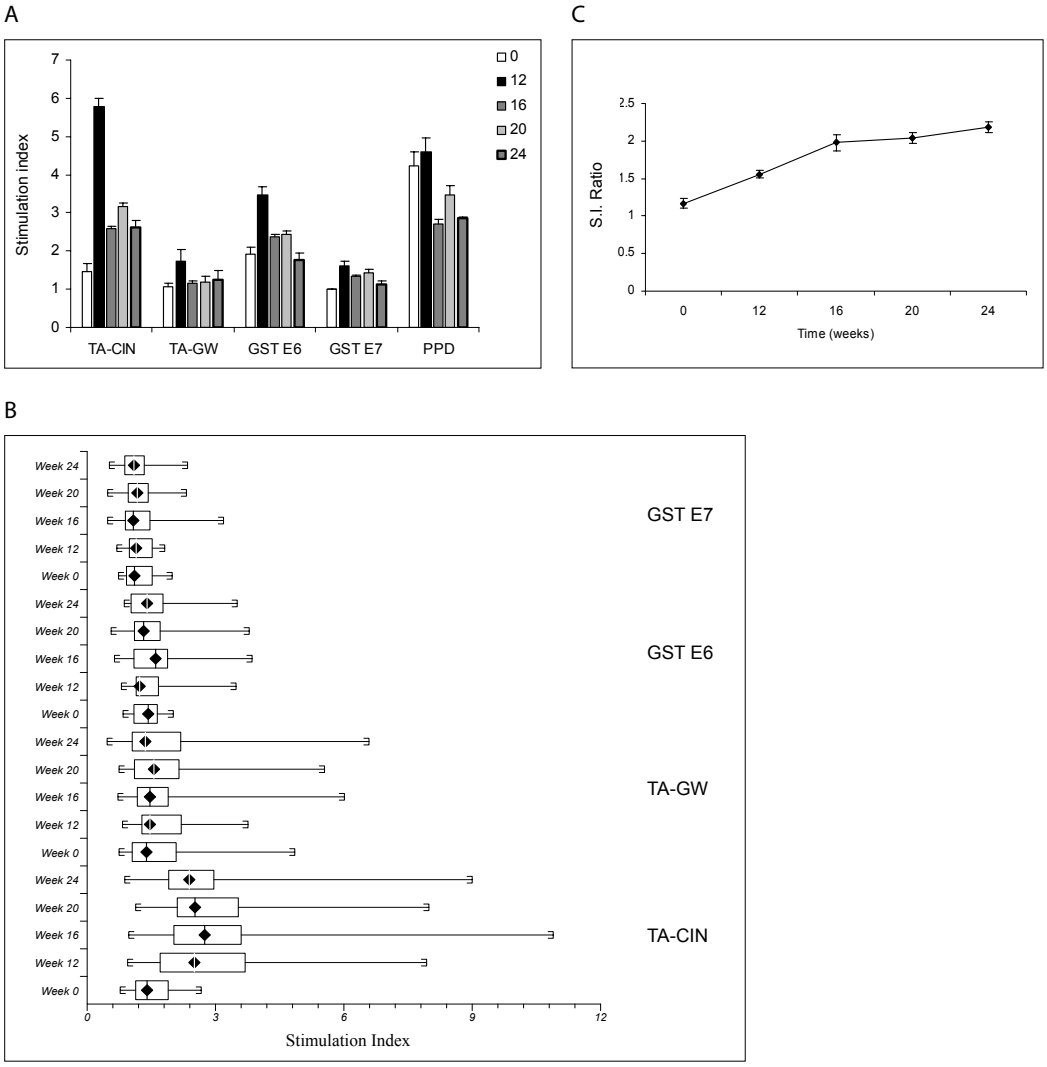


Figure 1A. PBMC proliferative responses of an individual patient to TA-CIN, TA-GW, GST-E6 and GST-E7 at weeks 0, 12, 16, 20 and 24. Stimulation index (SI) was calculated by dividing the mean number of counts for the antigen-stimulated PBMC by the mean number of counts for the medium only (for TA-CIN, TA-GW and PPD). GST-E6 and E7 counts were normalized to the mean number of counts to GST protein. A preexisting proliferative T-cell response to TA-CIN and HPV16 E6/E7 was defined as a SI of >2. A vaccine-specific proliferative T-cell response to TA-CIN was defined as a 3-fold increase in the stimulation index postvaccination compared with the prevaccination value.

Figure 1B. *Box-whisker plot* representing PBMC proliferative responses (SI) of the patient group at weeks 0, 12, 16, 20 and 24 to GST proteins E6 and E7, TA-CIN and TA-GW. *Boxes* represent upper and lower quartiles, *center point* is the median, and *whiskers* are the upper and lower range values.

Figure 1C. Ratio of SI change over time; HPV16 E6 SI values were divided by HPV16 E7 SI values to demonstrate the bias of vaccine-specific lymphoproliferative responses to HPV16 E6 over time. *Error bars* represent the SE.

baseline responses to HPV6 L2/E7. Of all patients, 17 showed evidence of a TA-CIN-specific response (at least a 2-fold increase) at some point after prime or prime-boost vaccination. Ten of these 17 patients showed a definite vaccine induced response (>3-fold increase in SI), with only 2 from those displayed a preexisting response to TA-CIN. No similar individual responses were seen to the control antigen HPV6 L2/E7. Patients with preexisting responses had an average increase in postvaccination proliferation of 1.49 +/-0.13 SE, whereas patients without preexisting immunity gave 2.16 +/-0.15 SE. It seems there are higher and more consistent responses in the patients with lower preexisting proliferative responses.

Examination of the proliferative responses against the two oncoproteins using recombinant HPV16 GST-E6 or GST-E7 proteins in the proliferation assay showed that group responses were significantly increased only for GST-E6 at weeks 16 (Table 1). The ratio of E6 to E7 responses over the trial period supports this bias for E6-specific T-cell proliferation (Figure 1C). The overall magnitude of response, reflected by the SI range and median values, is greater for TA-CIN than E6-GST (Figure 1), implying that the magnitude of the T-cell response reactive with non-E6 or E7-encoded T-cell epitopes is higher. A comparison of proliferative responses at weeks 12 (before TA-HPV) and 16 showed evidence for a booster effect of the TA-HPV vaccination for E6-GST reactivity ($p=0.045$) but not for TA-CIN ($p=0.16$). All patient lymphocytes showed a proliferative response to tuberculin purified protein derivative, indicating that there was no overt immune suppression in these patients as well as that the samples tested were of good quality.

Vaccine-induced IFNγ-producing T-cell reactivity to HPV16/18 E6 and E7

IFNγ ELISPOT assays were performed with PBMC provided from 25 patients, taken before vaccination (week 0) and following all vaccinations (week 16 and/or week 20/24). HPV16-specific T-cell responses were detected in 11 of 25 patients (Table 2). Preexisting HPV16-specific immunity was detected in 3 patients (201, 301, and 412), all of whom showed reactivity to HPV16 E6 peptides. None of the patients showed HPV16 E7-specific T-cells before vaccination. Vaccine-induced HPV16 E6-specific T-cells were found in 9 of 25 patients after all vaccinations (Table 2). All of the patients who displayed at least a 3-fold increase in E6-specific reactivity in the proliferation assays (201, 301, 304, 310 and 311) responded to one or more pools of E6 peptides in the ELISPOT assay. In two patients (301 and 405), HPV16 E7-specific T-cells were enhanced after all vaccinations (Table 2 and Figure 2).

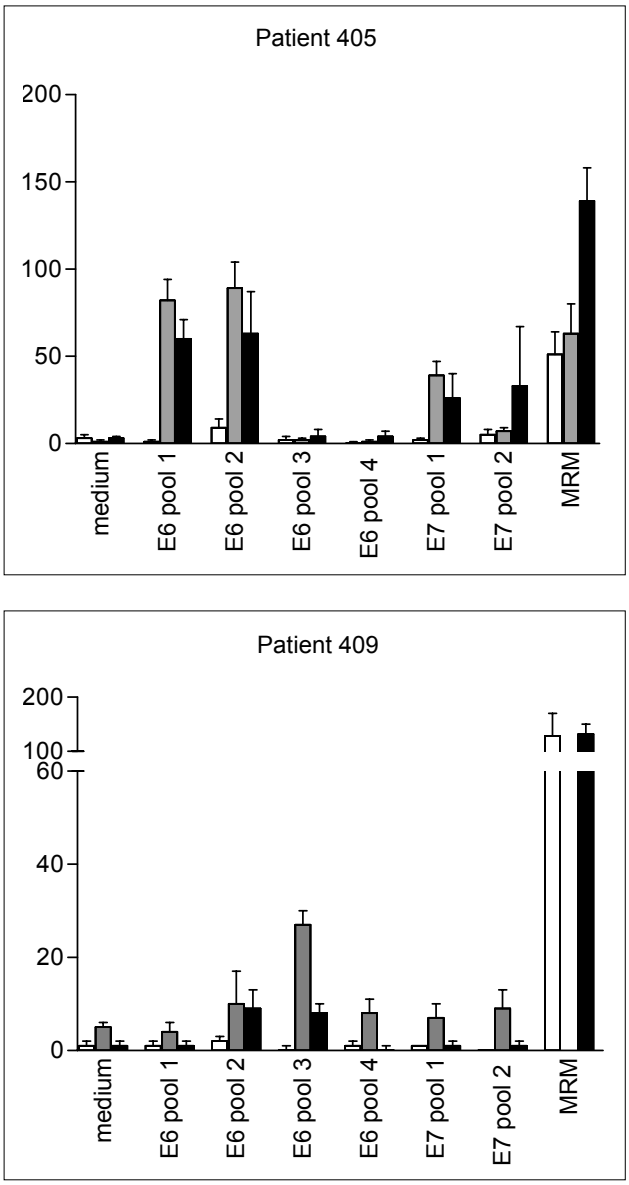
HPV18 E6 and E7 ELISPOT assays were performed with 10 of 25 patient lymphocytes pre- and postvaccination (201, 304, 305, 307, 311, 403, 407, 409, 411, and 413). One

Table 2. HPV16/18 E6- and E7-specific immune responses pre- and postvaccination determined by IFNγ enzyme-linked immunospot.

Patient		HPV16							HPV18						
	Time[a] (weeks)	E6 pool 1	E6 pool 2	E6 pool 3	E6 pool 4	E7 pool 1	E7 pool 2	MRM	E6 pool 1	E6 pool 2	E6 pool 3	E6 pool 4	E7 pool 1	E7 pool 2	MRM
201	0	-[b]	39	-	-	-	-	339	14	29	22	-	-	4	339
	16	-	54	-	-	-	-	353	-	-	-	-	1	-	350
	20	-	22	-	-	-	-	293	-	-	-	-	-	-	282
301	0	19	-	-	164	-	**7**	391							
	16	**187**	**45**	2	128	-	**39**	305							
	24	**109**	**61**	**72**	117	-	**119**	309							
304	0	-	-	-	-	-	-	117	-	**7**	-	-	-	-	113
	16	-	**20**	-	-	-	-	137	-	-	-	-	-	-	94
	24	NA	NA	NA	NA	NA	NA	NA							
305	0	-	2	-	1	-	-	56	-	-	-	-	-	-	44
	16	-	**10**	2	-	-	-	50	2	2	1	-	-	1	51
	20	-	4	-	-	-	2	74	-	-	1	1	-	2	62
307	0								-	-	-	-	-	-	75
	16								-	-	6	2	1	2	58
	20								3	-	**22**	**15**	-	4	103
311	0	-	-	-	-	-	-	98	-	3	-	-	-	-	104
	16	-	-	-	-	-	-	255							
	24	2	6	**11**	-	-	-	259	-	4	7	-	-	-	147
403	0								-	1	2	1	4	-	282
	16								-	-	-	-	-	-	35
	20								-	7	-	-	-	5	354
404	0	-	-	-	-	-	-	26							
	16	-	-	-	-	-	-	34							
	24	-	-	-	**14**	-	-	91							
405	0	-	1	-	-	-	-	43							
	16	**79**	**87**	-	-	**36**	5	60							
	24	**56**	**59**	-	-	**22**	**29**	135							
407	0								-	-	-	-	-	-	46
	16								-	-	-	-	-	-	45
	20								-	-	-	**12**	-	-	164
409	0	-	-	-	-	-	-	127	4	-	-	-	-	-	147
	16	-	3	**19**	-	-	-	87							
	24	-	6	5	-	-	-	129	-	-	-	-	-	-	182
411	0	-	-	-	-	-	-	129	-	-	-	-	-	-	29
	16	**12**	4	-	-	-	-	132	-	-	-	-	-	-	75
	24	7	9	-	-	-	-	170	-	-	-	-	-	-	100
412	0	-	-	10	-	-	-	200							
	16	-	-	-	-	-	-	247							
	24	5	-	-	-	2	5	217							
413	0	-	-	-	-	-	-	86	-	-	-	-	-	-	86
	16	-	**24**	9	-	-	-	239	-	-	-	-	-	-	197
	20	-	1	8	-	-	-	214	-	-	-	**25**	-	-	199

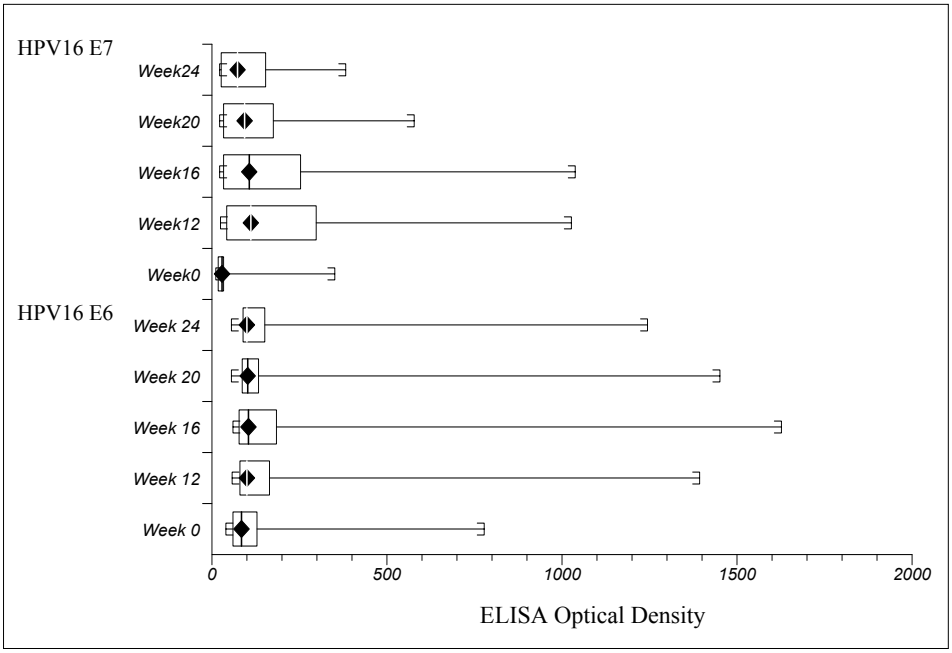
[a] Patient lymphocytes were assayed for IFNγ production on weeks 0, 16, and weeks 20 or 24. The 11 patients in whom no responses were detected against either HPV16 or HPV18 are not shown in this table. [b] Specific responses shown were calculated by subtracting the mean number of spots + 2 x SD of the medium control from the mean number of spots of experimental wells. The number of specific spots/ 100.000 PBMC is given. Peptide pool-specific T-cell frequencies ≥ 1/10.000 PBMC and at least 3-fold the prevaccination response were considered to be vaccine enhanced (new or enhanced responses indicated in bold). NA, not available for analysis; (-), no specific response to E6 or E7; MRM, memory recall mix was used as a positive control.

Figure 2. Vaccine-enhanced HPV16-specific IFNγ-producing T-cell responses in 2 clinical responding patients (405 and 409).



T-cell responses are shown at week 0 (prevaccination), week 16 (8 weeks after vaccination with HPV16 L2/E6/E7 protein, 4 weeks after vaccination with HPV16/18 E6/E7) and week 24. The prime-boost regimen induces HPV16 E6 and E7-specific IFNγ responses. The mean number of spots and *SE bars*/ 100,000 PBMC induced by the medium control or the peptides present in the E6 and E7 peptide pools are depicted. As positive control, a memory recall antigen mix was used.

Figure 3. Antibody responses of the patient group to HPV16 E6 and E7 throughout the prime-boost vaccination trial period.



Box-whisker plot representing serum antibody levels of the patient group before vaccination (week 0), after three prime vaccinations of HPV16 L2/E6/E7 protein (week12), after boost vaccination of HPV16/18 E6/E7 (week 16), and during clinical follow up (weeks 20 and 24).

Table 3. P-values from Wilcoxon's signed rank tests showing statistical evaluation of patient group antibody responses to HPV16/18 E6 and E7[a].

HPV type	Week 12	Week 16	Week 20	Week 24	Week 12 *versus* Week 16 (boost effect)
16 E6	**0.001**	**< 0.001**	**0.006**	**< 0.001**	0.158
16 E7	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.998
16 L1	0.46	0.89	0.99	0.19	0.42
18 E6	0.23	0.55	0.70	0.94	0.78
18 E7	0.51	0.43	0.44	0.63	0.10

[a] HPV16/18 E6/E7- and HPV16 L1-specific IgG levels at time 0 were compared with postvaccination HPV16 L2/E6/E7 (week 12), after boosting with HPV16/18 E6/E7 (week 16) and in the follow-up in a Wilcoxon's signed ranks test where $p \leq 0.05$ was considered significant. Comparisons were also made by the same method between weeks 12 and 16 to assess a HPV16/18 E6/E7 boost effect.

patient (201) had preexisting T-cells to several HPV18 E6 peptide pools and 3 others (307, 407, and 413) showed enhanced HPV18 E6-specific T-cell activity postvaccination at week 20. No preexisting or vaccine-induced HPV18 E7 reactivity was detected in this group of patients. Importantly, no indications were found for cross-reactivity between HPV16 and 18 peptides because patients responded to either HPV16 or HPV18 peptides or to different peptide pools of these two virus types. In summary, using IFNγ ELISPOT assays for the detection of HPV16- and HPV18 E6- and E7-specific T-cells, we found that 11 of 25 patients responded to vaccination.

Induction of HPV16 E6- and E7-specific antibodies

Compared with normal control subjects, the patients before vaccination had evidence of significantly higher levels of antibodies to HPV16 E7 ($p = 0.0001$), HPV18 E7 ($p = 0.036$), and HPV16 L1 ($p = 0.0001$) in a nonpaired non-parametric Mann-Whitney U test. Thirteen patients had HPV16 L1-specific antibodies at baseline greater than the arbitrary cutoff generated from the normal blood donors; these L1 antibody levels remained stable over the course of the study. Figure 3 shows box-whisker plots of group serological responses to HPV16 E6 and E7 after vaccination and statistical analysis. Table 3 shows significant group responses at all time points compared with baseline for both HPV16 E6 and E7 but not L1. No significant changes were seen in serological responses to HPV18 E6 or E7 after vaccination. As defined by a 3-fold increase from baseline and being above the normal cutoff level, 14 of 24 patients tested were categorized as showing positive IgG responses to HPV16 E7, 1 of whom also had positive responses to HPV16 E6 (Table 4). The responses to HPV16 E6 or E7 were transient, with levels peaking at week 16 and declining by weeks 20 and 24, consistent with the single booster vaccination with E6 and E7 encoding TA-HPV at week 12.

Table 4. Summary of patient immunological responses to vaccination[a]

Summary of immunological responses to HPV16

Patient no.	SI TA-CIN >3x increase	ELISPOT >3x increase	Antibodies to HPV16		Clinical responses
			E6	E7	
201	+	-	+	+	Progressor
301	+	+	+	-	Stable
302	-	NT	-	+	Stable
303	+	-	-	+	Stable
304	+	+	-	-	Stable
305	-	+	-	-	Progressor
306	-	NT	-	-	Progressor
307	-	-	-	-	Stable
308	-	-	-	+	Stable
309	-	-	-	+	Progressor
310	+	-	-	-	Stable
311	-	+	-	+	Responder
401	-	-	-	+	Responder
402	-	NT	-	+	Stable
403	-	-	-	-	Stable
404	D0 NT	+	-	+	Responder
405	D0 NT (x3 to boost)[b]	+	-	+	Responder
406	-	-	-	+	Responder
407	+	-	-	-	Stable
408	-	-	-	+	Stable
409	+	+	-	-	C. Responder
410	-	-	-	+	Stable
411	+	+	-	+	Stable
412	+	-	NT	NT	Stable
413	-	+	-	-	Stable
414	-	NT	NT	NT	Stable
415	-	-	NT	NT	Stable
416	-	-	NT	NT	Stable
417	-	-	NT	NT	Stable

[a] A vaccine-induced 3-fold increase in immunity at any postvaccination timepoint as measured by proliferation, enzyme-linked immunospot (ELISPOT), or ELISA is denoted by (+). (-) is < 3-fold and NT is not tested.

[b] Patient 404 and 405 samples were limited at time 0 for lymphoproliferation analysis; however, patient 405 did show a 3-fold SI increase at week 16 compared with week 12. Clinical responses are categorized: progressor, stable, responder, and complete (C.) responder.

Discussion

We have analyzed the immunogenicity of a combination of TA-CIN and TA-HPV candidate therapeutic vaccines for the treatment of HPV16-positive, high-grade AGIN in a Phase II clinical study. Our previous preclinical mouse model study had shown that the E7-specific T-cell antitumor activity was optimally boosted by a heterologous prime-boost protocol in which mice were primed by TA-CIN and boosted by TA-HPV[9]. In this study, there is clear evidence that the prime-boost vaccinations are immunogenic in AGIN patients, and the response appears to be biased to HPV16 E6. Evidence of a booster effect of the TA-HPV immunization is seen with HPV16 E6-specific proliferative responses, which were shown to be significantly higher at week 16, after the administration of TA-HPV. The HPV16 E6 bias in T-cell reactivity is also detected in the ELISPOT analysis of PBMC samples that were isolated after all vaccinations. *De novo* HPV18 E6 ELISPOT responses were found in 4 of 10 patients, consistent with the effectiveness of a single TA-HPV vaccination[5,6] and supporting immunogenicity of this component of the prime/boost regimen.

Strong proliferative TA-CIN-specific responses were found in the AGIN patients after the vaccinations with TA-CIN, and these are of similar magnitude as those found in TA-CIN vaccinated healthy subjects[7]. The higher level of TA-CIN-specific proliferation seen compared with that detected with E6 and the absence of E7-specific T-cell proliferation might be explained by a strong response to HPV16 L2 or an epitope derivative from the L2/E6/E7 fusion (and absent from the HPV6 L2E7 control). A preponderance of L2 directed T-cell reactivity may also explain the lack of detection of booster activity by injection with TA-HPV encoding the E6 and E7 oncoprotein only when tested in a proliferation assay with TA-CIN as stimulating antigen. Interestingly, those patients with the lowest preexisting TA-CIN-specific response were most likely to respond vigorously upon vaccination with TA-CIN. This might be indicative of a preexisting response masking vaccination-induced changes, with respect to the quality or type of T-cell response, not measured by the proliferation assays. Therefore, vaccine-induced proliferative responses may only be detected in those patients with no preexisting proliferative responses. Indeed, none of 5 patients in whom an E6-specific vaccine-induced proliferative response was detected had evidence of a preexisting E6 response. These observations point to some limitations of the TA-CIN proliferation assay.

In some of the cases, a nonprotective type of immunity (*e.g.,* Th2, Treg, or nonpolarized) preexisting T-cell response may exist or the E6- and E7-specific T-cells could have been anergized through the presentation of the two oncogenes to the immune system in a noninflammatory context, as was found in a HPV16 mouse model[13]. The long period over which the E6 and E7 proteins are presented to the immune system in the noninflammatory context of AGIN compared with the lack of L2 production in these lesions may hamper the induction of a strong type 1 T-cell reactivity against the two oncogenes when compared with L2. Indeed, the frequency of HPV16 E6-specific IFNγ-T-cell responses were found to be greater in patients with a preexisting Th1 type E6-response, whereas in the other patients, including those in which a vaccine-induced proliferative response was detected, a more modest response was induced (ref[5]; and this study).

Although priming of E6-specific T-cell reactivity is achieved by vaccination with TA-CIN (ref[7]; and this study), the major increase in E6-specific T-cell reactivity is due to the booster vaccination with TA-HPV. Analysis of the proliferative group responses revealed that the E6-specific T-cell responses were significantly increased at week 16, which is 4 weeks after vaccination with TA-HPV. Furthermore, our previous analysis of T-cell reactivity in high-grade VIN patients also revealed that vaccination with TA-HPV enhanced especially the numbers of IFNγ-producing E6-specific T-cells[5]. Such HPV16 E6-specific T-cell immunity is frequently detected in healthy subjects, and this is supportive of a role in protection against persistent HPV infection and associated development of malignancies[14]. The fact that the heterologous prime-boost protocol increases the numbers of these effectors should therefore offer potential therapeutic value in some of the high-grade VIN patients.

Clinically, we observed objective responses in 6 patients and 19 had stable disease, and in 4 patients, there was evidence of progression. Table 4 summarizes these and the immunological responses. Of the 6 clinical responders, 2 patients showed both a proliferative TA-CIN-specific response and an E6-specific IFNγ response, whereas 3 other patients displayed E6 reactivity only. Stable disease was recorded in 19 patients, 8 of whom showed a concomitant TA-CIN-specific proliferative and/or E6-specific T-cell response, whereas in the other 11 patients, no vaccine-induced response could be detected. Of the 4 progressors, 2 failed to make a T-cell response and 2 responded by either proliferation or E6 ELISPOT alone. There is no statistically demonstrable association between clinical response and immunological response measured by either TA-CIN proliferation or ELISPOT or anti-E7 antibodies (odd ratios of 0.36,

0.11, and 0.16, respectively). However, such individual immune responses of patients were defined by 3-fold increases in activity from baseline and are thus a reflection of the magnitude of measurable vaccine-induced change rather than inherent HPV immunity. Focusing on combinations of immunological activity, outcome of vaccination in patients and other factors may give another perspective. Our previous analysis of vaccinated VIN patients showed that clinical responsiveness to treatment was dependent on the presence of lesion-associated $CD4^+$, $CD8^+$, and $CD1a^+$-immune cells[6], suggesting that not only the numbers of systemically present HPV16-specific T-cells are important but also their capacity to reach the target site. In the design of new trials, local immune infiltration will be an important factor to consider in relation to outcome. Preselection of high-grade VIN patients with lesions displaying T-cell infiltrate before vaccination may identify a group of subjects who benefits the most from vaccination.

Acknowledgements

We thank all patient participants of the vaccine trial and normal sera donors. We also thank the TA-HPV project team at Xenova Research Ltd., Jane Sterling, and Peter Baldwin.

References

1. Stern PL, Faulkner R, Veranes EC, et al. The role of human papillomavirus vaccines in cervical neoplasia. Best Pract Res Clin Obstet Gynaecol 2001; 15: 783-799.
2. Stanley MA. Progress in prophylactic and therapeutic vaccines for human papillomavirus infection. Expert Rev Vaccines 2003; 2: 381-389.
3. Borysiewicz LK, Fiander A, Nimako M, et al. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet 1996; 347: 1523-1527.
4. Kaufmann AM, Stern PL, Rankin EM, et al. Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 E6 and E7 genes, in women with progressive cervical cancer. Clin Cancer Res 2002; 8: 3676-3685.
5. Baldwin PJ, van der Burg SH, Boswell CM, et al. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003; 9: 5205-5213.
6. Davidson EJ, Boswell CM, Sehr P, et al. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003; 63: 6032-6041.
7. de Jong A, O'Neill T, Khan AY, et al. Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T-cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. Vaccine 2002; 20: 3456-3464.
8. Davidson EJ, Faulkner RL, Sehr P, et al. Effect of TA-CIN (HPV 16 L2E6E7) booster immunisation in vulval intraepithelial neoplasia patients previously vaccinated with TA-HPV (vaccinia virus encoding HPV 16/18 E6E7). Vaccine 2004; 22: 2722-2729.
9. van der Burg SH, Kwappenberg KM, O'Neill T, et al. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine, in homologous and heterologous prime-boost regimens. Vaccine 2001; 19: 3652-3660.
10. Sehr P, Zumbach K, Pawlita MA. generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. J Immunol Methods 2001; 253: 153-162.
11. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II- restricted epitopes. Int J Cancer 2001; 91: 612-618.
12. Sehr P, Muller M, Hopfl R, et al. HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase. J Virol Methods 2002; 106: 61-70.
13. Frazer IH, De Kluyver R, Leggatt GR, et al. Tolerance or immunity to a tumor antigen expressed in somatic cells can be determined by systemic proinflammatory signals at the time of first antigen exposure. J Immunol 2001; 167: 6180-6187.
14. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.

CHAPTER 6

Detection of human papillomavirus (HPV)16-specific CD4+ T-cell immunity in patients with persistent HPV16-induced vulvar intraepithelial neoplasia in relation to clinical impact of imiquimod treatment

Mariëtte I.E. van Poelgeest
Manon van Seters
Marc van Beurden
Kitty M.C. Kwappenberg
Claudia Heijmans-Antonissen
Jan Wouter Drijfhout
Cornelis J.M. Melief
Gemma G. Kenter
Theo J.M. Helmerhorst
Rienk Offringa
Sjoerd H. van der Burg

Clinical Cancer Research 2005; 11: 5273-5280

Abstract

Purpose: Topical application of the immune response modifier imiquimod is an alternative approach for the treatment of human papillomavirus (HPV)-positive vulvar intraepithelial neoplasia (VIN) and aims at the immunologic eradication of HPV-infected cells. We have charted HPV16-specific immunity in 29 patients with high-grade VIN and examined its role in the clinical effect of imiquimod treatment.
Experimental design: The magnitude and cytokine polarization of the HPV16 E2-, E6-, and E7-specific CD4+ T-cell response was charted in 20 of 29 patients by proliferation and cytokine bead array. The relation between HPV16-specific type 1 T-cell immunity and imiquimod treatment was examined in a group of 17 of 29 patients.
Results: HPV16-specific proliferative responses were found in 11 of the 20 patients. In 8 of these patients, T-cell reactivity was associated with IFNγ production. Fifteen of the women treated with imiquimod were HPV16+, of whom 8 displayed HPV16 E2- and E6-specific T-cell immunity before treatment. Imiquimod neither enhanced nor induced such immunity in any of the subjects. Objective clinical responses (complete remission or >75% regression) were observed in 11 of the 15 patients. Of these 11 responders, 8 patients displayed HPV16-specific type 1 CD4+ T-cell immunity, whereas 3 lacked reactivity. Notably, the 4 patients without an objective clinical response also lacked HPV16-specific type 1 T-cell immunity.
Conclusions: HPV16-specific IFNγ-associated CD4+ T-cell immunity, although not essential for imiquimod-induced regression of VIN lesions, may increase the likelihood of a strong clinical response ($p = 0.03$).

Introduction

Genital infections with high-risk human papillomaviruses (HPV) are very common[1-3]. Fortunately, the majority of infected subjects clear the infection[4,5]. A persistent infection with a high-risk HPV, mostly HPV16, can lead to neoplasia of the anogenital tract, of which cervical intraepithelial neoplasia (CIN) and cervical carcinoma are the most well known[6,7]. HPV16 infection may also cause a chronic skin disorder of the vulva known as vulvar intraepithelial neoplasia (VIN)[8-10]. In contrast to CIN, which in general is effectively treated by eradication of the area involved, VIN is a chronic disease with high relapse rates after standard treatments[11-13].

Imiquimod therapy has been put forward as an alternative approach for the treatment of VIN. This immune response modifier acts through Toll-like receptor 7 of the innate immune system resulting in the secretion of a multitude of proinflammatory cytokines. There is recent evidence that imiquimod also possesses direct proapoptotic activity against tumor cells[14-16]. Topical application preserves the anatomy and function of the vulva, whereas surgical excision or ablation of affected skin may be extensive and disfiguring and can carry considerable psychosexual morbidity. Clinical success rates differ and are estimated on 30% to 87%[17-21].

The HPV16 early antigens E2, E6, and E7 are among the first of proteins that are expressed in HPV-infected epithelia. Our previous studies on HPV-specific T-cell immunity against these early antigens showed that type 1 (IFNγ) T-cell memory against the early antigens can be detected in the majority of healthy sexually active individuals but is weak or absent in patients with HPV16-induced cervical neoplasia[22-24]. In combination with earlier reports that point at a role for CD4+ T-cells in the protection against progressive HPV infection (reviewed in[25]), our data argue that the CD4+ type 1 T-cell response against the early antigens of HPV16 plays an important role in the protection against progressive HPV16-induced disease.

To examine the role of HPV16-specific CD4+ T-cell immunity in the success or failure of treatment with imiquimod, we have done a detailed analysis with respect to the magnitude and cytokine polarization of the HPV16-specific CD4+ T-cell response in patients with high-grade VIN. Furthermore, HPV16-specific type 1 immunity was analyzed before, during, and after topical treatment with imiquimod. Our data indicate that chronic exposure of the immune system to the HPV16 viral proteins results in the induction of type 1 T-cell immunity in about half of the patients. Importantly, the presence of these type 1 T-cell responses is likely to be associated with a more favorable clinical response to imiquimod treatment.

Methods

Patients

Twenty-nine women with high-grade VIN (age range, 24-73 years; median age, 47 years) were recruited from the departments of gynecology of the Academic Medical Center and Leiden and Erasmus University Medical Center, The Netherlands. On the average, these patients had been diagnosed with VIN 3 5.4 years before enrollment in the study (range, 6 months to 15 years). Eighteen women had undergone previous treatments for VIN 3 (surgical excision, laser therapy, or imiquimod treatment (patients 20, 21, 24, 27)) before study entry.

Seventeen of these 29 subjects (age, 29-60 years; median, 43 years) were experimentally treated with a 5% imiquimod cream. The patients were asked to apply the cream to the affected areas on the vulva twice-weekly overnight for a maximum period of 16 weeks. To analyze the effect of imiquimod treatment on the HPV16-specific immune response, we collected serial blood and serum samples before the start of imiquimod treatment (T=0), after 8 weeks of treatment (T=8), and at the end of treatment (T=16). Vulvar lesions were assessed by direct measurement and photographic records at entry and after 8 and 16 weeks of treatment. Clinical responses were defined as a complete response; a partial response type 1, as defined by a reduction in lesion diameter from 76% to 99%; a partial response type 2, as defined by a reduction in lesion diameter from 26% to 75%; or no clinical response.

From 20 of 29 women peripheral blood mononuclear cells (PBMC) were isolated and directly used to analyze HPV16-specific proliferative T-cell reactivity. Of these 20 women, 8 patients had also participated in the imiquimod study. In 6 cases blood was taken 3 months (patient 1), 4 months (patient 10), 10 months (patient 5) to over 1 year (patients 12, 13 and 15) after the end of the imiquimod study, in the other 2 cases (patients 2 and 4) blood was taken within 4 weeks after the start of treatment. Serum was collected to study the presence of virus-like particle L1 (VLP)-specific antibodies.

All subjects were typed for HPV by GP5+/6+ PCR followed by reverse line blot analysis as described previously[26]. The study design was approved by the Medical Ethical Committees and all women gave written informed consent.

Antigens

A set of peptides spanning the whole HPV16 E2, E6, and E7 protein were used for the T-cell proliferation assays. The E2 peptides consisted of twenty-two 30-mer peptides with a 15-amino-acid overlap and the COOH-terminal peptide with a length of 35 amino acids. For the T-cell proliferation assays, the E2 peptides, 32-mer peptides of

the E6 protein, and the 35-mer peptides of the E7 protein with an overlap of 14 amino acids were used in pools of two peptides per pool. For the IFNγ enzyme-linked immunospot (ELISPOT) assays, the peptides used spanned the HPV 16 E2, E6, and E7 protein and consisted of the most immunogenic regions of the E2 30-mer peptides[22] and 15 E6 and 9 E7 overlapping 22-mer peptides. The peptides were synthesized and dissolved as decribed previously[27]. The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (*e.g.,* $E2_{1-45}$, residues 1-30 and 16-45). Memory response mix (MRM), consisting of a mixture of tetanus toxoid (0.75 *limus flocculentius*/ml final concentration; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 µg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and *Candida albicans* (0.005%, HAL Allergenen Lab., Haarlem, The Netherlands), was used as a positive control.

Short-term T-cell proliferation assay

Freshly isolated PBMC were incubated with 12 pools of HPV16 E2-derived 30-mer peptides, 4 pools of E6 32-mer peptides, and 2 pools of E7 35-mer peptides (each pool consisted of two overlapping peptides). PBMC were seeded at a density of 1.5 x 10^5 cells per well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 125 µl of Iscove's medium (BioWhittaker, Verviers, Belgium) supplemented with 10% autologous serum. HPV16 E2-, E6-, and E7-derived peptides were added at a concentration of 10 µg/ml/peptide. Medium alone was taken along as a negative control, and memory response mix (dilution, 1:50) served as a positive control. For each peptide pool, eight parallel microcultures were incubated. Fifty microliters of supernatant from the microcultures were taken at day 6 after incubation and stored at -20°C until cytokine analysis. Peptide-specific proliferation was measured at day 7 by [^{3}H]thymidine incorporation. Cultures were scored positive when the proliferation of ≥ 75% of the test wells exceeded the mean proliferation + 3 x SD of the control wells containing medium only, and the stimulation index, defined as the mean of all test wells divided by the mean of the control wells, was ≥ 3[22].

Analysis of cytokines associated with HPV16-specific proliferative responses

The detection of cytokines in the supernatants of the short-term proliferation assays was done using the cytometric bead array (CBA) (Becton Dickinson, Erebodegem-Aalst, Belgium). This technique allows the simultaneous detection of six different Th1 and Th2 cytokines IFNγ, tumor necrosis factor α, interleukin (IL)-2, IL-4, IL-5, and IL-10. The CBA was done according to the manufacturer's instructions. Cutoff values

were based on the standard curves of the different cytokines (50 pg/ml for IFNγ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and > 2x the concentration of the medium control[23,28].

Analysis of HPV16-specific T-cell reactivity by IFNγ enzyme-linked immunospot

The number of IFNγ producing HPV-specific T-cells, present in the peripheral blood of the 17 patients treated with imiquimod, was quantified using ELISPOT that was done as described previously[29,30]. Briefly, PBMC were thawed, washed, and seeded at a density of 2 x 10^6 cells per well of a 24-well plate (Costar) in 1ml of Iscove's modified Dulbecco's medium (Bio Whittaker) enriched with 10% human AB serum, in the presence or absence of indicated HPV16 E2, E6, and E7 peptide pools. Peptides were used in pools of 4 to 5 peptides at a concentration of 5 µg/ml/peptide. The peptides, as indicated by their first and last amino acid in the protein, were used in the following pools: *E2-I*: 1-30, 16-45, 31-60, 46-75; *E2-II*: 61-90, 76-105, 91-120, 106-135; *E2-III*: 121-150, 136-165, 151-180, 166-195; *E2-IV*: 271-300, 286-315, 301-330, 316-345, 331-365; *E6-I*: 1-22, 11-32, 21-42, 31-52; *E6-II*: 41-62, 51-72, 61-82, 71-92; *E6-III*: 81-102, 91-112, 101-122, 111-132; *E6-IV*: 111-132, 121-142, 131-152, 137-158; *E7-I*: 1-22, 11-32, 21-42, 31-52; *E7-II*: 41-62, 51-72, 61-82, 71-92, 77-98. Following 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells per well in 100 µl Iscove's modified Dulbecco's medium enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ-catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT was done according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (Bio Sys). Specific spots were calculated by subtracting the mean number of spots + 2 x SD of the medium control from the mean number of spots in experimental wells provided that the mean number of spots of the medium control wells were either <10 or >10 with a SD <20% of the mean. Antigen-specific T-cell frequencies were considered to be increased when specific T-cell frequencies were ≥ 1/10,000 and at least ≥ 2x background[30]. The background number of spots was 2,6 ± 2,2 (mean ± SD), with one exception (patient 23, 51 ± 10 spots).

HPV16 virus-like particle ELISA

For the detection of HPV16-specific antibodies in serum we used an ELISA method previously described by Kirnbauer et al.[31]. Each serum sample was tested for reactiv-

ity against HPV16 VLPs (baculovirus-expressed capsids comprising the L1 protein) and against bovine papillomavirus capsids, the latter disrupted by treatment with 0.1 mol/L carbonate buffer to serve as a negative control. Both VLP and bovine papillomavirus were kindly provided by Prof. Dr. J. Dillner (LUNDS University, Sweden). The patients were tested for both HPV16-specific IgG and IgA. A set of sera of healthy children (n=8; mean age, 7.3 years; range, 4.3-14.1 years) was tested to determine background reactivity. For HPV16 L1-VLP IgG type responses a cutoff absorbance value of 0.230 was used (mean A = 0.060; range –0.056 to 0.150; mean + 2 x SD = 0.230). For IgA type responses a cutoff of A = 0.215 was used (mean A = 0.189; range, 0.171 to 0.205).

Statistical analysis

Statistical analysis of the HPV16-specific proliferative responses associated with cytokine production was done using Fisher's exact test. Fisher's exact test (two tailed) was used to analyze HPV-specific immunity to clinical response upon treatment with imiquimod.

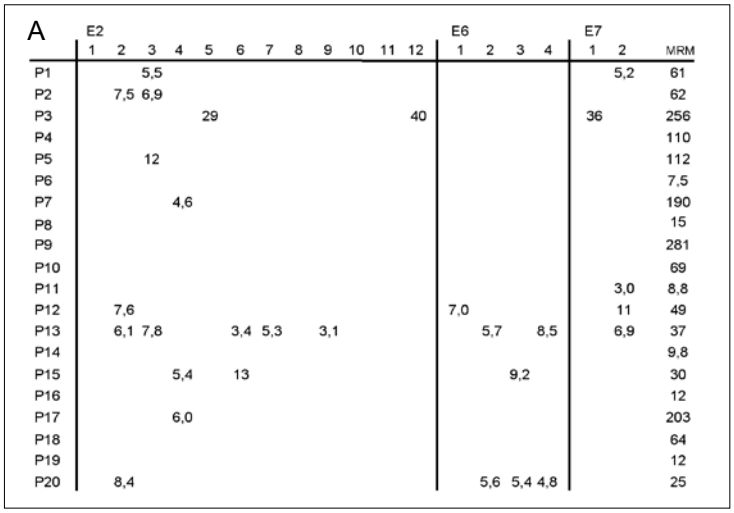
Statistical analyzes were done using Graphpad Instat Software (version 3.0).

Results

HPV16-specific cellular and humoral responses in patients with high-grade VIN

VIN forms a unique aspect of HPV-induced disease because patients are frequently treated, but the infection often persists. HPV16 is found most often. To gain a more profound insight in the CD4+ T-cell response against HPV16 in VIN, we charted the magnitude, specificity, and functionality of HPV16 E2, E6, and E7-specific proliferative T-cell responses in a group of 20 women with HPV16-associated high-grade VIN.

PBMC isolated from VIN patients were stimulated with peptides derived from HPV16 proteins E2, E6, and E7 as well as with a mix of common recall antigens (memory response mix, MRM), in a short-term proliferation assay. We have previously shown that this assay is geared towards the detection of CD4+ T-cell responses[23]. HPV16-specific proliferative T-cell responses against E2 and/or E6 were detected in 10 of 20 patients (Figure 1A). E7-specific responses were detected in 5 of 20 subjects. Analysis of the supernatants of these T-cell cultures for the presence of type 1 and type 2 cytokines revealed the secretion of the Th1 cytokine IFNγ in 8 of 20 patients. In some of the patients, the production of tumor necrosis factor α, IL-5, and IL-10 was occasionally detected (Figure 1B). Although the overall frequency of proliferative responses is



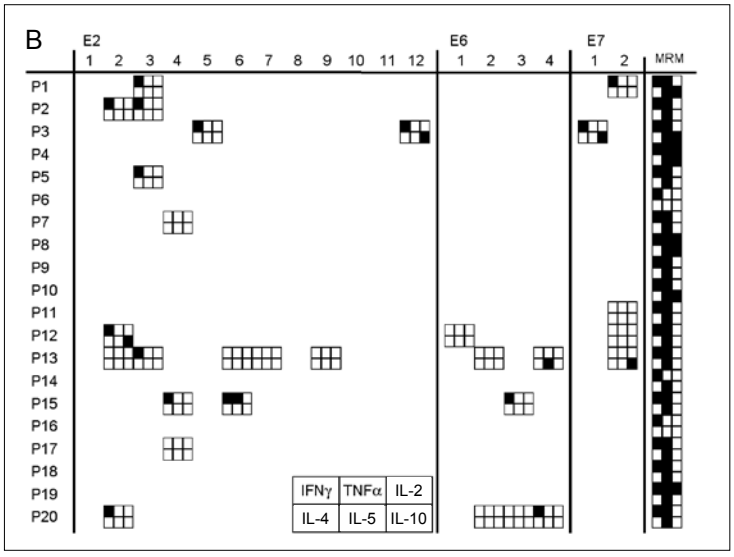


Figure 1A. HPV16-specific proliferative T-cell responses in VIN
Freshly isolated PBMC from 20 patients with high-grade HPV16-associated VIN were tested in short-term proliferation assays using a complete set of HPV16 E2-, E6-, and E7-derived peptide pools. Responses were scored positive when the proliferation (cpm) of ≥ 6 of 8 test wells exceeded the mean proliferation + 3 x SD of the control (medium only) wells, and the mean stimulation index of all test wells over control wells was ≥ 3. Memory response mix (MRM), consisting of a mixture of recall antigens, was used as a positive control. The stimulation indices of responses scored positive are indicated.

Figure 1B. Supernatants of the positive proliferative responses indicated in *A* were analyzed for the presence of IFNγ, tumor necrosis factor-α (TNFα), IL-2, IL-4, IL-5, and IL-10 by cytometric bead array. The indicated layout is used for the six measured cytokines; a filled square represents antigen-specific cytokine production. Cutoff values were based on the standard curves of the different cytokines (50 pg/ml for IFNγ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and >2x the concentration of the medium control.

similar when compared with that previously found for cervical cancer patients, the number of patients with IFNγ-associated HPV-specific T-cell responses in these VIN patients was higher (8 of 20 versus 4 of 17, respectively;[23]).

In addition to T-cell immunity, the humoral response to HPV16 was measured in 28 VIN patients by ELISA using HPV16 L1-VLP as antigen. Overall, HPV16 L1-VLP IgG and IgA antibodies were detected in 25 of 28 (89%) and 13 of 28 (46%) subjects, respectively (Table 1). Based on the absorbance values, the HPV16 L1-VLP-specific IgG response exceeded that of IgA (Table 1). In general, HPV16-specific IgA responses were detected when patients displayed relatively high levels of HPV16-specific IgG. If IgG absorbance values were ≥0.5, 11 of 19 (58%) of the samples contained HPV16 L1-specific IgA, whereas at IgG levels <0.5 only 2 of 9 samples were IgA seropositive.

In conclusion, HPV16 L1-specific humoral immunity was detected in the great majority of patients, whereas HPV16 E2-, E6-, and/or E7-specific IFNγ-associated type 1 T-cell reactivity was detected in about half of the patients tested.

Table 1. Distribution of absolute absorbance values among IgG- and IgA-seropositive samples

	$\Delta A_{415\ nm}$	
Immunoglobulin type (*n* positive/28)	Mean (SD)	Median (range)
IgG seropositive (*n*=25)	1.10 (0.61)	1.22 (0-1.93)
IgA seropositive (*n*=13)	0.34 (0.26)	0.31 (0-0.88)

HPV16 L1 IgG and IgA antibodies detected in the sera of 28 VIN 3 patients. Serum antibody responses were measured by VLP-ELISA. Depicted are the absolute absorbance values at 415 nm. The absorbance values were calculated by subtraction of the background response value and the mean absorbance value of the young children's sera.

HPV16-specific immunity is associated with a more favorable clinical response upon immunomodulatory treatment with imiquimod

Our analysis of HPV16-specific proliferation indicates that a high number of the proliferative T-cell responses is associated with IFNγ production. To examine the role of these HPV16-specific type 1 T-cell responses in the success or failure of treatment with the immunomodulator imiquimod, we studied this immune response in a group of patients with high-grade HPV16+ VIN. PBMC were isolated before (T=0), during (T=8), and after (T=16) treatment and stored in liquid nitrogen. HPV-specific T-cell reactivity against HPV16 peptides E2, E6, and E7 was analyzed by IFNγ ELISPOT. This is a sensitive method for the analysis of antigen-specific type 1 T-cell reactiv-

ity on frozen material[32,33]. Three of these patients had been treated with imiquimod in the year before inclusion in our study (Table 2, patients 21, 24, and 27). Of these 17 patients, 15 were HPV16-positive. Preexisting IFNγ-associated T-cell responses (T=0) were detected in 8 of 15 patients by IFNγ ELISPOT. In 5 of 15 patients, HPV16-specific T-cell reactivity against E2 was detected, whereas 4 of 15 patients displayed

Table 2. HPV16-specific T-cell responses in patients treated with imiquimod

Patient*	HPV type	clinical response†	VLP‡	T	E2-peptides				E6-peptides				E7-peptides		MRM
					1§ 75	61 135	121 195	271 365	1 52	41 92	81 132	111 158	1 52	41 98	
1	16	no	++	8	-	-	-	-	-	-	-	-	-	-	**179**
2	16	PR1	+	0	**52**	**54**	**15**	**31**	-	-	*3*	-	*9*	-	**295**
4	16	CR	++	0	-	-	-	-	-	*1*	-	-	-	-	**124**
5	16	CR	++	0	-	**32**	*9*	*3*	-	-	-	-	-	-	**32**
10	16	PR1	-	0	-	*4*	-	-	-	-	**84**	-	*5*	-	**54**
12	16	PR1	++	0	**15**	-	-	-	-	-	-	-	-	-	**32**
13	16	CR	++	0	-**	-	-	-	-	-	**104**	-	-	-	**105**
15	16	PR1	+	0	-	-	-	-	-	-	-	-	-	-	**179**
21	neg	PR1	++	0	-	-	-	-	-	-	-	-	-	-	**364**
22	16	PR2	++	8	*8*	-	-	*4*	-	-	-	-	-	-	**25**
23	16	PR1	++	0	-	*1*	**59**	*5*	-	**74**	-	-	*21*§§	*11*	**52**
24	16	no	++	0	-	-	-	-	-	-	-	-	-	-	**172**
25	neg	CR	+	0	-	-	-	*4*	-	-	-	-	-	-	**444**
26	16	CR	++	0	-	-	-	-	-	-	-	-	-	-	**21**
27	16	no	++	0	-	*4*	-	-	-	-	-	-	-	-	**70**
28	16	CR	++	0	*6*	-	**45**	**18**	-	*7*	*3*	*2*	*3*	-	**20**
29	16	CR	-	0	-	*2*	*7*	-	-	**40**	-	-	-	*2*	**157**

Abbreviations: CR, complete response; PR, partial response; MRM, memory response mix.

* PBMC from 17 VIN 3 patients were tested for type 1 T-cell reactivity against HPV16 peptides. PBMC were stimulated with different pools of HPV16 E2, E6, and E7 peptides and tested for antigen-specific IFNγ production by ELISPOT.

† Clinical responses were defined as no clinical response, a partial response type 1, as defined by a reduction in lesion diameter from 76% to 99%; a partial response type 2, as defined by a reduction in lesion diameter from 26% to 75%; and a complete response (CR).

‡ Sera of the patients were tested for the presence of HPV16 L1-VLP specific IgG antibodies. Indicated is the presence (+) or absence (-) of antibodies.

§ The first and last amino acid in the indicated protein of the peptide pool used are indicated.

** Per patient, T-cell responses on T=0 are shown. In case of a missing T=0 sample, data from T=8 are shown. Specific responses were calculated by subtracting the mean number of spots + 2 x SD of the medium control from the mean number of spots of experimental wells. The number of specific spots per 100,000 PBMC are given. Responses were considered positive if peptide pool-specific T-cell frequencies were ≥ 10/100,000 PBMC. These values are indicated in bold. Values below this threshold are shown in italics. (-), no specific response to E6 or L1. MRM was used as a positive control.

§§ Responses considered negative because values did not exceed ≥ 2x the medium control.

a response against E6 (Table 2). None of these patients showed preexisting T-cell responses against HPV16 E7. In 2 cases the T=0 sample was not available and the reaction in PBMC from T=8 are shown (Table 2, patients 1 and 22).

Despite that for some patients one of the two follow-up samples was not available (patients 5, 13, 27, and 28), it was clear that we could not detect a direct influence of imiquimod on the numbers of HPV-specific T-cells. In none of the patients was a clear-cut increase of HPV16-specific T-cells detected upon imiquimod treatment (Figure 2A,B). In some cases, patients had already been treated with a course of imiquimod before this study, but even this repeated treatment did not result in an

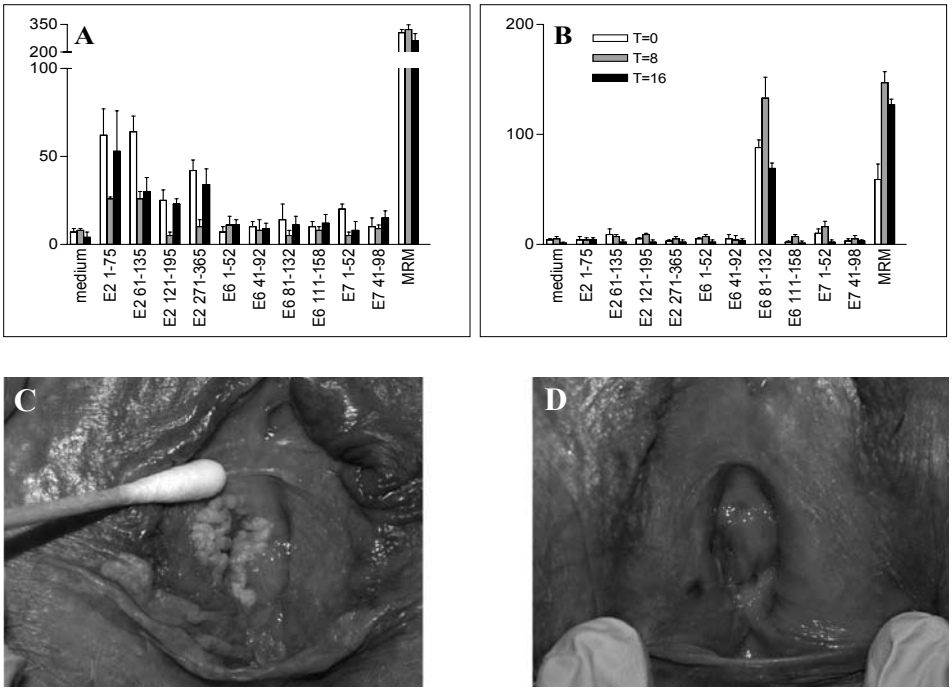


Figure 2A and B. HPV16-specific IFNγ-associated T-cell responses in two representative patients with high-grade VIN (patient 2, *left* and patient 10, *right*). T-cell responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). Local application of 5% imiquimod containing cream does not result in enhanced systemic HPV16-specific T-cell responses. Note that the magnitude of the T-cell responses varies slightly over the different time points. The mean number of spots and SE induced by the medium control or the peptides present in the E2, E6, and E7 pools per 100,000 PBMC are depicted. As positive control, the memory recall mix (MRM) was used.

Figure 2C and D. patients with preexisting HPV16-specific T-helper type 1 responses show objective clinical responses after imiquimod treatment. A typical example is shown. *C.* biopsy-proven VIN 3 lesion of patient 5 before imiquimod treatment. *D.* the same vulvar area of patient 5 after 16 weeks of treatment.

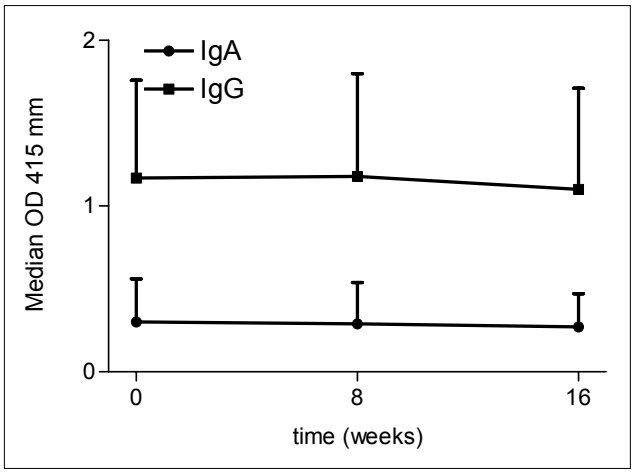


Figure 3. IgG and IgA reactivity to HPV16 VLPs over time in 17 VIN 3 patients treated with imiquimod. At least two serum specimens were tested in every patient. Serologic responses are shown at week 0 (before imiquimod treatment), week 8 (during imiquimod treatment), and at week 16 (after imiquimod treatment). The absorbance (OD) values are depicted as median ± SD of positive responses. The absorbance values were calculated by subtraction of the background response value and the mean absorbance value of the young children's sera.

increase of HPV16-specific T-cells (Table 2, patients 21 and 24). In addition, the HPV16 VLP-specific IgG and IgA response did not overly change when patients were treated with imiquimod (Figure 3).

Thirteen of the 17 women treated (76%) displayed an overt clinical response upon treatment with imiquimod as indicated by 76% to 100% reduction in the size of their lesion (complete response or partial response 1, Table 2 and Figure 2C,D). Three patients showed no reduction in size of the affected area of vulvar disease and one woman showed only minimal improvement upon treatment.

Importantly, when the group of HPV16+ patients (n=15) was divided in patients either with or without an HPV-specific Th1 immune response, all 8 patients with an HPV-specific immune response displayed a complete or near complete clinical response (complete response or partial response 1) upon imiquimod treatment (Table 2). In contrast, patients without an HPV-specific immune response were less likely to show such a clinical improvement (p = 0.03, two-sided Fisher's exact test).

Taken together, chronic viral antigen exposure can induce type 1 CD4+ T-cell immunity against the HPV16 early antigens E2, E6, or E7 in patients with VIN 3. The presence of these HPV16-specific Th1 cells as detected by IFNγ ELISPOT, although not

essential for imiquimod-induced regression of VIN lesions, does increase the likelihood of a strong clinical response. The presence of L1-specific humoral reactivity was not correlated with imiquimod-induced regressions.

Discussion

We have analyzed the HPV16 E2-, E6-, and E7-specific CD4+ T-cell responses in a group of 29 patients with high-grade VIN, 17 of whom were treated with the immunomodulator imiquimod. HPV16-specific type 1 (IFNγ) CD4+ T-cell proliferative immunity is present in about half of patients with VIN 3 (8 of 20). Virus-specific CD4+ Th1-type T-cells have emerged as an essential component in the immune response to chronic viral infection, fulfilling a multifactorial role, including the activation of antigen-presenting cell maturation for efficient CD8+ priming, the release of cytokines important in CD8+ T-cell proliferation and differentiation, and in the recruitment of other effector cells such as eosinophils and macrophages. Indeed, a substantial number of patients with VIN 3 were reported to display high frequencies of HPV16-specific CD8+ T-cells[34-36]. In contrast, only in a few occasions HPV16-specific CD8+ T-cell reactivity was detected in patients with CIN 3 and cervical carcinoma[37-40]. However, these latter types of patients display an impaired HPV16-specific CD4+ T-cell response[23].

Topical application of imiquimod neither enhanced the preexistent HPV16-specific CD4+ T-cell responses nor resulted in the induction of such responses in any of the other subjects. Todd et al. made a similar observation with respect to HPV16-specific CD8+ T-cells[36]. Notably, we found that a preexisting HPV-specific type 1 T-cell response was associated with a more favorable clinical outcome upon topical imiquimod treatment of VIN 3. This indicates that a combination therapy, in which the HPV16-specific T-cell response is induced or boosted by vaccination and the affected skin is treated with imiquimod, may increase the number of patients that benefit from treatment.

Compared with normal vulvar skin, a number of VIN lesions display increased infiltration of CD4+ and CD8+ T-cells[41-43]. The clinical consequences of the infiltration of immune cells in these VIN lesions are poorly understood, but the immunological make-up of the vulvar microenvironment may determine the clinical outcome[43]. The local cytokine microenvironment in high-grade cervical neoplasia is associated with a decreased expression of the proinflammatory Th1 cytokines tumor necrosis factor α and IFNγ[44-46]. It is conceivable that similar to CIN, the vulvar micro-

environment also lacks proinflammatory cytokines. Imiquimod is known to directly stimulate Langerhans cells and macrophages[17,47], of which the latter are increased in VIN lesions[42]. Furthermore, it stimulates natural killer cells and T-helper type 1 cells via indirect mechanisms[17,47]. Upon stimulation, the antigen-presenting cells release proinflammatory cytokines, predominantly IFNα, tumor necrosis factor α, and IL-12[14,15 17,47]. This may restore an inducive environment in which the innate effector cells, macrophages, and natural killer cells, as well as activated HPV16-specific T-cells may act in concert to form an effective immune response. The requirement for these additional signals to activate T-cells is sustained by recent observations in animal models. In the HPV16 E7-transgenic skin transplantation model Matsumoto et al.[48] showed that despite the presence of large numbers of E7-specific memory T-cells, E7+ skin transplants were not rejected, except when these E7-specific memory T-cells were activated through vaccination. This suggested that the presence of the HPV16 E7 antigen itself is not sufficient to evoke a strong skin-destroying immune response but that additional activating signals were required. Similarly, Van Mierlo et al.[49] showed that adenovirus-specific CD8+ T-cells developed in the draining lymph nodes of mice bearing adenovirus-positive tumors, indicating that tumor-antigen was detected by T-cells of the immune system. The tumor was rejected only when strong proinflammatory signals were provided. Likewise, HPV16-induced VIN 3 lesions may fail to endow the immune system with strong inflammatory signals and exogenously provided signals will be required to provide a state of inflammation. These signals can be delivered by imiquimod, electrocoagulation[50] or by vaccines[32,33,43].
Currently, it is not clear whether immune activation causes the HPV16-specific IFNγ-producing CD4+ T-cells to migrate into the HPV-infected tissue or whether these T-cells should simply provide help to activate effector cells in the draining lymph nodes. Therefore, we are currently examining both local and systemic immune response in patients with high-grade VIN.

Acknowledgments

We thank all patients who have participated in this study, the staff of the Laboratory of Experimental Immunology (head: Prof. Dr. R.A.W. van Lier) at the Academic Medical Center in Amsterdam for the isolation of PBMC, the Department of Pathology (head: Prof. Dr. C.J.L.M. Meijer) at the Free University Medical Center in Amsterdam for human papillomavirus testing, and Prof. J. Dillner for providing HPV16 VLP-L1 and BPV-L1 and helpful advice in the analysis of the HPV16-specific humoral responses.

References

1. Burk RD, Kelly P, Feldman J, et al. Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. Sex Transm Dis 1996; 23: 333-341.
2. Koutsky L. Epidemiology of genital human papillomavirus infection. Am J Med 1997; 102: 3-8.
3. Schiffman M, Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. J Natl Cancer Inst Monogr 2003; 14-19.
4. Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis 1995; 171: 1026-30.
5. Ho GY, Bierman R, Beardsley L, et al. Natural history of cervicovaginal papillomavirus infection in young women. N Engl J Med 1998; 338: 423-428.
6. Remmink AJ, Walboomers JM, Helmerhorst TJ, et al. The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease: natural history up to 36 months. Int J Cancer 1995; 61: 306-311.
7. Kjaer SK, van den Brule AJ, Paull G, et al. Type specific persistence of high risk human papillomavirus (HPV) as indicator of high grade cervical squamous intraepithelial lesions in young women: population based prospective follow up study. BMJ 2002; 325: 572.
8. van Beurden M, ten Kate FJ, Smits HL, et al. Multifocal vulvar intraepithelial neoplasia grade III and multicentric lower genital tract neoplasia is associated with transcriptionally active human papillomavirus. Cancer 1995; 75: 2879-2884.
9. Buscema J, Naghashfar Z, Sawada E, et al. The predominance of human papillomavirus type 16 in vulvar neoplasia. Obstet Gynecol 1988; 71: 601-606.
10. Hording U, Junge J, Poulsen H, et al. Vulvar intraepithelial neoplasia III: a viral disease of undetermined progressive potential. Gynecol Oncol 1995; 56: 276-279.
11. Sykes P, Smith N, McCormick P, et al. High-grade vulvar intraepithelial neoplasia (VIN 3): a retrospective analysis of patient characteristics, management, outcome and relationship to squamous cell carcinoma of the vulva 1989-1999. Aust N Z J Obstet Gynaecol 2002; 42: 69-74.
12. Andreasson B, Bock JE. Intraepithelial neoplasia in the vulvar region. Gynecol Oncol 1985; 21: 300-305.
13. Rettenmaier MA, Berman ML, DiSaia PJ. Skinning vulvectomy for the treatment of multifocal vulvar intraepithelial neoplasia. Obstet Gynecol 1987; 69: 247-250.
14. Schon MP and Schon M. Immune modulation and apoptosis induction: two sides of the antitumoral activity of imiquimod. Apoptosis 2004; 9: 291-298.
15. Geisse J, Caro I, Lindholm J, et al. Imiquimod 5% cream for the treatment of superficial basal cell carcinoma: results from two phase III, randomized, vehicle-controlled studies. Am Acad Dermatol 2004; 50: 722-733.
16. Sauder DN. Imiquimod: modes of action. Br J Dermatol 2003; 149 Suppl 66: 5-8.
17. Stanley MA. Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential. Clin Exp Dermatol 2002; 27: 571-577.
18. Marchitelli C, Secco G, Perrotta M, et al. Treatment of bowenoid and basaloid vulvar intraepithelial neoplasia 2/3 with imiquimod 5% cream. J Reprod Med 2004; 49: 876-882.
19. Todd RW, Etherington IJ, Luesley DM. The effects of 5% imiquimod cream on high-grade vulvar intraepithelial neoplasia. Gynecol Oncol 2002; 85: 67-70.
20. van Seters M, Fons G, van Beurden M. Imiquimod in the treatment of multifocal vulvar intraepithelial neoplasia 2/3. Results of a pilot study. J Reprod Med 2002; 47: 701-705.
21. Wendling J, Saiag P, Berville-Levy S, et al. Treatment of undifferentiated vulvar intraepithelial neoplasia with 5% imiquimod cream: a prospective study of 12 cases. Arch Dermatol 2004; 140: 1220-1224.
22. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.
23. de Jong A, van Poelgeest MI, van der Hulst JM, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004; 64: 5449-5455.
24. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory T-helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.
25. Palefsky JM, Holly EA. Chapter 6: Immunosuppression and co-infection with HIV. J Natl Cancer Inst Monogr 2003; 41-46.

26. van den Brule AJ, Pol R, Fransen-Daalmeijer N, et al. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J Clin Microbiol 2002; 40(3): 779-787.
27. van der Burg SH, Kwappenberg KM, Geluk A, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that is processed and presented to HIV-specific CD4+ T-cells by at least four unrelated HLA-DR molecules. J Immunol 1999; 162: 152-160.
28. van der Burg SH, Menon AG, Redeker A, et al. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. Int J Cancer 2003; 107: 425-433.
29. de Jong A, O'Neill T, Khan AY, et al. Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T-cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. Vaccine 2002; 20: 3456-3464.
30. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II- restricted epitopes. Int J Cancer 2001; 91: 612-618.
31. Kirnbauer R, Hubbert NL, Wheeler CM, et al. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J Natl Cancer Inst 1994; 86: 494-499.
32. Baldwin PJ, van der Burg SH, Boswell CM, et al. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulvar and vaginal intraepithelial neoplasia. Clin Cancer Res 2003; 9: 5205-5213.
33. Smyth LJ, van Poelgeest MI, Davidson EJ, et al. Immunological responses in women with human papillomavirus type 16 (HPV-16)-associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV-16 oncogene vaccination. Clin Cancer Res 2004; 10: 2954-2961.
34. Todd RW, Roberts S, Mann CH, et al. Human papillomavirus (HPV) type 16-specific CD8+ T-cell responses in women with high grade vulvar intraepithelial neoplasia. Int J Cancer 2004; 108: 857-862.
35. Davidson EJ, Sehr P, Faulkner RL, et al. Human papillomavirus type 16 E2- and L1-specific serological and T-cell responses in women with vulvar intraepithelial neoplasia. J Gen Virol 2003; 84: 2089-2097.
36. Todd RW, Steele JC, Etherington I, eet al. Detection of CD8+ T-cell responses to human papillomavirus type 16 antigens in women using imiquimod as a treatment for high-grade vulvar intraepithelial neoplasia. Gynecol Oncol 2004; 92: 167-174.
37. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. Int J Cancer 2000; 88: 92-98.
38. Ressing ME, van Driel WJ, Celis E, et al. Occasional memory cytotoxic T-cell responses of patients with human papillomavirus type 16-positive cervical lesions against a human leukocyte antigen-A *0201-restricted E7-encoded epitope. Cancer Res 1996; 56: 582-588.
39. Nimako M, Fiander AN, Wilkinson GW, et al. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. Cancer Res 1997; 57: 4855-4861.
40. Youde SJ, Dunbar PR, Evans EM, et al. Use of fluorogenic histocompatibility leukocyte antigen-A*0201/HPV 16 E7 peptide complexes to isolate rare human cytotoxic T-lymphocyte-recognizing endogenous human papillomavirus antigens. Cancer Res 2000; 60: 365-371.
41. Gul N, Ganesan R, Luesley DM. Characterizing T-cell response in low-grade and high-grade vulvar intraepithelial neoplasia, study of CD3, CD4 and CD8 expressions. Gynecol Oncol 2004; 94: 48-53.
42. Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, et al. Immunological and viral factors associated with the response of vulvar intraepithelial neoplasia to photodynamic therapy. Cancer Res 2001; 61: 192-196.
43. Davidson EJ, Boswell CM, Sehr P, et al. Immunological and clinical responses in women with vulvar intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003; 63: 6032-6041.
44. Mota F, Rayment N, Chong S, et al. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. Clin Exp Immunol 1999; 116: 33-40.
45. Giannini SL, Hubert P, Doyen J, et al. Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development of squamous intraepithelial lesions of the cervix. Int J Cancer 2002; 97: 654-659.
46. Pao CC, Lin CY, Yao DS, et al. Differential expression of cytokine genes in cervical cancer tis-

sues. Biochem Biophys Res Commun 1995; 214: 1146-1151.
47. Hengge UR, Benninghoff B, Ruzicka T, et al. Topical immunomodulators-progress towards treating inflammation, infection, and cancer. Lancet Infectious Dis 2001; 1: 189-198
48 Matsumoto K, Leggatt GR, Zhong J, et al. Impaired antigen presentation and effectiveness of combined active/passive immunotherapy for epithelial tumors. J Natl Cancer Inst 2004; 96: 1611-1619.
49. van Mierlo GJ, Boonman ZF, Dumortier HM, et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol 2004; 173: 6753-6759.
50. Villada IB, Barracco MM, Ziol M, et al. Spontaneous regression of grade 3 vulvar intraepithelial neoplasia associated with human papillomavirus-16-specific CD4(+) and CD8(+) T-cell responses. Cancer Res 2004; 64: 8761-8766.

CHAPTER 7

High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with voluminous early-stage cervical cancer

Mariëtte I.E. van Poelgeest
Sytse J. Piersma
Ekaterina S. Jordanova
Kitty M.C. Kwappenberg
Jeanette M. van der Hulst
Jan Wouter Drijfhout
Cornelis J.M. Melief
Gemma G. Kenter
Gert Jan Fleuren
Rienk Offringa
Sjoerd H. van der Burg

Cancer Research 2007; 67: 354-361

Abstract

In a prospective study, we have examined the tumor-specific immune response in a group of 59 patients with human papillomavirus (HPV) 16-positive (HPV16+)-induced or HPV18+ induced cervical cancer. Local antitumor immunity was analyzed by the enumeration of tumor-infiltrating dendritic cells and CD4+, CD8+, and regulatory T-cells as well as by calculation of the ratio of CD8+/CD4+ T-cells and CD8+/regulatory T-cells. Systemic tumor-specific immunity was assessed by determination of the HPV E6- and/or E7-specific T-cell response in the blood of these patients. Finally, these variables were evaluated with respect to known histopathologic prognostic variables, including the absence (LN-) or presence (LN+) of lymph node metastases.

Stratification according to the lymph node status of patients revealed a significantly stronger CD8+ T-cell tumor infiltration, a higher CD8+/CD4+ T-cell ratio, and higher CD8+/regulatory T-cell ratio in the group of patients in which the tumor failed to metastasize to the tumor-draining lymph node. Subdivision according to the presence (IR+) or absence (IR-) of circulating HPV-specific T-cells disclosed that the highest number of tumor-infiltrating CD8+ T-cells was found in the group of LN- patients displaying a concomitant systemic tumor-specific immune response (LN-IR+). CD8+ T-cell infiltration in LN-IR- patients was comparable to that of LN+ patients.

In cervical cancer, the absence of lymph node metastases is strongly associated with a better prognosis. Our data indicate that, especially in a subgroup of LN- patients, a strong and effective interaction between immune system and tumor exists. This subgroup of cervical cancer patients may have the best prognosis.

Introduction

Cervical cancer is caused by high-risk types of human papillomavirus (HPV), particularly types 16 (HPV16) and 18 (HPV18), which account for approximately two-third of all cervical carcinomas[1,2]. It is the second most common cancer in women worldwide[3,4]. Overall cure rates of early-staged tumors approach 85%. However, a spectrum of relapse risk exists depending on several prognostic factors. Clinical stage and lymph node status are the most powerful predictors of outcome in cervical cancer, but other histopathologic factors, including size of primary tumor, infiltration depth, and vasoinvasion, have effect on the prognosis too[5-8].

Cervical cancer cells highly express two well-known tumor-specific oncoproteins that are encoded by HPV. These proteins, E6 and E7, are constitutively expressed because they are required to maintain the malignant phenotype[9]. The tumor can be infiltrated by lymphocytes[10,11], and both CD8+ and CD4+ T-cells isolated from tumors are able to recognize the E6 and E7 tumor antigens[12-14] (and Piersma, manuscript in preparation) as well as to kill tumor cells in vitro[15,16]. Tumor-specific T-cells, directed against the two HPV-encoded oncoproteins, can be detected in the peripheral blood of almost 50% of the patients albeit at low levels[17-23]. Therapeutic vaccination strategies aiming at the activation of large numbers of E6- and/or E7-specific T-cells in patients with precursor cancer lesions showed promising results[24-28], but no clear association between the presence of either a spontaneously induced or a vaccine-induced systemic tumor-specific immune response and clinical outcome was established.

Thus far, the only immunological determinant associated with a better prognosis is a pronounced infiltration of cervical carcinoma by lymphocytes[10,11]. However, thorough evaluations on the prognostic significance of tumor-infiltrating lymphocytes (TIL) in other human cancers revealed that especially intraepithelial infiltrating CD3+CD8+ T-cells contributed to a better prognosis[29-31]. On the other hand, the infiltration by CD3+CD4+ T-cells[32,33] or a subpopulation of CD4+ T-cells with immunosuppressive properties, so called regulatory T-cells that were detected by staining for Foxp3[34], was reported to counteract the beneficial effect of CD8+ T-cells[33-35]. High ratios between CD8+ T-cells and the other cell types were associated with improved survival[32,33]. These types of analyses have not been done in patients with cervical carcinoma.

In the present study, we did an in-depth analysis of the tumor-specific immune response in patients with HPV-induced cervical cancer. Local antitumor immunity

was analyzed by the enumeration of tumor-infiltrating dendritic cells and CD4+, CD8+, and regulatory T-cells as well as by calculation of the ratio of CD8+/CD4+ T-cells and that of CD8+/regulatory T-cells. The presence of systemic tumor immunity was assessed by examination of HPV E6- and/or E7-specific T-cell immunity in the peripheral blood of these patients. Finally, these variables were evaluated with respect to the lymph node status of patients because of its strong prognostic value in cervical cancer.

Methods

Subjects

Women presenting with histologically proven early-stage cervical carcinoma at the department of gynecology of the Leiden University Medical Center (Leiden, The Netherlands) and scheduled for radical hysterectomy were enrolled in the CIRCLE study that investigates cellular immunity against HPV16-positive cervical lesions after providing informed consent. The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center. Patient characteristics are given in Table 1. Blood was drawn at day of treatment before surgery. Peripheral

Table 1. Patient characteristics

No. of patients	59	*Tumor size (cm)*	
		< 4	35
Age (years)		≥ 4	24
Median	44		
Range	29-76	*Infiltration depth (mm)*	
		< 15	34
FIGO stage		≥ 15	21
1B	49	Unknown	4
2A	8		
2B	2	*Vasoinvasion*	
		Yes	36
HPV type		No	15
HPV16+	45	Unknown	8
HPV18+	14		
Lymph node metastases			
Positive	23		
Negative	36		

Abbreviation: FIGO, Fédération Internationale des Gynaecologistes et Obstetristes

blood mononuclear cells (PBMC) were obtained for the analysis of HPV-specific T-cell reactivity. The subjects were typed for HPV16 and HPV18 using HPV16- and 18-specific primers on DNA isolated from surgical resection specimens[36]. HPV16+ and HPV18+ subjects were included in the immunologic and immunohistochemical analyzes. Due to the fact that the obtained materials of these patients are the subject of many different studies within the Leiden University Medical Center, a selection bias occurred for the inclusion of patients diagnosed with a bigger tumor volume. Because a larger tumor is associated with the presence of lymph node metastases[37], a larger fraction of the selected patients harbored metastatic disease in the pelvic lymph nodes than would be expected at these early disease stages (Table 1).
Histologic specimens of normal cervices from women (n=9; median age, 46 years; range, 31-60), who underwent hysterectomies for benign uterine diseases with no cervical abnormalities served as control for the cervical carcinoma group. These specimens were obtained as paraffin-embedded blocks from the Department of Pathology of the Leiden University Medical Center.

Antigens and T-cell assay
A set of peptides spanning the whole HPV16 and 18 E6 and E7 proteins was used for the T-cell proliferation assays. For these assays, 32-mer peptides of the HPV16 E6 protein and the 35-mer peptides of the HPV16 E7 protein with an overlap of 14 amino acids were used in pools of two peptides per pool. The HPV18 peptides consisted of 15 E6 and 9 E7 22-mer peptides (overlap 12 amino acids) and were used in pools of four peptides per pool. The peptides were synthesized and dissolved as described previously[38]. The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (*e.g.*, $E6_{1-50}$, residues 1-32 and 19-50). To examine the presence of circulating HPV-specific T-cells, freshly isolated PBMCs were incubated with the indicated peptide pools in eight parallel microcultures as described previously[17]. Medium alone was taken along as a negative control, and the memory response mix or MRM (dilution, 1: 50) served as a positive control[17]. Supernatant (50 µl) from the microcultures was taken at day 6 after incubation and stored at -20°C until cytokine analysis. Peptide-specific proliferation was measured at day 7 by [^{3}H]thymidine incorporation. Cultures were scored positive when the proliferation of ≥ 75% of the test wells exceeded the mean proliferation + 3 x SD of the control wells containing medium only, and the stimulation index, defined as the mean of all test wells divided by the mean of the control wells, was ≥ 3[39].

Cytokine analysis

The detection of cytokines in the supernatants of the short-term proliferation assays was done using the cytometric bead array (CBA; Becton Dickinson, Etten-Leur, the Netherlands). This technique allows the simultaneous detection of six different T-helper (Th) 1 and Th2 cytokines: IFNγ, tumor necrosis factor α, interleukin (IL)-2, IL-4, IL-5, and IL-10. The CBA was done according to the manufacturer's instructions. Cutoff values were based on the standard curves of the different cytokines (100 pg/ml for IFNγ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and more than twice the concentration of the medium control[17,40].

Quadruple immunostaining of tumor tissues and infiltrating lymphocytes

A newly developed technique for simultaneous immunohistochemical staining of four different epitopes was applied to 4 μm formalin-fixed, paraffin-embedded tissue sections[41]. The AE1/AE3 antibody (IgG1; DAKO, Glostrup, Denmark) was added and slides were incubated overnight. Slides were washed and incubated first with cationic BSA (Aurion, Wageningen, the Netherlands). After 2 h of incubation with ultrasmall gold-labeled goat anti-mouse antibody (Aurion), silver enhancement was done using the Aurion enhancement kit.

The silver-stained slides were incubated overnight with a mix of either ab828 (anti-CD3, rabbit polyclonal antibody; Abcam, Cambridge, United Kingdom), hNK-1 (anti-CD57, mouse IgM, culture supernatant of a hybridoma grown in our own laboratory), and 4B11 (anti-CD8, mouse IgG2b; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-CD4 (rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) and CD45RO (mouse monoclonal IgG2a, DAKO). Slides were washed and incubated with the appropriate combination of fluorescent antibody conjugates (goat anti-rabbit IgG-Alexa Fluor 546, goat anti-mouse IgM-Alexa Fluor 488, and goat anti-mouse IgG2b-Alexa Fluor 647 or goat anti-rabbit IgG-Alexa Fluor 546 and goat anti-mouse IgG2a-Alexa Fluor 647). Alexa Fluor conjugates were obtained from Molecular Probes (Leiden, The Netherlands).

Our pilot study revealed that, without counterstaining with anti-CD3, the enumeration of infiltrating CD4+ T-cells based only on their morphologic appearance was difficult. In a selected set of tumors, we compared the numbers of CD4+ T-cells by direct staining with anti-CD4 or by indirect identification using a double staining of anti-CD3 and anti-CD8 by counting the CD3+CD8- cells. Because the indirect technique resulted in similar numbers of T-cells, we used CD3+CD8- cells as a marker for CD4+ T-cells in all other tumor sections.

The images were captured with a confocal laser scanning microscope (Zeiss LSM510, Zeiss, Jena, Germany). Fifteen images were scanned per slide. For each case, one successive negative control slide was included. These controls were silver stained but the primary lymphocyte-specific antibody step was omitted to ensure the specificity of the secondary antibody binding. Three images were taken at different positions on the control slide using exactly the same confocal laser scanning microscope multitrack settings as for the respective case. Intraepithelial and stromal infiltrating lymphocyte cell counts were done by two independent investigators (MvP and ESJ) and represented as the number of cells per mm^2.

Immunostaining of immature/activated dendritic cells and regulatory Foxp3+ T-cells
Paraffin sections (4 μm) were used for standard immunohistochemical staining of infiltrating antigen-presenting cells. The mouse monoclonal antibodies directed against CD1a (IgG1; Neomarkers-Lab Vision, Fremont, CA) and DC-Lamp (IgG1; Immunotech, Marseille, France) were used for staining immature or activated dendritic cells, respectively. The mouse monoclonal Foxp3 antibody (clone 236A/E7; Abcam, Cambridge, MA) was used for detecting regulatory T- cells. Briefly, slides were deparaffinized and antigen retrieval was done using EDTA for the DC-Lamp staining and trypsin pretreatment for the CD1a staining. After an overnight incubation, the biotin-labeled rabbit anti-mouse immunoglobulins and a biotinylated horseradish peroxidase-streptavidin complex (both were from Dako) were applied. To visualize immune complexes, a 0.05% solution of diaminobenzidine (Sigma, St. Louis, MO) containing 0.0018% H_2O_2 in a 0.05 mol/L Tris-HCl buffer (pH 7.6) was used. Counterstaining of the slides was done with Mayer's hematoxylin. Cells were quantified by counting the tumor-infiltrating Langerhans cells and the stromal DC-Lamp+ cells per 10 randomly selected high-power fields (x 400 magnification). Foxp3+ T-cells were counted separately both in the tumor fields and in the stromal compartment and represented as the number of cells per mm^2.

Statistical analysis
A two-tailed *t* test with Welch correction or Mann-Whitney test (when applicable) was applied for the analysis of the numbers of infiltrating immune cells or ratios in the different patient groups divided into categories that were based on the following clinical prognostic factors: invasion depth of tumor in cervical tissue (< 15 or ≥ 15 mm), tumor size (< 4 or ≥ 4 cm), vasoinvasion, and lymph node metastases[5,7,8].
Chi-square analyses for trend were used to test the relationship between different categories of CD8+ T-cell infiltration versus lymph node status. Categories were based

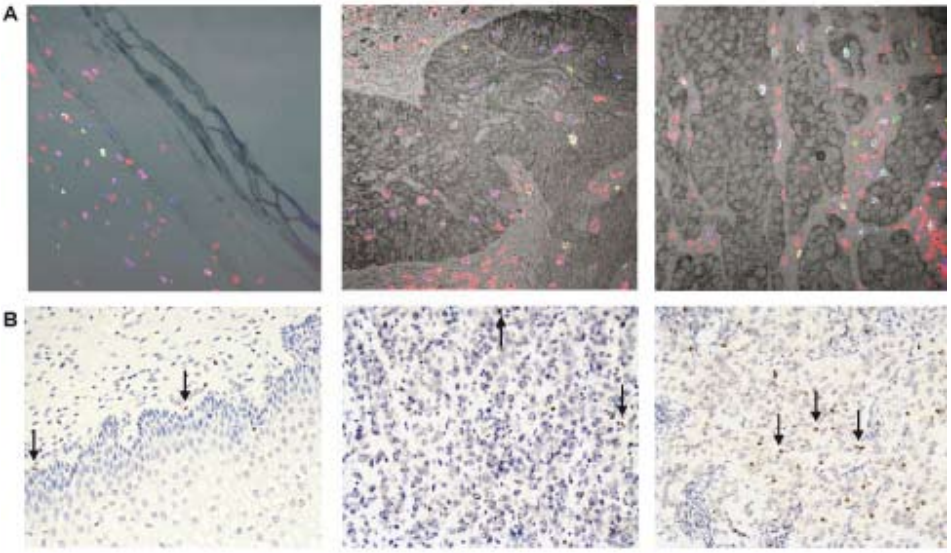
on 25th percentile and 75th percentile. Spearman rank analysis was done to test the association between the different types of infiltrating immune cells. Fisher's exact test (two-tailed) was used to analyze both the HPV-specific immunity and lymph node metastases and the relationship between different categories of infiltrating CD8+ T-cells, CD4+ T-cells and regulatory T-cells and combinations hereof versus lymph node status. Statistical analyses were done using Graphpad Instat software (version 3.0) and GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Results

The tumors of cervical carcinoma patients display a wide variety in the number of tumor-infiltrating T-cells

A detailed examination of the antitumor response was done in a group of 59 patients with HPV16+ or HPV18+ cervical cancer (Table 1). The numbers of CD3+ T-cells and regulatory T-cells (Foxp3+) present in stroma or between epithelial tumor cells were counted. Because previous reports strongly indicated that, especially intraepithelial infiltration by T-cells was correlated with prognosis in cervical cancer[10,11], the numbers of CD3+CD8+ T-cells, CD3+CD8+CD57+ T-cells, and CD3+CD8- (CD4) T-cells specifically infiltrating the tumor epithelium were examined. Additionally, tumor-infiltrating immature dendritic cells (CD1a+) and activated dendritic cells (DC-Lamp+) in the tumor were enumerated. To be able to assess to which degree immune infiltration was due to tumor-specific immunity, we also analyzed the infiltration of immune cells in sections of normal cervical epithelium that had been obtained from a group of nine patients with no evidence of cervical abnormalities. In figure 1A, an example of the analysis of CD3+ (red) and CD3+CD8+ (purple) T-cell infiltration by quadruple-color confocal miscroscopy is shown. CD3+ T-cells were present both in normal cervical tissue and in tumors. More stroma resident CD3+ T-cells were detected in tumor tissue than in normal cervical tissue (patients: mean, 966±635 cells per mm^2; controls: mean, 385±96 cells per mm^2; $p<0.0001$). The mean number of intraepithelial CD3+CD8+ or CD3+CD8- (CD4) T-cells that infiltrated tumors of all patients was about as twice as high when compared with those infiltrating normal cervices, but the number of intraepithelial tumor-infiltrating T-cells varied enormously (range 2.9-592 per mm^2) between patients (Figure 2). The number of both intraepithelial (range 0.6-56 mm^2) and stroma (range 22-249 per mm^2) resident regulatory (Foxp3+) T-cells was significantly enhanced in cervical carcinoma when compared to control cervical tissue ($p<0.001$; Figures 1 and 2). Furthermore, small numbers of intraepithelial

Figure 1. Intraepithelial infiltrating T-cells in cervical cancer patients and in healthy controls.



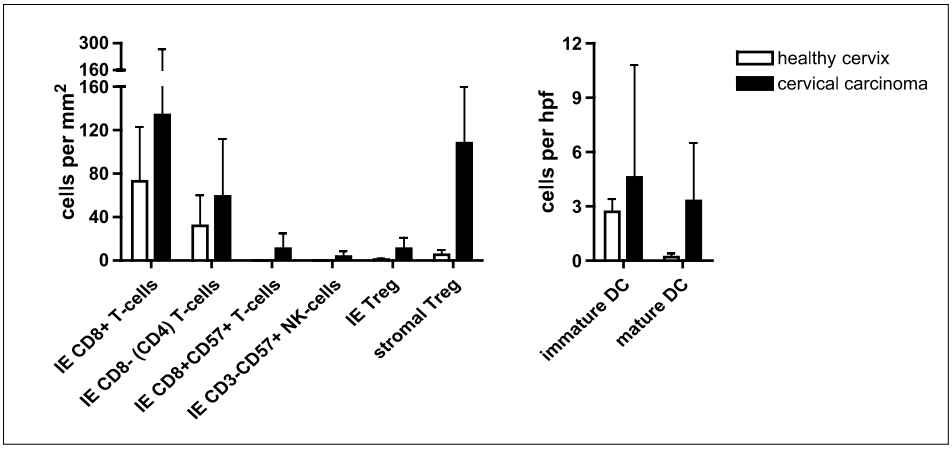
A. To detect tumor-infiltrating T-cells, 4 µm tissue sections were stained with anti-CD3 (red), anti-CD8 (blue), and anti-CD57 (green) followed by the appropriate combination of fluorescent antibody conjugates. Images were captured with a confocal laser scanning microscope (Zeiss LSM510) and used to count all T-cells that were located intraepithelial and in the stroma of 15 different (tumor) fields in each tissue section. The number of T-cells per tumor area (T-cells/ mm^2 tumor epithelium) was calculated. Three representative examples are shown. *Left,* example of a normal cervix. *Middle,* tumor tissue of a patient without lymph node metastases displaying a systemic immune response to HPV (patient 161) that shows intraepithelial infiltration by CD3+CD8- (CD4) T-cells (red) and CD3+CD8+ (purple) T-cells. *Right,* tumor tissue of a patient with lymph node metastases and no systemic immune response (patient 148).

B. To detect regulatory T-cells, 4 µm paraffin sections were stained with the mouse monoclonal Foxp3 antibody (clone 236A/E7). The number of positive cells per 10 randomly selected high-power fields (x400 magnification) was counted separately both in the tumor fields and in the stromal compartment and represented as the number of cells per mm^2. Example of a normal cervix *(left)* and lymph node-negative patient 161 *(middle)* showing rare infiltration by regulatory T-cells *(arrow)*. *Right,* tumor tissue of a patient with lymph node metastases and strong infiltration by regulatory T-cells (patient 148).

CD3+CD8+CD57+ terminally activated effector T-cells[42], and natural killer (NK)-like cells (CD3-CD57+) were observed in cervical tumors (Figure 2).

The degree of intraepithelial infiltration by CD8+ T-cells was paralleled by other types of immune cells infiltrating the tumor. Spearman rank analysis revealed significant correlations between the number of intraepithelial CD3+CD8+ T-cells and the number of CD3+CD8- T-cells ($r=0.64$, $p<0.0001$), CD3+CD8+CD57+ T-cells ($r=0.71$,

Figure 2. Comparison of immune cells infiltrating normal cervical tissue or cervical carcinoma.



(Left) Cell densities of only the intraepithelial tumor-infiltrating T-cells (expressed as cell number/mm^2 ± SD) were enumerated because of previous reports stating that the infiltration of cervical cancer cell nests displays the strongest correlation with prognosis[10,11]. The number of both intraepithelial (IE) and stromal tumor-infiltrating regulatory T (Treg) cells, identified through Foxp3+ staining, was quantified because there were no data to support that either location is more important.
(Right) Cell densities of intraepithelial and stromal tumor-infiltrating CD1a and DC-Lamp+ dendritic cells (DC) depicted per 10 high-power fields ± SD.

p<0.0001), CD3-CD57+ NK-like cells (r=0.43, p<0.001) and regulatory T-cells (r=0.45, p<0.001) that infiltrated the tumor of each patient.
The number of tumor-infiltrating immature (CD1a+) dendritic cells approximated two to three cells per 10 high-power fields both in the controls and in the patients. Small numbers of activated dendritic cells (DC-Lamp+) were detected in all tumors but were virtually absent in the normal cervices (p<0.001; Figure 2). In general, the number of tumor-infiltrating immature and activated dendritic cells was correlated per tumor (Spearman rank analysis r=0.34, p=0.008). Together, these data indicate that there is great variation in the number of immune cells that can infiltrate HPV-induced cervical tumors. Furthermore, on the recruitment of higher numbers of CD8+ T-cells, there is also a more effective infiltration of the tumor by other immune cells, suggesting an intense interaction between tumor and immune system.

Strong tumor immunity is present in a subset of patients without lymph node metastases

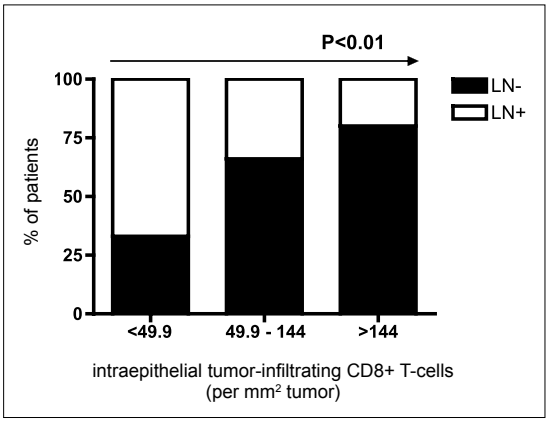
Histopathologic factors known to affect the prognosis of patients are vasoinvasion,

the size of a tumor, and the depth of tumor invasion into the normal cervical stroma[5-8]. Especially, the presence of lymph node metastases is a strong independent prognostic factor associated with a decrease in overall and disease-specific survival and an increase of recurrence in cervical cancer[5,6]. Stratification of the patient group based on clinical prognosis, as predicted by these histopathologic factors, revealed that the mean number of intraepithelial CD8+ T-cells was higher in the group of patients without lymph node metastases (LN-) than in the group of patients with a poor prognosis (LN+; 151±126 versus 108±147, respectively; p=0.02). No differences were observed when other types of infiltrating immune cells, including CD3-CD57+ (NK) and CD8CD57+ cells known to play a role in controlling tumor metastases in other cancers[43, 44], were analyzed. For the other histopathologic factors, we found a correlation neither between vasoinvasion (p=0.10), infiltration depth of tumor (p=0.14), or tumor size (p=0.31) and CD8+ T-cell infiltration nor for the infiltration by other immune cells.

To confirm the relationship between intratumoral CD8+ T-cell infiltration and lymph node status, we reanalyzed our patient group and stratified patients according to either weak infiltration (25 percentile: ≤49.9 intraepithelial CD8+ T-cells per mm^2; n=5 LN-; n=10 LN+), intermediate infiltration (25-75 percentile, n=19 LN-; n=10 LN+) or strong infiltration (75 percentile: ≥144 intraepithelial CD8+ T-cells per mm^2; n=12 LN-; n=3 LN+) with intraepithelial CD8+ T-cells. Based on the fraction of LN- patients within these groups, there was a significant association between the number of infiltrating CD8+ T-cells and the absence of lymph node metastases (Chi-square for trend 6.9, p<0.01; Figure 3).

In other cancers, a high ratio between the CD8+ T-cells and CD4+ T-cells or regulatory T-cells is associated with favorable prognosis[32,33], indicating that especially the proportion between the different subtypes of immune cells within the tumor of each individual patient is important. In view of the wide ranges observed for the numbers of infiltrating CD3+CD8+, CD3+CD8- and regulatory T-cells cells, the mean number of each type of infiltrating immune cells may not differ considerably between subgroups of patients, whereas the ratio between these subtypes can. Therefore, the ratios between CD3+CD8+, CD3+CD8-, and regulatory T-cells cells were calculated for each patient. The mean CD8+/CD8- (CD4) T-cell ratio did not differ between normal tissue and that of the group of all patients (3.1±2.4 vs. 2.8±2.8, respectively). However, the CD8/CD4 T-cell ratio was considerably lower in the LN+ patient group (1,9±1,7) than in the LN- patient group (p=0.01), who displayed a CD8+/CD8- (CD4) T-cell ratio (3,3±3,2) similar to what is found in normal cervices. The ratio between

Figure 3. The prognostic value of intraepithelial tumor-infiltrating CD8+ T-cells, CD4+ T-cells, and regulatory T-cells.



Patients (n=59) were stratified according to weak infiltration (25 percentile: ≤49.9 intraepithelial CD8+ T-cells per mm^2), intermediate infiltration (25-75 percentile), or strong infiltration (75 percentile: ≥144 intraepithelial CD8+ T-cells per mm^2) with intraepithelial CD8+ T-cells. The percentage of patients with lymph node metastases of the tumor (LN+, n=23) or without lymph node metastases (LN-, n=36) is indicated per subgroup. The group of patients with weak infiltration consisted of 5 LN- and 10 LN+ patients, the group of patients with intermediate infiltration consisted of 19 LN- and 10 LN+ patients, and the group of patients with strong infiltration consisted of 12 LN- and 3 LN+ patients. There was a significant association between a higher number of infiltrating CD8+ T-cells and the absence of lymph node metastases (Chi-square 6.9; $p<0.01$).

intraepithelial CD8+ T-cells and either stromal (1.4±1.4) or intraepithelial (27±56) regulatory T-cells was much lower in cancer patients than in normal cervical tissue (22±18 and 118±100, respectively; $p<0.001$). Importantly, a higher CD8+/stromal regulatory T-cell ratio was found in the LN- patient group than for the LN+ patient group (1.6±1.5 *vs.* 0.9±1.0; $p<0.05$). Thus, evaluation of cervical cancer patients with respect to their lymph node status revealed that the LN- patient group, which in general has a good prognosis, displays significantly higher number of intraepithelial CD8+ T-cells, a higher CD8+/CD4+ T-cell ratio, and a higher CD8+/regulatory T-cell ratio than the group of patients with lymph node metastases.

To assess the influence of CD4+ T-cells or regulatory T-cells on the association of infiltrating CD8+ T-cells with lymph node status, two groups of patients that represented the two most extreme situations of tumor-infiltrating T-cells were analyzed. In these two groups, patients who displayed either a favorable constitution of tumor infiltration (higher number of intraepithelial CD8+ T-cells, a higher CD8+/CD4+ T-

cell ratio, and a higher CD8+/regulatory T-cell ratio) or a nonfavorable constitution of infiltration (lower CD8+ T-cell count and a low CD8+/CD4+ and CD8+/regulatory T-cell ratio) were included. Although the fraction of LN- patients was higher among the patients with a higher CD8+ T-cell infiltration (≥95.2 cells per mm^2; 50 percentile), this was not significant (p=0.06). Selection based on CD8+ T-cells and both the CD8+/CD4+ T-cell ratio (50 percentile ratio, 2.235) and CD8+/stromal regulatory T-cell ratio (50 percentile ratio, 0.747) resulted in significantly increased fraction of LN- patients within the group of patients with a favorable tumor infiltration when compared with the group of patients with a less favorable infiltration (p=0.01), but this increase was not significant when compared with the fraction of LN- patients within the group of patients selected only based on a higher CD8+ T-cell infiltration. These data suggest that coinfiltrating CD4+ T-cells and regulatory T-cells have a subtle influence on the lymph node status of patients but only in cases that they match the number of infiltrating CD8+ T-cells. However, the absence of lymph node metastases is most prominently associated with a robust CD8+ tumor-infiltration.

Strong CD8+ T-cell infiltration is predominantly observed in the subgroup of LN- patients with circulating HPV-specific T-cells

From 50 patients, sufficient amounts of PBMC were available to study the systemic HPV-specific T-cell response using a methodology that detects CD4+ T-cell responses against the HPV early antigens[17,19,45]. In this group, 24 patients displayed a systemic HPV-specific T-cell response against one or more of the E6 and/or E7 peptide pools while 26 patients failed to produce a detectable T-cell response, a frequency that is in line with our previous findings[17]. Examples shown in Figure 4A reveal that some patient PBMC samples show proliferative T-cell reactivity against only a single peptide pool, whereas other samples display a broader response against up to three peptide pools. As illustrated in Figure 4B, IFNγ-production is found in some of the culture supernatants, but this did not depend on the strength of the proliferative response (compare patients 239 and 161 in Figure 4). Ten of the 24 patients with a systemic HPV-specific immune response produced IFNγ on stimulation with at least one of the peptide pools. There was no direct relationship between the presence (IR+) or absence (IR-) of circulating HPV-specific T-cells and the lymph node status of patients (p=1.0) or with the magnitude of tumor-infiltrating T-cells (e.g., IR status versus CD8+ T-cells; p=0.31).

Therefore, the group of patients was further subdivided according to both the HPV immune status and lymph node status (Table 2). Considerable differences were found in the groups of patients that displayed a systemic antitumor response. The group

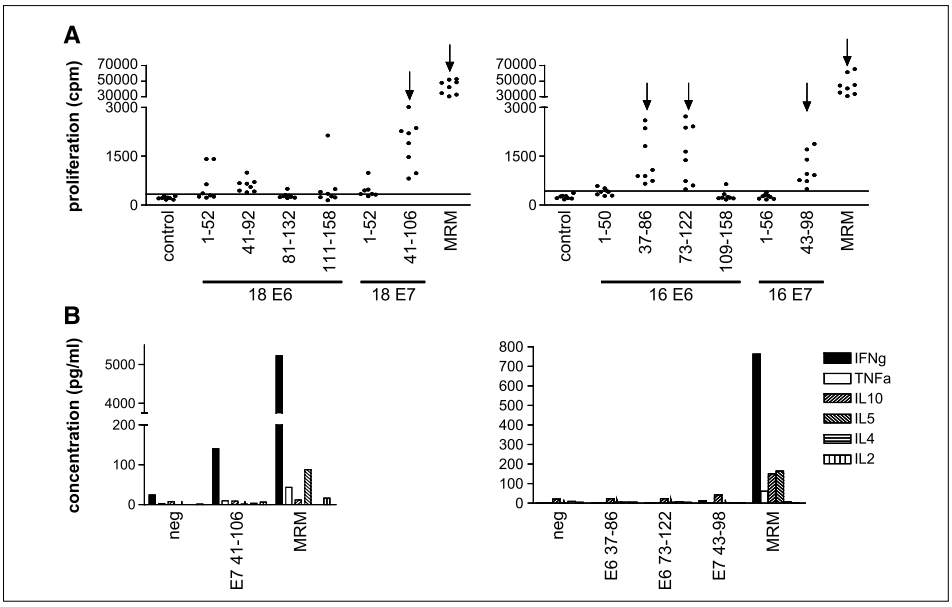


Figure 4. Two representative examples of patient-derived T-cell cultures that recognize HPV-specific peptide pools of HPV16 or 18 but display different cytokine profiles.

A. proliferative responses of patients 239 *(left)* and 161 *(right)*. Each *circle* represents the proliferation of an individual microculture (n=8); supernatants of the positive responses (*arrows*) were tested for cytokine production.

B. corresponding cytokine levels produced on antigenic stimulation of these microcultures are depicted, indicating that IFNγ production is found in some but not all of the supernatants isolated from the HPV-specific T-cell proliferative cultures.

of LN-IR+ patients displayed a significant higher number of intraepithelial tumor-infiltrating CD8+ T-cells than LN- patients without a concomitant systemic immune response (p=0.02; Table 2). Furthermore, within the group of LN- patients, not only intraepithelial CD8+ T-cells but also the number of CD8+CD57+ terminally activated effector T-cells and regulatory (Foxp3+) T-cells as well as CD8+/CD4+ T-cell ratio were increased in the group of LN-IR+ patients (p=0.03; Table 2). These data suggest that, within the group of LN- patients who in general display a better prognosis, a subgroup can be identified. The patients within this subgroup mount a significantly stronger antitumor response, which is reflected by circulating tumor-specific T-cells, a robust CD8+ tumor infiltration, and a higher CD8+/CD4+ T-cell ratio.

Unexpectedly, the LN+IR+ group displayed the lowest numbers of tumor-infiltrating immune cells (Table 2). In addition, this group displayed a significantly lower CD8+/CD8- (CD4) ratio (p=0.003) when compared with the LN-IR+ group (Table 2).

Table 2. Summary of the number of tumor-infiltrating immune cells in groups of patients stratified according to the absence (LN–) or presence (LN+) of lymph node metastases and circulating HPV-specific T-cells.

	LN–		LN+	
	IR– (n=17)	IR+ (n=14)	IR– (n=9)	IR+ (n=10)
CD8+ T-cells[a]	90±65	200±145	129±190	58±46
CD8- (CD4+) T-cells	42±29	62±46	54±75	61±69
CD3+CD8+CD57+ T-cells	9,8±12	13±15	8,4±16	5,6±8,5
CD3-CD57+ NK-like cells	4,4±6,7	3,1±2,5	3,8±5,9	2,0±2,2
IE Foxp3+ T-cells[b]	7,5±7,1	15±9,5	10±15	7,1±5,8
stromal Foxp3+ T-cells	100±69	136±69	100±44	104±42
CD8+/CD8- (CD4) T-cell ratio[c]	2,3±1,3	4,4±4,5	2,6±2,1	1,5±1,6
CD8+/IE regulatory T-cell ratio[c]	27±47	28±40	51±112	9,7±11
CD8+/stromal regulatory T-cell ratio[c]	1,3±1,3	1,8±1,6	1,2±1,3	0,4±0,2

[a] Cell densities of only the intraepithelial tumor-infiltrating T-cells (expressed by cell number/mm^2 ± SD) were enumerated because of previous reports stating that the infiltration of tumor cell nests displays the strongest correlation with prognosis[10,11,29,30,31,33].

[b] The number of both intraepithelial (IE) and stromal tumor-infiltrating regulatory (Foxp3+) T-cells was quantified because there is no evidence that either location is more important.

[c] The ratio of intraepithelial CD8+ and intraepithelial CD8- (CD4+) T-cells was determined as well as the ratio between intraepithelial CD8+ T-cells and regulatory (Foxp3+) T-cells.

Furthermore, regulatory T-cells residing in the tumor stroma readily outnumbered infiltrating CD8+ T-cells, resulting in a low CD8+/ regulatory T-cell ratio (Table 2). In contrast, in the group of LN-IR+ patients, the number of infiltrating CD8+ T-cells surpassed that of regulatory T-cells in the stroma despite the fact that in this group also more regulatory T-cells were recruited into the tumor. Consequently, the ratio between intraepithelial CD8+ T-cells and regulatory T-cells in the stroma is higher in the group of LN-IR+ than in the LN+IR+ group ($p<0.001$). There were no significant differences between the groups of LN- or LN+ patients without circulating HPV-specific T-cells (IR-).

Discussion

In cervical carcinoma, infiltration of intratumoral lymphocytes has been associated with an improved clinical outcome[10,11]. In the present study, we did a detailed analysis of the immune response in patients with HPV-induced cervical cancer. Although infiltration of most TIL subtypes positively correlated with each other, intraepithelial CD8+ T-cells were the only subtype that correlated with a lack of pelvic lymph node spread and, therefore, might have a better prognosis. This observation is consistent with other studies, indicating that strong intraepithelial CD8+ T-cell infiltration is associated with a favorable course of disease in colorectal cancer[29,30], ovarian cancer[33,46] and endometrial carcinoma[31]. In addition, we showed that failure of CD8+ T-cells to substantially infiltrate the tumor results in a significantly lower CD8+/CD4+ T-cell ratio in patients with lymph node metastases when compared with patients without metastatic disease or to normal cervical tissue. Similar observations were made in colorectal and ovarian cancer[32,33]. Importantly, recently, we detected and isolated HPV-specific CD4+ Th1 cells as well as HPV-specific CD4+Foxp3+ regulatory T-cells from several lymph nodes and tumors derived from patients with cervical cancer[49], indicating that part of the cervical tumor-infiltrating CD4+ T-cells can suppress local immunity. The number of regulatory T-cells in cervical carcinoma was directly examined by detection of Foxp3, serving as specific marker for at least a subset of regulatory T-cells[34]. In healthy cervices, such regulatory T-cells were almost absent. In contrast, cervical tumor tissue was strongly infiltrated by Foxp3+ T-cells (Figure 2). We did not find a direct correlation between the presence of regulatory T-cells and the lymph node status of patients, but patients without such metastases displayed a significantly more favorable intraepithelial CD8+/regulatory T-cell ratio. As expected, the strongest correlation was found when the CD8+/regulatory T-cell ratio was determined with Foxp3+ T-cells present in the tumor stroma, where also most of the CD4+ T-cells reside (Figure 1). This suggests that a robust CD8+ T-cell infiltration can overcome the negative effects of tumor resident CD4+ regulatory T-cells resulting in a failure of the tumor to metastasize to the lymph nodes. A recent comprehensive study of the percentage of CD8+ T-cells, CD4+ T-cells, and regulatory T-cells in both tumor-draining lymph nodes and tumor of mice suggests that this concept may indeed be true. In comparison with lymph nodes, CD8+ T-cells are underrepresented in the tumor when compared with CD4+ T-cells and regulatory T-cells[47]. Releasing the breaks on T-cells by anti-CTLA-4 therapy strongly increased the number of tumor-infiltrating CD8+ T-cells, resulting in a significant increase of the CD8+/ regulatory T-cell ratio, and this correlated with tumor rejection. Interestingly,

a therapy-induced decrease in this ratio was observed in the tumor draining lymph node[47].

In addition to the local immune response, we also analyzed systemic antitumor immunity. For this, we exploited the T-cell response against the tumor-specific antigens E6 and E7 encoded by HPV. Previous studies indicated that the expression of E6 and E7 in cervical carcinoma results in the induction of a detectable HPV-specific proliferative CD4+ T-cell response in approximately half of all cases[17-19,22,23,48]. Here, circulating HPV-specific proliferative T-cells were detected in 24 of 50 cases. There was no direct correlation between the presence of HPV-specific systemic immunity and lymph node metastases, which were present in approximately half of the 24 patients displaying HPV-immunity. However, subdivision according to both lymph node status and HPV-immune status revealed an intriguing picture.
First, the higher number of CD8+ T-cells, higher CD8+/CD4+ T-cell ratio and higher CD8+/regulatory T-cell ratio that was found in the group of LN- patients, when compared with the group of LN+ patients, could be attributed to those LN- patients displaying a concomitant systemic tumor-specific immune response (LN-IR+; Table 2). The number of infiltrating CD8+ T-cells was higher in this subgroup of patients than in the LN- patients without such a response. Strikingly, CD8+ T-cell infiltration in the LN-IR- patient group was comparable with that of LN+ patients. In ovarian, endometrial, and colorectal cancer, strong intraepithelial CD8+ T-cell infiltration has a favorable prognostic effect[29-31,33,46] suggesting that especially the LN-IR+ subgroup of lymph node-negative cervical cancer patients will display a better disease course.
Second, we observed that the most extreme differences occurred among the patients with a positive HPV immune status. Whereas in the LN- patient group the presence of circulating HPV-specific T-cells was associated with higher numbers of infiltrating T-cells (LN-IR+), the opposite was observed in the LN+ patient group (Table 2). The LN+IR+ group displayed the lowest number of intratumoral CD8+ T-cells, and the lowest CD8+/regulatory T-cell ratio. These data suggests that the presence of circulating HPV-specific T-cells not necessarily reflects a proper antitumor response and implies that the presence of tumor cell metastases in the draining lymph nodes may direct the development of unwanted tumor-specific T-cell responses that can counteract the recruitment of effector cells in the tumor. Indeed, HPV16 E6-specific CD4+ Foxp3+ regulatory T-cells isolated from tumor-positive lymph nodes can suppress the action of antigen-specific T-cells at a one-to-one ratio[49]. Notably, ratios of one to one and even two to one (regulatory T-cells/ CD8+ T-cells) are present in the tumors of LN+ patients (Table 2).

In conclusion, our results show that the presence of intraepithelial CD8+ T-cells infiltrating tumor cells is associated with the lack of tumor metastases in the draining lymph nodes of cervical cancer patients. Because the absence of lymph node metastases is strongly associated with a better prognosis, patients with strong CD8+ T-cells infiltrating tumor cells are likely to display an improved clinical outcome in cervical cancer. Moreover, our data suggest that a subgroup of patients within the cohort of LN- patients, who may experience the relatively best course of disease, exists. In view of our current data on the relationships among lymph node status, systemic antitumor immunity, and tumor-infiltration, studies evaluating immunocorrelations between vaccine-induced tumor-specific T-cell responses and clinical outcome should include an examination of the constitution of the local immune response.

Acknowledgments

We thank all patients who have participated in this study. We also thank F. Prins for his excellent help in the analysis of tissue sections by confocal microscopy.

References

1. Bosch FX, Manos MM, Munoz N, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. J Natl Cancer Inst 1995; 87: 796-802.
2. Munoz N, Bosch FX, de Sanjose S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348(6): 518-527.
3. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002; 2: 342-350.
4. Bosch FX, de Sanjose S. Human papillomavirus and cervical cancer--burden and assessment of causality. J Natl Cancer Inst Monogr 2003: 3-13.
5. Singh N, Arif S. Histopathologic parameters of prognosis in cervical cancer--a review. Int J Gynecol Cancer 2004; 14: 741-750.
6. Thomas GM. Adjuvant therapy after primary surgery for stage I-IIA carcinoma of the cervix. J Natl Cancer Inst Monogr 1996: 77-83.
7. Snijders-Keilholz A, Hellebrekers BW, Zwinderman AH, et al. Adjuvant radiotherapy following radical hysterectomy for patients with early-stage cervical carcinoma (1984-1996). Radiother Oncol 1999; 51: 161-167.
8. Kenter GG, Hellebrekers BW, Zwinderman KH, et al. The case for completing the lymphadenectomy when positive lymph nodes are found during radical hysterectomy for cervical carcinoma. Acta Obstet Gynecol Scand 2000; 79: 72-76.
9. zur Hausen H. Papillomavirus infections - a major cause of human cancers. Biochimica et Biophysica Acta 1996; 1288: F55-F78.
10. Bethwaite PB, Holloway LJ, Thornton A, et al. Infiltration by immunocompetent cells in early stage invasive carcinoma of the uterine cervix: a prognostic study. Pathology 1996; 28: 321-327.
11. Chao HT, Wang PH, Tseng JY, et al. Lymphocyte-infiltrated FIGO Stage IIB squamous cell carcinoma of the cervix is a prominent factor for disease-free survival. Eur J Gynaecol Oncol 1999; 20: 136-140.
12. Evans EM, Man S, Evans AS, et al. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res 1997; 57: 2943-2950.
13. Hohn H, Pilch H, Gunzel S, et al. Human papillomavirus type 33 E7 peptides presented by HLA-DR*0402 to tumor-infiltrating T cells in cervical cancer. J Virol 2000; 74: 6632-6636.
14. Hohn H, Pilch H, Gunzel S, et al. CD4+ tumor-infiltrating lymphocytes in cervical cancer recognize HLA-DR- restricted peptides provided by human papillomavirus-E7. J Immunol 1999; 163: 5715-5722.
15. Hilders CG, Ras L, van Eendenburg JD, et al. Isolation and characterization of tumor-infiltrating lymphocytes from cervical carcinoma. Int J Cancer 1994; 57: 805-813.
16. Santin AD, Bellone S, Palmieri M, et al. Induction of tumor-specific cytotoxicity in tumor infiltrating lymphocytes by HPV16 and HPV18 E7-pulsed autologous dendritic cells in patients with cancer of the uterine cervix. Gynecol Oncol 2003; 89: 271-280.
17. de Jong A, van Poelgeest MI, van der Hulst JM, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004; 64: 5449-5455.
18. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 2001; 91: 612-618.
19. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.
20. Welters MJ, van der Logt P, van den Eeden SJ, et al. Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. Int J Cancer 2006; 118: 950-956.
21. Ressing ME, van Driel WJ, Celis E, et al. Occasional memory cytotoxic T-cell responses of patients with human papillomavirus type 16-positive cervical lesions against a human leukocyte antigen-A*0201-restricted E7-encoded epitope. Cancer Res 1996; 56: 582-588.
22. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. Int J Cancer 2000; 88: 92-98.
23. Luxton JC, Rowe AJ, Cridland JC, et al. Proliferative T cell responses to the human papillomavirus type 16 E7 protein in women with cervical dysplasia and cervical carcinoma and

in healthy individuals. J Gen Virol 1996; 77: 1585-1593.
24. Frazer IH, Quinn M, Nicklin JL, et al. Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIX adjuvant in women with cervical intraepithelial neoplasia. Vaccine 2004; 23: 172-181.
25. Davidson EJ, Boswell CM, Sehr P, et al. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003; 63: 6032-6041.
26. Muderspach L, Wilczynski S, Roman L, et al. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. Clin Cancer Res 2000; 6: 3406-3416.
27. Smyth LJ, Van Poelgeest MI, Davidson EJ, et al. Immunological responses in women with human papillomavirus type 16 (HPV-16)-associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV-16 oncogene vaccination. Clin Cancer Res 2004; 10: 2954-2961.
28. Baldwin PJ, van der Burg SH, Boswell CM, et al. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003; 9: 5205-5213.
29. Naito Y, Saito K, Shiiba K, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 1998; 58: 3491-3494.
30. Menon AG, Fleuren GJ, Alphenaar EA, et al. A basal membrane-like structure surrounding tumour nodules may prevent intraepithelial leucocyte infiltration in colorectal cancer. Cancer Immunol Immunother 2003; 52: 121-126.
31. Kondratiev S, Sabo E, Yakirevich E, et al. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin Cancer Res 2004; 10: 4450-4456.
32. Diederichsen AC, Hjelmborg JB, Christensen PB, et al. Prognostic value of the CD4+/CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. Cancer Immunol Immunother 2003; 52: 423-428.
33. Sato E, Olson SH, Ahn J, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci USA 2005; 102: 18538-18543.
34. Wolf D, Wolf AM, Rumpold H, et al. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. Clin Cancer Res 2005; 11: 8326-8331.
35. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004; 10: 942-949.
36. Claas EC, Melchers WJ, van der Linden HC, et al. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. Am J Pathol 1989; 135: 703-709.
37. Chen AC, Sung WH, Wang PH, et al. Correlation of three-dimensional tumor volumetry with cervical cancer prognostic parameters. Eur J Gynaecol Oncol 2002; 23: 401-404.
38. van der Burg SH, Kwappenberg KM, Geluk A, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. J Immunol 1999; 162: 152-160.
39. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.
40. van der Burg SH, Menon AG, Redeker A, et al. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. Int J Cancer 2003; 107: 425-433.
41. van Vlierberghe RL, Sandel MH, Prins FA, et al. Four-color staining combining fluorescence and brightfield microscopy for simultaneous immune cell phenotyping and localization in tumor tissue sections. Microsc Res Tech 2005; 67: 15-21.
42. Mollet L, Sadat-Sowti B, Duntze J, et al. CD8hi+CD57+ T lymphocytes are enriched in antigen-specific T cells capable of down-modulating cytotoxic activity. Int Immunol 1998; 10: 311-323.
43. Tachibana T, Onodera H, Tsuruyama T, et al. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. Clin Cancer Res 2005; 11: 7322-7327.
44. Menon AG, Janssen-van Rhijn CM, Morreau H, et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. Lab Invest 2004; 84(4): 493-501.
45. de Jong A, van der Hulst JM, Kenter GG, et al. Rapid enrichment of human papillomavirus

(HPV)-specific polyclonal T cell populations for adoptive immunotherapy of cervical cancer. Int J Cancer 2005; 114: 274-282.
46. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348: 203-213.
47. Quezada SA, Peggs KS, Curran MA, et al. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. J Clin Invest 2006; 116: 1935-1945.
48. van der Burg SH, de Jong A, Welters MJ, et al. The status of HPV16-specific T-cell reactivity in health and disease as a guide to HPV vaccine development. Virus Res 2002; 89: 275-284.
49. van der Burg SH, Piersma SJ, de Jong A, et al. Association of cervical cancer with the presence of CD4+ regulatory T-cells specific for human papillomavirus antigens. Proc Natl Acad Sci USA 2007; 104: 12087-12092.

CHAPTER 8

Summary and general discussion

Summary

The studies in this thesis analyzed the spontaneous and vaccine-induced HPV16-specific CD4+ T-cell responses in healthy individuals and in women with HPV-induced anogenital disease. The impact of type 1 Th-cell immunity on the clinical outcome of topical treatment with an immune modifier in patients with high-grade VIN was described. In addition, a detailed analysis of the local immune microenvironment in patients with cervical cancer and its relationship with the presence of systemic HPV-specific T-cell responses and clinically important prognostic factors was performed. We conclude that in contrast to healthy individuals, patients with HPV-induced anogenital neoplasia display either impaired or absent systemic type 1 HPV-specific Th-cell responses against the early antigens (*chapters 2,3, and 4*). Strong type 1 T-cell immunity against the early antigens can be induced or restored in patients with anogenital intraepithelial neoplasia (AGIN) by HPV vaccination, and in combination with a local supportive environment HPV-specific Th-cell responses may improve clinical outcomes in the treatment of patients with high-grade VIN (*chapters 5 and 6*). In *chapter* 7, we showed that cervical cancer patients without lymph node metastases, which is a well-known positive prognostic factor, display a high number of infiltrating CD8+ T-cells, and provided evidence that regulatory T-cells, like in other cancers, play an important role in the natural course of cervical cancer.
Below, the main conclusions of the presented studies are put into perspective and a personal view is given on the implications of these findings for future immunotherapeutic strategies in HPV-induced diseases.

HPV16-specific type 1 CD4+ T-cell immunity against early antigens but not the late antigen L1 is important in preventing the development or progression of anogenital neoplasia

Animal models showed a role of HPV-specific type 1 T-cell immunity against early antigens in the protection against HPV-induced disease. Vaccination with HPV16 E6 and E7 long peptides induced strong HPV-specific CD4+ and CD8+ T-cell responses and HPV16-induced tumors were effectively eradicated in mice[1]. In the cottontail-rabbit papillomavirus (CRPV) model it was shown that therapeutic vaccination with a similar vaccine was effective in the prevention of CRPV induced papillomas and latent infections[2]. Vaccination of mice with HPV16 E7 protein in combination with CpG, which is known to induce a Th1 like pattern of cytokine production[3], resulted in a strong IFNγ-associated CD4+ T-cell response and suppression of tumor growth in the majority of animals[4]. Furthermore, in the canine oral papillomavirus (COPV) model, beagle dogs that were injected with a vaccine expressing the E2 and E7 genes

were protected from developing papillomas, and this protection was accompanied by significant IFNγ responses upon stimulation of PBMC with peptides of these early genes[5].

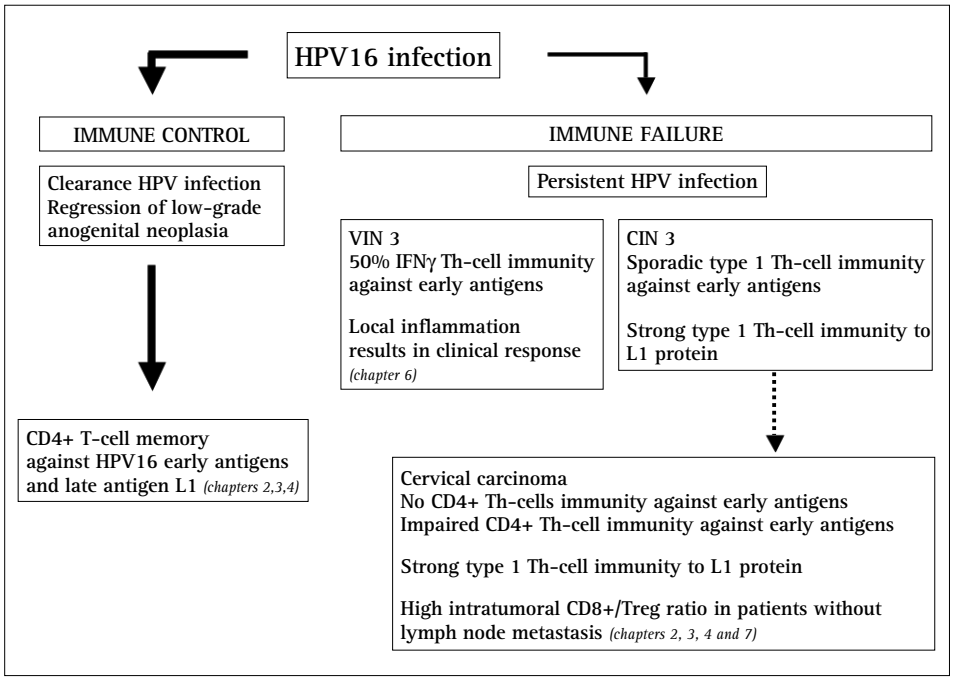
One main conclusion of the studies presented in this thesis is that unlike HPV16-specific T-cell responses against the structural protein L1, type 1 T-cell immunity against the early proteins E2 and E6 is more likely to protect against the development and progression of HPV-induced disease in humans. This is illustrated by the presence of IFNγ-producing HPV16-specific CD4+ T-cell responses against the early antigens E2 and E6 in the blood of the majority of healthy subjects while such responses are weak or absent in patients with HPV16-positive cervical carcinoma (*chapters 2,3, and 4*). Instead, HPV16 L1 peptide-specific IFNγ-associated T-cell responses were found in the majority of both patients with cervical lesions and healthy individuals, and with similar magnitude (*chapter 3*), suggesting that type 1 T-cell immunity against the structural protein L1 does not correlate with health or disease. Indeed, COPV-infected beagle dogs that were therapeutically vaccinated with the L1 protein developed papillomas at multiple sites of the body in 100% of cases[5]. The loss of expression of L1 in progressive CIN lesions and cervical carcinoma[6,7] may explain why L1 peptide-specific T-cell responses do not protect against the development or progression of established HPV16-induced anogenital lesions.

Despite all our studies we still have to show formally that the CD4+ T-cell response is important and as such that a failure to either mount such a response (*chapters 2,3, and 4*), or a lack of infiltration of T-cells in the lesion (*chapters 6 and 7*), may explain why a small proportion of HPV-infected patients is not capable to combat persistent infections and subsequent HPV-induced anogenital diseases (Figure 1). However, as already noted, the failure of immunodeficient HIV-infected subjects with low CD4 counts to fight HPV-infections[8,9] and the presence of strong HPV-specific memory responses in healthy subjects[10,11] provide strong evidence for the notion that HPV-specific CD4+ T-cell reactivity is important in the protection against HPV-induced anogenital disease.

Cervical cancer patients are not profoundly predisposed to an overall Th2 immune response

Human cancers and infectious diseases are frequently associated with an imbalance of the equilibrium between the production of type 1 and type 2 cytokines, in favor of the latter[12,13]. This is called the Th1/Th2 paradigm. Production of type 2 cytokines, in particular IL-10, was reported to be abnormally elevated in different human cancers,

Figure 1. Hypothesis: summary of HPV16-specific immunity in health and disease.



The strong HPV16-specific CD4+ T-cell responses found against the early proteins and late protein L1 in healthy individuals *(left)* may explain why the majority of infected subjects clear HPV infections and HPV-induced anogenital disease. In contrast, patients with HPV-induced anogenital disease *(right)* frequently fail to mount T-cell responses against the early antigens and this may lead to the development or progression of anogenital neoplasia.

including renal cell cancers, lymphomas, and melanomas[14-16]. Similar, IL-10 production was reported to be abnormally elevated in PBMC of patients with HPV-associated CIN and cervical cancer[17-20]. Clerici et al. showed that extensive HPV-induced cervical intraepithelial neoplasia was strongly associated with a pronounced shift from the production of type 1 to type 2 cytokines, suggesting that cervical cancer may occur in patients predisposed to develop Th2-like immune responses[17]. We showed that the HPV-specific CD4+ T-cell responses detected in healthy subjects comprise IFNγ and IL-5 producing T-cells reactive to the early antigens E2 and E6, as well as IFNγ-producing T-cells directed at L1, indicating that HPV infections naturally induce a mixed Th1/Th2 immune response (*chapter 2*). In patients with cervical cancer we observed an HPV-specific T-cell response to these early antigens that was associated

with a lack of the production of type 1 cytokines, but also with a lack of the production of the prototypic Th2 cytokine IL-5, suggesting that the HPV-specific immune response in these patients was not just characterized by a shift to production of type 2 cytokines (*chapter 2*). Importantly, analysis of the reactivity of PBMC to the HPV16 late gene product L1 showed a strong IFNγ-associated L1-specific Th-cell responses in these patients (*chapter 3*). The strong type 1 L1-specific immune responses are likely to represent memory CD4+ T-cell responses as previously shown for the Th-cell responses against the early antigens of HPV[10]. Taken together, the presence of both strong HPV16 L1-specific Th1 responses and impaired T-cell responses to the early antigens shows that these patients are not predisposed to an overall type 2 immune response (*chapter 3*). Thus now that it seems unlikely that the patients are predisposed to a Th2 response, is it then possible that gradually the Th2 response becomes more dominant?

We observed that only a small minority of patients with high-grade CIN develop HPV-specific T-cell responses against the early antigens, while more than half of patients with cervical cancer display HPV-specific T-cell reactivity. These observations do not correspond with the idea of a gradual shift of HPV16 early antigen-specific Th1-type to early antigen-specific Th2 type T-cell responses during disease progression (*chapter 2* and unpublished data). A potential explanation for such a shift may be the loss of expression of L1 in progressive HPV-induced anogenital neoplasia[7]. When the lesion stops to provide L1 antigen that can be taken up and presented by antigen presenting cells, L1-specific T-cells are not likely to become activated to produce IFNγ anymore. In contrast to L1, the expression of the oncogenes E6 and E7 is increased in progressive anogenital neoplasia, but the T-cell response against these antigens is not associated with the production of IFNγ. Thus, it is not likely that a Th1/Th2 shift occurs among T-cells responding to the same antigen, but more likely reflects a gradual loss of Th1 cells responding to L1 and the activation of non-proinflammatory T-cells responding to the early antigens of HPV. As a result Th2 type cytokines may dominate.

Problems in the restoration of an effective type 1 HPV16-specific T-cell response

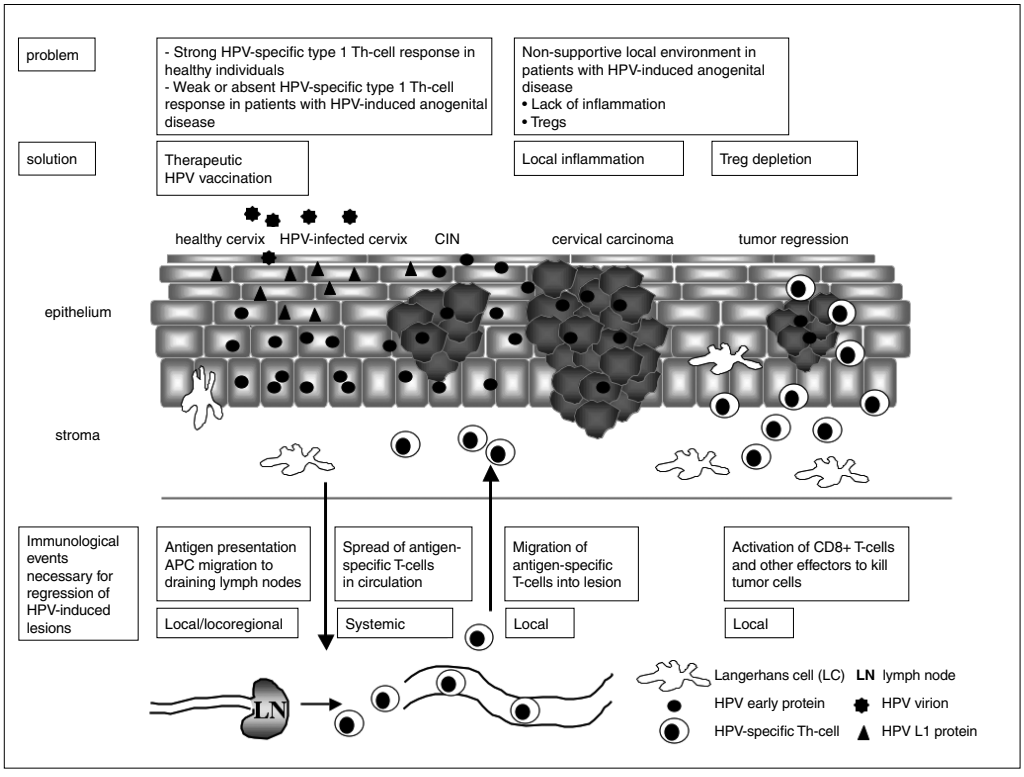
The immunological events leading to the rejection of (pre)malignant tissue can be divided into 4 steps[21]. First, activation and migration of antigen-presenting cells to the loco-regional lymph nodes is necessary to initiate the adaptive immune response. Second, activation and systemic spread of antigen-specific T-cells. Third, migration of these T-cells into the target lesion. Fourth, at the target site CD4+ T-cells should activate tumoricidal cells of the innate immune system and the CD8+ T-cells to kill

the tumor cells[22,23]. The consecutive steps involved in the rejection of HPV-induced lesions in relation to the findings from the studies decribed in this thesis and from other studies are depicted in Figure 2. Problems in the induction or restoration of HPV-specific immunity and immune escape mechanisms of HPV-infected and tumor cells are discussed in the text and possible solutions are given.

Induction and restoration of systemic HPV-specific Th-cell responses

Spontaneously induced circulating T-cells directed against various tumor antigens have been described in patients with melanomas, leukemias, and in various epithelial tumors[24-26]. Most studies analyzing T-cell responses to tumor antigens have focused on CD8+ T-cells, however, underlying CD4+ T-cell responses are highly likely to play a role because they are of pivotal importance in the induction and activation

Figure 2. Immunological events leading to the rejection of (pre)malignant tissue (adapted from[21], and this thesis).



Problems in the induction or restoration of HPV-specific immunity and possible solutions are discussed throughout the text.

of effector CD8+ T-cells. Spontaneous CD4+ T-cell responses against tumor antigens have also been described for instance for prostate-specific antigen in prostate cancer and for her-2/ neu in breast cancer[27,28]. These studies indicate that tumor-antigen is taken up and presented by APC to T-cells. So far, little is known about whether these circulating tumor-specific CD4+ T-cells influence the clinical course of disease. Type 1 Th-cell immunity was shown to prevent or delay relapses in patients with different hematological malignancies who underwent peripheral blood stem cell transplantation[29]. Although data from a number of other studies in selected patients suggest a favorable clinical course in patients with antigen-specific T-cells[25,30,31], no study has systematically compared patients with and without tumor-specific T-cell responses.
Others and we have shown that the spontaneously induced HPV-specific T-cell response does not fulfill all criteria necessary for an effective immune response because it is usually absent or weak in patients with HPV-induced disease (*chapters 2, 3, 5, and 6)*. Therapeutic vaccination aiming at the induction of HPV-specific T-cells against the early antigens should overcome the first two criteria. A number of studies have shown the immunogenicity of different HPV oncogene vaccines in patients with anogenital neoplasia[32-35] (and Kenter, van der Burg and Melief, unpublished results). We have shown that the combination of an HPV16 L2E6E7 fusion protein (TA-CIN) and a recombinant vaccinia virus expressing HPV16 and 18 E6E7 (TA-HPV) in women with AGIN was effective in the induction of strong HPV16-specific type 1 CD4+ T-cell responses (*chapter 5*). Furthermore, even in late-stage cervical cancer, the administration of a therapeutic HPV16 E6 and E7 long peptide vaccine leads to IFNγ-producing CD4+ T-cell responses (Kenter, vd Burg, Melief, unpublished results). In addition, vaccination of a patient with advanced HPV18-positive cervical cancer with E7-peptide pulsed autologous DC and infusions of T-cells stimulated with E7-pulsed DC was associated with a delayed-type hypersensitivity response to HPV18 E7 and disease stabilisation for 18 months[36]. These studies indicate that vaccines can restore the HPV-specific CD4+ and CD8+ T-cell response. The adoptive transfer of HPV16-specific T-cells may form an alternative treatment modality for patients with advanced cervical cancer. Clinical responses to adoptive transfer of autologous CD8+ T-cells or a mixture of CD4+ and CD8+ T-cells have been described for patients with advanced renal cell carcinoma[37] and metastatic melanoma[38]. One of the main obstacles for the large-scale and effective implementation for adoptive immunotherapy may be the difficulty of obtaining sufficient numbers of antigen-specific autologous T-cells. A recent study reported on the successful isolation and expansion of large amounts of HPV16-specific T-cell populations with broad specificity from healthy donors within a time span of 2-3 weeks[39]. It can be envisaged that healthy donor-

derived HPV16-specific type 1 T-cells are transferred into cervical cancer patients following allogeneic stem cell transplantation and eradication of host immune cells. The reactivity of these adoptively transferred T-cells may then be boosted by specific vaccination.

From all HPV vaccination studies in patients with anogenital disease that have been performed so far, it is clear that there is no simple relationship between the induction or restoration of large numbers of HPV-specific T-cells and clinical responses. Some patients even had progressive disease in the presence of very high levels of HPV-specific T-cells (*chapter 5,*[32,33]), and this is in line with the results of peptide vaccination studies in various human cancers, like melanomas[40,41], renal cell carcinomas[42], and advanced malignant gliomas[43]. Together, these data indicate that the induction of large numbers of circulating tumor-specific T-cells is not sufficient for the regression of developing lesions in a substantial part of patients with human cancers. The efficacy of strong antitumor T-cell immunity is highly likely to depend on a supportive local environment, which is the battlefield between the immune system and developing neoplasia.

The impact of the local microenvironment on clinical outcome in patients with AGIN
The epithelial dendritic cell (Langerhans cell, LC) is the key antigen-presenting cell involved in the induction and modulation of HPV-specific immunity because HPV infections are strictly confined to the epithelium and HPV-infected keratinocytes cannot reach the draining lymph nodes[44]. In the absence of any inflammation or pathogenic elements, most dendritic cells have a resting, immature phenotype characterized by low surface expression of MHC- and costimulatory molecules[45]. The activation and migration of DC requires strong inflammatory signals[46]. Upon interaction with proinflammatory cytokines, CpG, or CD40-ligand, DCs rapidly acquire an activated phenotype, and mature DCs are very efficient in the activation of T-cells. Davidson et al. showed that clinical responses upon vaccination with TA-HPV in patients with high-grade VIN were significantly associated with a preexisting infiltrate by CD4+ and CD8+ T-cells as well as CD1a+ dendritic cells. The presence of these immune cells suggests that in these patients the local environment supports the infiltration of T-cells, including vaccine-induced HPV-specific T-cells[33]. Poor response rates to photodynamic therapy in women with VIN have been attributed to decreased numbers of Langerhans cells and CD8+ T-cells within the lesions[47]. In several types of human cancers patients display huge differences in the number of intraepithelial immune cells that infiltrated the tumor cell nests[48-50]. Either some

patients did not develop a tumor-specific immune response or the tumor-specific T-cells failed to infiltrate the tumor. We showed that even in patients with circulating tumor-specific T-cells, strong tumor-infiltration does not always occur (*chapter 7*) or that the type of tumor-specific T-cell response prevents infiltration of effector T-cells. This suggests that the local environment supports infiltration in some patients but not in others. Notably, the presence of high numbers of tumor-infiltrating T-cells was shown to be a prognostic factor correlating with improved survival of patients[48-50]. A change in the local environment may alter the course of disease. In a murine model, it was shown that adenovirus specific CD8+ T-cells developed in draining lymph nodes of mice with adenovirus-positive tumors, but that rejection of tumors only occurred when strong inflammatory agents were locally injected[51].

Earlier studies have suggested that topical application of inflammatory agents may alter the natural course of anogenital disease. Intralesional treatment with beta-interferon was significantly associated with the regression of CIN 2 lesions[52]. A large, randomized phase III trial in 301 women with CIN 2/3 showed that topical application of all-trans-retinoic acid (RA), a chemopreventive agent that inhibits cellular proliferation, increased the complete histologic regression rate of CIN 2 from 27% to 43% compared to the placebo group[53]. Interestingly, another study showed that low-dose topical administration of RA was not more effective than placebo in the regression of lesions, suggesting that the serial biopsies that had been taken from all women and its induction of associated inflammation may have been an effective treatment by itself[54]. A recent study has shown that local invasive procedures strongly enhance systemic HPV16-specific type 1 T-cell responses in patients with HPV16-induced cervical cancer[55]. Especially those patients who had undergone previous invasive procedures showed the strongest enhancement of HPV16-specific IFNγ-associated T-cell responses, suggesting a boosting effect of the second invasive procedures[55]. The strong enhancement of T-cell responses was not seen in CIN patients. Similar, we could not detect a direct influence of topical application of Imiquimod on the numbers of circulating HPV-specific Th cells in our patients with high-grade VIN (*chapter 6*).

A number of studies in human (pre)malignant diseases have shown that the presence of circulating antigen-specific T-cells in combination with local inflammation was associated with better clinical responses. In patients with stage III melanoma, pre-existing IFNγ-producing CD4+ T-cell responses against melanoma antigens in combination with a strong inflammatory agent were associated with overall survival[56].

After electrocoagulation of her VIN 3 lesions, clinical regression was observed in a woman displaying strong systemic preexisting HPV16 E6 and E7-specific T-cell responses and local infiltration of CD4+ and CD8+ T-cells[57]. We found that topical application of the immune response modifier Imiquimod was significantly associated with a better clinical outcome in a group of patients with HPV-induced high-grade VIN, especially in those who display a systemic HPV-specific type 1 T-cell response (*chapter 6*). This may be explained by a number of immunological mechanisms. First, Imiquimod induces a type 1 adaptive immune response through activation of macrophages and dendritic cells via binding to cell surface receptors like TLR (toll-like receptors) 7 and 8[58-60]. Second, Imiquimod has been shown to increase antigen presentation and maturation of Langerhans cells[61,62]. Third, topical application of Imiquimod was shown to increase the number of infiltrating CD4+ and CD8+ T-cells in cutaneous squamous cell carcinoma specimens[60]. Taken together, it is likely that topical application of Imiquimod leads to the induction of a more favorable local environment by counteracting type 2 cytokines, a better presentation of HPV antigens to the adaptive immune system, and increased infiltration of HPV-specific T-cells to lesions, leading to objective clinical responses in patients with high-grade VIN after treatment with this agent. Overall the results of these studies indicate that the induction of an inflammatory reaction in the lesion or tumour is necessary to induce tumor-specific T-cell infiltration and clinical success.

Imiquimod may also be useful in HPV vaccination schedules as vaccine adjuvant. Subcutaneous injection at the vaccination site of Imiquimod in mice was found to increase the number and maturation status of dendritic cells in draining lymph nodes, and to enhance IFNγ-associated CD4+ and CD8+ T-cell responses[63]. A limited number of phase I studies in humans have reported that oral or subcutaneous administration of Imiquimod seems safe and leads to increased levels of pro-inflammatory cytokines in serum[64,65]. Furthermore, the use of imiquimod as adjuvant supported the induction of melanoma-specific T-cells when skin areas were pretreated with imiquimod by topical application to the vaccine sites 24 h and 2 h prior to peptide vaccination[66].

Regulatory CD4+CD25+ T-cells

In 1995, Sakaguchi et al. identified so-called "regulatory" T-cells (Treg cells) by identifying a population of CD4+ T-cells highly expressing the IL-2 receptor α-chain (CD25) and preventing autoimmunity in a murine model[67]. More recent studies have shown that the transcription factor forkhead box P3 (FOXP3) is a crucial developmental and functional factor for CD4+CD25+ Tregs[68,69]. Numerous reports in the

following years have shown that regulatory T-cells suppress the activation and function of other T-cells[70,71]. Their physiological role is to protect the host against the development of autoimmunity by regulating immune responses against antigens expressed by normal tissues[72,73]. Since tumor antigens are mainly self-antigens, Tregs may also prevent the tumor-bearing host from mounting an effective antitumor response. Previous studies have shown that elevated numbers of circulating and tumor CD4+CD25+ Tregs can be found in patients with different types of cancer[74-77]. Regulatory T-cells producing the type 2 cytokine TGFβ were increased in tumor specimens from patients with late stage ovarian cancer as compared with control CD4+ T-cells from healthy donor peripheral blood[75]. Furthermore, high numbers of intratumoral Tregs were found to be associated with reduced survival in patients with ovarian cancer[78]. It is likely that there is a balance between tumor-promoting and tumor-inhibiting lymphocytes within tumor microenvironments, and therefore the local presence of Tregs may explain why HPV-specific T-cells do not succeed in rejection of HPV-induced cervical tumors. Earlier studies in cervical cancer patients have found that CD4+CD25+ T-cells are predominantly detected in tumor draining lymph nodes[79]. We have shown that cervical cancers were infiltrated stronger with Foxp3+ T-cells compared to healthy cervices (*chapter 7*). Importantly, a higher CD8+/Treg ratio was detected in cervical cancer patients without lymph node metastases, which is in general a positive prognostic factor. Interestingly, we found that the patients with lymph node metastases displaying a systemic HPV-specific T-cell response showed the lowest CD8+/Treg ratio when compared to all other groups. However, a direct relationship between the presence or absence of systemic HPV-specific T-cells and the lymph node status was not detected. These data suggest that the presence of circulating HPV-specific T-cells not necessarily reflect an effective antitumor response and that evasion of tumor cells, especially in the lymph nodes, is a prerequisite for the induction of HPV-specific T-cell responses that can counteract the recruitment of effector cells in the tumor. Indeed, recent data from our group have shown that HPV16 E6-specific CD4+ Tregs are present in the tumor and lymph nodes of patients with cervical cancer and are able to suppress the action of naive CD4+ T-cells[97]. In addition, a low CD8+/ regulatory T-cell ratio was significantly associated with worse survival of patients with cervical cancer (Jordanova ES, personal communication).

Note that not only tumors but possibly also therapeutic vaccination strategies, although designed to enhance CD4+ and CD8+ T-cell effector immunity against the E6 and E7 oncoproteins of HPV16 and/or 18, may lead to the unwanted induction and/or activation of regulatory T-cells. Recently, Hussain et al. showed that

an HPV16 E7 vaccine induced high numbers of IL-10 and TGFβ-secreting tumor-infiltrating CD4+CD25+ Tregs in mice[80]. Notably these animals showed no or little regression of their established E7-expressing tumors [80]. A similar study by Zhou et al. elegantly showed that vaccination of tumor-bearing hosts harboring a mixture of antigen-specific responder and regulatory T-cells resulted in concomitant expansion of both T-cell populations. Importantly, the net effect of vaccination in this setting was failure of the antitumor immune response[81].

Preclinical studies in mice have indicated that depletion of Tregs using anti-CD25 antibodies can induce effective antitumor immunity and positively affect survival[82-84]. Two recent studies in humans have shown that in vivo elimination of CD25-expressing Tregs by ONTAK (recombinant IL-2 diphteria toxin conjugate DAB_{389}IL-2) enhanced the magnitude of vaccine-mediated tumor-specific T-cell responses in the blood of patients with metastatic renal cell carcinoma and ovarian cancer[85,86]. Cyclophosphamide is known to decrease the number of circulating CD4+CD25+ regulatory T-cells in both animals and humans[87,88]. In a rat colon cancer model, administration of cyclophosphamide depleted Treg cells and delayed the outgrowth of tumors[89]. Interestingly, combining cyclophosphamide and immunotherapy even cured the mice, whereas both strategies applied alone had no curative effect. In melanoma patients, the combination of adoptive transfer of ex vivo activated tumor-specific T-cells to patients after chemotherapy with cyclophosphamide and fludarabine induced tumor regression in up to 50% of patients treated[90].
Rather than depleting Tregs, interfering with their regulatory function is an alternative approach to improve effector T-cell responses in the treatment of human cancers. Peng et al. have reported the expression of TLR-8 on human Tregs, and they have identified a simple poly-guanine oligonucleotide that acts directly on Tregs to prevent suppression of effector T-cells[91]. Because Imiquimod acts through TLR-7 and 8, part of its action may rely on the counteraction of the negative regulation of Tregs at the local environment. In combination with circulating HPV-specific T-cells, this may contribute to better clinical responses in patients with high-grade VIN after imiquimod therapy (*chapter 6*).
Current knowledge about Treg migration is still limited. In patients with ovarian cancer, Tregs expressing the CCR4 receptor migrated preferentially and predominantly to the CCR4-ligand (CCL22) positive tumor and the associated malignant cells in the ascites rather than the draining lymph nodes[78]. Also in lung cancer tissues increased percentages of CXCR4 positive CD4+CD25+Foxp3+ T-cells were found when compared with normal lung tissue[92]. So far, it is unknown what chemokine receptor/

ligand pair is important for the migration and homing of Tregs in HPV-induced anogenital disease, and whether Tregs would exclusively use such a receptor/ligand pair. This has to be examined before blocking the migration of regulatory T-cells by chemokine receptor antagonists becomes an option for immunotherapeutic intervention strategies in HPV-associated anogenital disease.

Future prospects

Effective antitumor immune mechanisms can be limited simply by the absence of proinflammatory local environment or more sophisticated by immunoregulatory components such as regulatory T-cells and type 2 cytokines like IL-10 and TGFβ. Immunotherapeutic combination strategies in which these negative local aspects are counteracted are currently tested in mouse tumor models and in the clinic (read above). Another mechanism by which tumor cells can escape the immune system is by defects in MHC class I antigen presentation. Loss of HLA class I expression and mutations in TAP genes are frequently observed in cervical cancer and high-grade VIN[47,93-95]. Recently, a category of CTLs was described that may be able to prevent the escape of tumor cells with defects in MHC class I antigen processing[96]. These CD8+ T-cells are directed against a repertoire of peptides (T-cell epitopes associated with impaired peptide processing, TEIPPs) that are presented at the surface of processing-deficient cells. Addition of these CD8+ peptide-epitopes to current vaccines may prevent the escape of TAP down-regulated tumor cells.

Based on the results of the studies described in this thesis, it is not likely that vaccination alone will be sufficient for the effective eradication of established (pre)malignant HPV-induced lesions. Therapies should a) aim at the depletion of regulatory T-cells, b) induce local inflammation, for example by topical application of immune modifiers, local destructive therapies, or a combination of chemotherapy and radiation, and c) induce both CD4+ and CD8+ T-cells specific for HPV16 and maybe even the induction of TEIPP-specific CD8+ T-cells, to have the best chance in establishing clinical efficacy at early and late stages of anogenital disease.

References

1. Zwaveling S, Mota SC, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol 2002; 169: 350-358.
2. Vambutas A, Devoti J, Nouri M, et al. Therapeutic vaccination with papillomavirus E6 and E7 long peptides results in the control of both established virus-induced lesions and latently infected sites in a pre-clinical cottontail rabbit papillomavirus model. Vaccine 2005; 23: 5271-5280.
3. Klinman DM, Yi AK, Beaucage SL, et al. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci USA 1996; 93: 2879-2883.
4. Kim TY, Myoung HJ, Kim JH, et al. Both E7 and CpG-oligodeoxynucleotide are required for protective immunity against challenge with human papillomavirus 16 (E6/E7) immortalized tumor cells: involvement of CD4+ and CD8+ T cells in protection. Cancer Res 2002; 62: 7234-7240.
5. Johnston KB, Monteiro JM, Schultz LD, et al. Protection of beagle dogs from mucosal challenge with canine oral papillomavirus by immunization with recombinant adenoviruses expressing codon-optimized early genes. Virology 2005; 336: 208-218.
6. Melsheimer P, Kaul S, Dobeck S, et al. Immunocytochemical detection of HPV high-risk type L1 capsid proteins in LSIL and HSIL as compared with detection of HPV L1 DNA. Acta Cytol 2003; 47: 124-128.
7. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. Clin Sci (Lond), 2006; 110: 525-541.
8. Palefsky JM, Minkoff H, Kalish LA, et al. Cervicovaginal human papillomavirus infection in human immunodeficiency virus-1 (HIV)-positive and high-risk HIV-negative women. J Natl Cancer Inst 1999; 91: 226-236.
9. Palefsky JM, Holly EA. Chapter 6: Immunosuppression and co-infection with HIV. J Natl Cancer Inst Monogr 2003; 41-46.
10. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002; 62: 472-479.
11. Welters MJ, de Jong A, van den Eeden SJ, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003; 63: 636-641.
12. Clerici M, Shearer GM, Clerici E. Cytokine dysregulation in invasive cervical carcinoma and other human neoplasias: time to consider the TH1/TH2 paradigm. J Natl Cancer Inst 1998; 90: 261-263.
13. Lucey DR, Clerici M, Shearer GM. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. Clin Microbiol Rev 1996; 9: 532-562.
14. Bost KL, Bieligk SC, Jaffe BM. Lymphokine mRNA expression by transplantable murine B lymphocytic malignancies. Tumor-derived IL-10 as a possible mechanism for modulating the antitumor response. J Immunol 1995; 154: 718-729.
15. Kruger-Krasagakes S, Krasagakis K, Garbe C, et al. T. Expression of interleukin 10 in human melanoma. Br J Cancer 1994; 70: 1182-1185.
16. Nakagomi H, Pisa P, Pisa EK, et al. Lack of interleukin-2 (IL-2) expression and selective expression of IL-10 mRNA in human renal cell carcinoma. Int J Cancer 1995; 63: 366-371.
17. Clerici M, Merola M, Ferrario E, et al. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. J Natl Cancer Inst 1997; 89: 245-250.
18. Jacobs N, Giannini SL, Doyen J, et al. Inverse modulation of IL-10 and IL-12 in the blood of women with preneoplastic lesions of the uterine cervix. Clin Exp Immunol 1998; 111: 219-224.
19. Chopra V, Dinh TV, Hannigan EV. Circulating serum levels of cytokines and angiogenic factors in patients with cervical cancer. Cancer Invest 1998; 16: 152-159.
20. Bais AG, Beckmann I, Lindemans J, et al. A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions. J Clin Pathol 2005; 58: 1096-1100.
21. Marincola FM, Wang E, Herlyn M, et al. Tumors as elusive targets of T-cell-based active immunotherapy. Trends Immunol 2003; 24: 335-342.
22. Hung K, Hayashi R, Lafond-Walker A, et al. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med 1998; 188: 2357-2368.
23. Melief CJ, Toes RE, Medema JP, et al. Strategies for immunotherapy of cancer. Adv Immunol 2000; 75: 235-282.
24. Scheibenbogen C, Lee KH, Stevanovic S, et al. Analysis of the T cell response to tumor and viral peptide antigens by an IFNgamma-ELISPOT assay. Int J Cancer 1997; 71: 932-936.

25. Scheibenbogen C, Letsch A, Thiel E, et al. CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. Blood 2002; 100: 2132-2137.
26. Lee PP, Yee C, Savage PA, et al. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med 1999; 5: 677-685.
27. Disis ML, Knutson KL, Schiffman K, et al. Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res Treat 2000; 62: 245-252.
28. McNeel DG, Nguyen LD, Ellis WJ, et al. Naturally occurring prostate cancer antigen-specific T cell responses of a Th1 phenotype can be detected in patients with prostate cancer. Prostate 2001; 47: 222-229.
29. Mitra DK, Singh HP, Singh M, et al. Reconstitution of naive T cells and type 1 function after autologous peripheral stem cell transplantation: impact on the relapse of original cancer. Transplantation 2002; 73: 1336-1339.
30. Valmori D, Scheibenbogen C, Dutoit V, et al. Circulating Tumor-reactive CD8(+) T cells in melanoma patients contain a CD45RA(+)CCR7(-) effector subset exerting ex vivo tumor-specific cytolytic activity. Cancer Res 2002; 62: 1743-1750.
31. Karanikas V, Colau D, Baurain JF, et al. High frequency of cytolytic T lymphocytes directed against a tumor-specific mutated antigen detectable with HLA tetramers in the blood of a lung carcinoma patient with long survival. Cancer Res 2001; 61: 3718-3724.
32. Baldwin PJ, van der Burg SH, Boswell CM, et al. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003; 9: 5205-5213.
33. Davidson EJ, Boswell CM, Sehr P, et al. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003; 63: 6032-6041.
34. Davidson EJ, Faulkner RL, Sehr P, et al. Effect of TA-CIN (HPV 16 L2E6E7) booster immunisation in vulval intraepithelial neoplasia patients previously vaccinated with TA-HPV (vaccinia virus encoding HPV 16/18 E6E7). Vaccine 2004; 22: 2722-2729.
35. Frazer IH, Quinn M, Nicklin JL, et al. Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIXtrade mark adjuvant in women with cervical intraepithelial neoplasia. Vaccine 2004; 23: 172-181.
36. Santin AD, Bellone S, Gokden M, et al. Vaccination with HPV-18 E7-pulsed dendritic cells in a patient with metastatic cervical cancer. N Engl J Med 2002; 346: 1752-1753.
37. Kawai K, Saijo K, Oikawa T, et al. Clinical course and immune response of a renal cell carcinoma patient to adoptive transfer of autologous cytotoxic T lymphocytes. Clin Exp Immunol 2003; 134: 264-269.
38. Dudley ME, Wunderlich JR, Yang JC, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 2005; 23: 2346-2357.
39. de Jong A, van der Hulst JM, Kenter GG, et al. Rapid enrichment of human papillomavirus (HPV)-specific polyclonal T cell populations for adoptive immunotherapy of cervical cancer. Int J Cancer 2005; 14: 274-282.
40. Powell DJ Jr., Rosenberg SA. Phenotypic and functional maturation of tumor antigen-reactive CD8+ T lymphocytes in patients undergoing multiple course peptide vaccination. J Immunother 2004; 27: 36-47.
41. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol 2005; 175: 6169-6176.
42. Uemura H, Fujimoto K, Tanaka M, et al. A phase I trial of vaccination of CA9-derived peptides for HLA-A24-positive patients with cytokine-refractory metastatic renal cell carcinoma. Clin Cancer Res 2006; 12: 1768-1775.
43. Yajima N, Yamanaka R, Mine T, et al. Immunologic evaluation of personalized peptide vaccination for patients with advanced malignant glioma. Clin Cancer Res 2005; 11: 5900-5911.
44. Offringa R, de Jong A, Toes RE, et al. Interplay between human papillomaviruses and dendritic cells. Curr Top Microbiol Immunol 2003; 276: 215-240.
45. van Duivenvoorde LM, van Mierlo GJ, Boonman ZF, et al. Dendritic cells: vehicles for tolerance induction and prevention of autoimmune diseases. Immunobiology 2006; 211: 627-632.
46. Heath WR, Carbone FR. Cross-presentation in viral immunity and self-tolerance. Nat Rev Immunol 2001; 1: 126-134
47. Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, et al. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. Cancer Res 2001; 61: 192-196.

48. Kondratiev S, Sabo E, Yakirevich E, et al. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin Cancer Res 2004; 10: 4450-4456.
49. Menon AG, Fleuren GJ, Alphenaar EA, et al. A basal membrane-like structure surrounding tumour nodules may prevent intraepithelial leucocyte infiltration in colorectal cancer. Cancer Immunol Immunother 2003; 52: 121-126.
50. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348: 203-213.
51. van Mierlo GJ, Boonman ZF, Dumortier HM, et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol 2004; 173: 6753-6759.
52. Rotola A, Costa S, Di Luca D, et al. Beta-interferon treatment of cervical intraepithelial neoplasia: a multicenter clinical trial. Intervirology 1995; 38: 325-331.
53. Meyskens FL Jr, Surwit E, Moon TE, et al. Enhancement of regression of cervical intraepithelial neoplasia II (moderate dysplasia) with topically applied all-trans-retinoic acid: a randomized trial. J Natl Cancer Inst 1994; 86: 539-543.
54. Ruffin MT, Bailey JM, Normolle DP, et al. Low-dose topical delivery of all-trans retinoic acid for cervical intraepithelial neoplasia II and III. Cancer Epidemiol Biomarkers Prev 2004; 13: 2148-2152.
55. Visser J, van Baarle D, Hoogeboom BN, et al. Enhancement of human papilloma virus type 16 E7 specific T cell responses by local invasive procedures in patients with (pre)malignant cervical neoplasia. Int J Cancer 2006; 118: 2529-2537.
56. Hsueh EC, Famatiga E, Shu S, et al. Peripheral blood CD4+ T-cell response before postoperative active immunotherapy correlates with clinical outcome in metastatic melanoma. Ann Surg Oncol 2004; 11: 892-899.
57. Villada IB, Barracco MM, Ziol M, et al. Spontaneous regression of grade 3 vulvar intraepithelial neoplasia associated with human papillomavirus-16-specific CD4(+) and CD8(+) T-cell responses. Cancer Res 2004; 64: 8761-8766.
58. Stanley MA. Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential. Clin Exp Dermatol 2002; 27: 571-577.
59. Sauder DN. Imiquimod: modes of action. Br J Dermatol 149 Suppl 2003; 66: 5-8.
60. Smith KJ, Hamza S, Skelton H. Topical imidazoquinoline therapy of cutaneous squamous cell carcinoma polarizes lymphoid and monocyte/macrophage populations to a Th1 and M1 cytokine pattern. Clin Exp Dermatol 2004; 29: 505-512.
61. Burns RP Jr, Ferbel B, Tomai M, et al. The imidazoquinolines, imiquimod and R-848, induce functional, but not phenotypic, maturation of human epidermal Langerhans' cells. Clin Immunol 2000; 94: 13-23.
62. Suzuki H, Wang B, Shivji GM, et al. Imiquimod, a topical immune response modifier, induces migration of Langerhans cells. J Invest Dermatol 2000; 114: 135-141.
63. Thomsen LL, Topley P, Daly MG, et al. Imiquimod and resiquimod in a mouse model: adjuvants for DNA vaccination by particle-mediated immunotherapeutic delivery. Vaccine 2004; 22: 1799-1809.
64. Witt PL, Ritch PS, Reding D, et al. Phase I trial of an oral immunomodulator and interferon inducer in cancer patients. Cancer Res 1993; 53: 5176-5180.
65. Goldstein D, Hertzog P, Tomkinson E, et al. Administration of imiquimod, an interferon inducer, in asymptomatic human immunodeficiency virus-infected persons to determine safety and biologic response modification. J Infect Dis 1998; 178: 858-861.
66. Shackleton M, Davis ID, Hopkins et al. The impact of imiquimod, a Toll-like receptor-7 ligand (TLR7L), on the immunogenicity of melanoma peptide vaccination with adjuvant Flt3 ligand. Cancer Immun 2004; 4: 9.
67. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155: 1151-1164.
68. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003; 4: 330-336.
69. Khattri R, Cox T, Yasayko SA, et al. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003; 4: 337-342.
70. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004; 22: 531-562.
71. Shevach EM. Certified professionals: CD4(+)CD25(+) suppressor T cells. J Exp Med 2001; 193: F41-F46.
72. Jonuleit H, Schmitt E, Schuler G, et al. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature

human dendritic cells. J Exp Med 2000; 192: 1213-1222.
73. Read S, Powrie F. CD4(+) regulatory T cells. Curr Opin Immunol 2001; 13: 644-649.
74. Wang HY, Peng G, Guo Z, et al. Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4+ regulatory T cells. J Immunol 2005; 174: 2661-2670.
75. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 2001; 61: 4766-4772.
76. Woo EY, Yeh H, Chu CS, et al. Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. J Immunol 2002; 168: 4272-4276.
77. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 2002; 169: 2756-2761.
78. Curiel TJ, Coukos G, Zou L, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004; 10: 942-949.
79. Fattorossi A, Battaglia A, Ferrandina G, et al. Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients. Gynecol Oncol 2004; 92: 106-115.
80. Hussain SF, Paterson Y. CD4+CD25+ regulatory T cells that secrete TGFbeta and IL-10 are preferentially induced by a vaccine vector. J Immunother 2004; 27: 339-346.
81. Zhou G, Drake CG, Levitsky HI. Amplification of tumor-specific regulatory T cells following therapeutic cancer vaccines. Blood 2006; 107: 628-636.
82. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol 1999; 163: 5211-5218.
83. Onizuka S, Tawara I, Shimizu J, et al. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 1999; 59: 3128-3133.
84. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 2001; 194: 823-832.
85. Barnett B, Kryczek I, Cheng P, et al. Regulatory T cells in ovarian cancer: biology and therapeutic potential. Am J Reprod Immunol 2005; 54: 369-377.
86. Dannull J, Su Z, Rizzieri D, et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005; 115: 3623-3633.
87. Ghiringhelli F, Menard C, Martin F, et al. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. Immunol Rev 2006; 214: 229-238.
88. Ercolini AM, Ladle BH, Manning EA, et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. J Exp Med 2005; 201: 1591-1602.
89. Ghiringhelli F, Larmonier N, Schmitt E, et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004; 34: 336-344.
90. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002; 98: 850-854.
91. Peng G, Guo Z, Kiniwa Y, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science 2005; 309: 1380-1384.
92. Wald O, Izhar U, Amir G, et al. CD4+CXCR4high-CD69+ T cells accumulate in lung adenocarcinoma. J Immunol 2006; 177: 6983-6990.
93. Evans M, Borysiewicz LK, Evans AS, et al. Antigen processing defects in cervical carcinomas limit the presentation of a CTL epitope from human papillomavirus 16 E6. J Immunol 2001; 167: 5420-5428.
94. Hilders CG, Munoz IM, Nooyen Y, et al. Altered HLA expression by metastatic cervical carcinoma cells as a factor in impaired immune surveillance. Gynecol Oncol 1995; 57: 366-375.
95. Fowler NL, Frazer IH. Mutations in TAP genes are common in cervical carcinomas. Gynecol Oncol 2004; 92: 914-921.
96. van Hall T, Wolpert EZ, van Veelen P, et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. Nat Med 2006; 12: 417-424.
97. van der Burg SH, Piersma SJ, de Jong A, et al. Association of cervical cancer with the presence of CD4+ regulatory T-cells specific for human papillomavirus antigens. Proc Natl Acad Sci USA 2007; 104: 12087-12092.

CHAPTER 9

Samenvatting

Humaan papillomavirus (HPV) en ziekten van de vrouwelijke geslachtsorganen
Baarmoederhalskanker is wereldwijd de op een na meest voorkomende vorm van kanker onder vrouwen. In Nederland worden jaarlijks ongeveer 700 nieuwe gevallen vastgesteld en overlijden tussen de 250 en 300 vrouwen aan de gevolgen van deze ziekte. Het bevolkingsonderzoek, waarbij van alle vrouwen tussen de 30 en 60 jaar eens in de 5 jaar een uitstrijkje wordt gemaakt, is gericht op de opsporing van voorstadia van baarmoederhalskanker, ook wel cervicale intraepitheliale neoplasie of CIN genaamd. Recent is duidelijk geworden dat in vrijwel alle gevallen van baarmoederhalskanker en CIN, maar ook bijvoorbeeld voorstadia van kanker van de schaamlippen, vagina of de anus, het humane papillomavirus (HPV) wordt aangetroffen.

Humane papillomavirussen kunnen worden onderverdeeld in laagrisico en hoogrisico typen. De eerstgenoemde veroorzaken bijvoorbeeld genitale wratten. De hoog-risico typen zijn in staat om (voorstadia van) kanker te veroorzaken wanneer zij langdurig in het lichaam verblijven. Het belangrijkste voorbeeld uit deze groep is HPV type 16 (HPV16). In ongeveer 60% van de gevallen van baarmoederhalskanker wordt dit type aangetroffen. De ongecontroleerde groei van cellen, kenmerkend voor (voorstadia van) kanker, wordt veroorzaakt door binding van bepaalde eiwitten van HPV aan structuren van geinfecteerde cellen. De E6 en E7 eiwitten van HPV zijn hiervan de belangrijkste voorbeelden. Het zogenaamde L1 eiwit van HPV is het belangrijkste onderdeel van het kapsel of omhulsel van het virus. In tegenstelling tot de E6 en E7 eiwitten, die in alle gevallen van kanker gevonden worden, komt het L1 eiwit voornamelijk tot uiting in geinfecteerde cellen en in vroege voorstadia van kanker, en juist niet meer in de latere stadia van infectie en afwijkingen.

HPV is een zeer veel voorkomend virus dat via sexueel contact van mens op mens kan worden overgebracht. Naar schatting komt ongeveer 80% van alle mensen eens in zijn of haar leven in contact met HPV. De overgrote meerderheid van deze mensen is in staat om het virus op te ruimen uit het lichaam. Het is aannemelijk dat het immuunsysteem een belangrijke rol hierin speelt omdat bekend is dat mensen met een verminderde afweer, zoals bijvoorbeeld patiënten met HIV, vaker HPV infecties hebben die ook vaak ernstiger verlopen dan mensen met een goed werkend immuunsysteem.

Het immuunsysteem
Het immuunsysteem kan worden onderverdeeld in het zogenaamde aangeboren immuunsysteem en het verworven immuunsyteem. Voorbeelden van het aangeboren

immuunsysteem zijn bijvoorbeeld de huid en slijmvliezen die als eerste verdediging dienen tegen ongewenste indringers zoals bacteriën en virussen. Het verworven immuunsysteem heeft als belangrijkste kenmerk dat het specifiek is, dat wil zeggen dat het zeer sterk kan reageren tegen bepaalde lichaamsvreemde indringers, en dat het in staat is om geheugen op te bouwen, zodat een volgend contact met dezelfde indringer sneller en effectiever zal leiden tot het opruimen ervan. Het verworven immuunsysteem bestaat uit 2 componenten: het humorale immuunsysteem en het cellulaire immuunsysteem. Het humorale immuunsysteem bestaat uit zogenaamde B-cellen die antilichamen kunnen produceren. Voor virusinfecties geldt dat deze antilichamen gericht zijn tegen het virale kapsel of omhulsel, dus in het geval van HPV infecties tegen het L1 eiwit. Antilichamen dienen ervoor om te verhinderen dat een bepaalde indringer een cel binnentreedt, met andere woorden: zij voorkomen het optreden van een bepaalde infectie. Wanneer een virus zich eenmaal in een gastheercel heeft genesteld kunnen antilichamen er niet meer bij omdat zij niet in staat zijn om cellen binnen te komen. Het cellulaire immuunsysteem bestaat uit zogenaamde T-cellen of T-lymfocyten en richt zich met name op intracellulaire infecties, waaronder HPV infecties vallen, maar ook op tumorcellen. Er zijn verschillende soorten T-cellen te onderscheiden: T-helper cellen of CD4+ T-cellen, cytotoxische T-cellen of CD8+ T-cellen, en ook de recent beschreven regulatoire T-cellen. T-helper cellen spelen een centrale rol in de afweer tegen virusinfecties en bepaalde vormen van kanker, omdat zij andere cellen van het immuunsysteem kunnen aansturen en activeren. Zo zijn zij van cruciaal belang voor het aansturen van B-cellen, maar ook voor de activatie van cytotoxische T-cellen, die virus-geinfecteerde cellen en tumorcellen kunnen doden. Regulatoire T-cellen zijn in staat om andere T-cellen te onderdrukken en worden daarom gezien als remmers van een effectieve afweer. T-cellen maken verschillende stoffen, cytokines genaamd, die van belang zijn voor het reguleren van afweerprocessen. Type 1 cytokines, waarvan interferon-gamma (IFNγ) het belangrijkste voorbeeld is, zijn belangrijk voor de activatie van de cellulaire afweer. Type 2 cytokines, zoals interleukine-4 (IL-4), IL-5 en IL-10 zijn belangrijk voor de humorale afweer. Daarnaast kan IL-10 ook remmend werken op de immuunrespons.

De studies in dit proefschrift hebben voornamelijk betrekking op de cellulaire Th-cel immuniteit tegen HPV16 omdat deze de spil lijkt van een effectieve afweer respons tegen infecties die het virus veroorzaakt en gerelateerde ziekten van de vrouwelijke geslachtsorganen.

De cellulaire immuunrespons tegen HPV16 in gezonde mensen en in mensen met HPV-geassocieerde ziekte; dit proefschrift

Zoals hierboven reeds genoemd, is de overgrote meerderheid van de bevolking eens in zijn of haar leven in contact geweest met HPV en weten wij dat slechts een klein percentage hiervan afwijkingen ontwikkelt die door het virus worden veroorzaakt. Is er iets mis met de T-cel afweer tegen HPV in vrouwen met HPV-geassocieerde aandoeningen, en zo ja wat? Om deze vraag te kunnen beantwoorden onderzochten wij T-cellen uit het bloed van gezonde mensen en vergeleken deze met die van vrouwen met CIN en baarmoederhalskanker. Wij vonden dat bloed van gezonde mensen in bijna alle gevallen T-helper cellen bevatte gericht tegen vroege eiwitten van HPV16, en dat deze T-helper cellen zowel type 1 als type 2 cytokines produceerden. In bloed van patiënten met baarmoederhalskanker werden T-helper cellen gericht tegen HPV16 maar in de helft van de gevallen aangetroffen, en deze T-cellen maakten veel minder type 1 en 2 cytokines. Deze resultaten suggereren sterk dat mensen met baarmoederhalskanker niet goed in staat zijn om T-helper cellen gericht tegen HPV (HPV-specifieke Th-cellen) te maken, en dat T-helper cellen gericht tegen de vroege eiwitten van HPV een belangrijke rol spelen in het onder controle houden van HPV infecties in gezonde mensen. In tegenstelling tot Th-cellen gericht tegen de vroege eiwitten van HPV werden in het bloed van mensen met CIN of baarmoederhalskanker even vaak Th-cellen gericht tegen het L1 eiwit van HPV gevonden als in het bloed van gezonde personen. Dit betekent dat Th-cel afweer tegen het L1 eiwit waarschijnlijk geen belangrijke rol speelt in de bescherming tegen het ontstaan van HPV-geassocieerde aandoeningen.

Vaccinatie is een manier om afweer op te bouwen tegen een bepaalde ziekteverwekker. Het rijksvaccinatieprogramma is gericht op de bescherming van kinderen tegen gevaarlijke infectieziektes. Ook tegen HPV zijn er verschillende soorten vaccins ontwikkeld. In tegenstelling tot de zogenaamde preventieve HPV vaccins, gericht op de produktie van antilichamen tegen het kapseleiwit L1 en het voorkomen een infectie met HPV, zijn therapeutische HPV vaccins gericht op het verkrijgen van HPV-specifieke T-cellen die van belang zijn voor de afweer tegen reeds geinfecteerde cellen en tumorcellen. Wij vonden dat een vaccin dat de E6 en E7 eiwitten van HPV bevatte zeer effectief was in de inductie van HPV-specifieke Th-cellen in een groep vrouwen met afwijkingen van de baarmoedermond, vagina en schaamlippen. Hoewel in sommige gevallen de afwijkingen na vaccinatie kleiner werden, was er geen duidelijk verband tussen het voorkomen van HPV-specifieke T-cellen in het bloed en uitkomst na behandeling in deze groep vrouwen. In een andere groep patiënten met voorstadia

van kanker van de schaamlippen vonden wij dat vrouwen met in het bloed aanwezige HPV-specifieke Th-cellen reageerden met het kleiner worden van de afwijkingen na behandeling met een ontstekingsbevorderende crème op de aangedane plekken. Uit deze twee studies kan worden geconcludeerd dat het voorkomen van HPV-specifieke Th-cellen in het bloed van belang lijkt voor het opruimen van aandoeningen die door HPV zijn veroorzaakt, maar dat het circuleren van zulke T-cellen alleen waarschijnlijk niet voldoende is. De afweerreacties ter plaatse van de afwijkingen lijken van groot belang voor de uiteindelijke uitkomst na behandeling. Het is mogelijk dat HPV-specifieke T-cellen hun werk niet kunnen doen ter plaatse van de afwijking in mensen met HPV geassocieerde aandoeningen. Mogelijke oorzaken hiervoor zijn het ontbreken van T-cellen tegen HPV of het onvermogen van deze T-cellen om vanuit het bloed de afwijking binnen te komen, bijvoorbeeld door het ontbreken van stoffen die T-cellen kunnen aantrekken, zoals ontstekingsbevorderende cytokines. Ook is het mogelijk dat regulatoire T-cellen de werking van de HPV-specifieke T-cellen teniet doen.

Om de plaatselijke afweerreacties te bestuderen hebben wij gekeken naar het voorkomen van de verschillende T-cel types in stukjes tumorweefsel van een groep vrouwen met baarmoederhalskanker. Er waren meer T-cellen aanwezig in tumorweefsel van patiënten dan in baarmoederhalsweefsel van een groep gezonde vrouwen. Verder vonden wij dat niet alleen het aantal T-cellen, maar juist de verhouding tussen de verschillende T-cel types in de tumor verband hield met een prognostisch betere variant van baarmoederhalskanker. Dit was al eerder aangetoond voor andere vormen van kanker in mensen, zoals darm- en eierstokkanker.

Samenvattend spelen HPV-specifieke T-helper cellen waarschijnlijk een belangrijke rol in de bescherming tegen ziekten van de vrouwelijke geslachtsorganen die door HPV worden veroorzaakt. Als mensen van nature niet in staat zijn om deze T-cellen te maken, is het mogelijk om ze door middel van vaccinatie te verkrijgen. Vaccinatie alleen is waarschijnlijk onvoldoende. Combinatietherapieën, gericht op het enerzijds induceren van HPV-specifieke T-cellen door vaccinatie, en anderzijds op het creëren van een gunstige plaatselijke situatie, bijvoorbeeld door toediening van ontstekingsbevorderende stoffen, zijn mogelijk zeer effectief in de behandeling van aandoeningen die door HPV worden veroorzaakt.

Abbreviations

AGIN	anogenital intraepithelial neoplasia
APC	antigen-presenting cell
CBA	cytometric bead array
CIN	cervical intraepithelial neoplasia
CTL	cytotoxic T lymphocyte
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked immunospot
FIGO	Fédération Internationale des Gynaecologistes et Obstetristes
HPV	human papillomavirus
IFNγ	interferon-gamma
IL-2	interleukin-2
MRM	memory response mix
PBMC	peripheral blood mononuclear cells
Th cell	T-helper cell
Treg	regulatory T-cell
VIN	vulvar intraepithelial neoplasia
VLP	virus-like particles

Authors and affiliations

From the Department of Gynecology, Leiden University Medical Center, Leiden, the Netherlands:
Mariëtte I.E. van Poelgeest, Muriel van den Hende, Gemma G. Kenter

From the Department of Clinical Oncology, Leiden University Medical Center, Leiden, the Netherlands:
Sytse J. Piersma, Kitty M.C. Kwappenberg, Jeanette M. van der Hulst, Sjoerd H. van der Burg

From the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands:
Jan Wouter Drijfhout, Rienk Offringa, Cornelis J.M. Melief

From the Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, the Netherlands:
Annemieke de Jong, Amon Wafelman

From the Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands:
Ekaterina S. Jordanova, Gert Jan Fleuren

From the Department of Gynecologic Oncology, Groningen University Medical Center, Groningen, the Netherlands:
Esther R. Nijhuis, Ineke E. Hamming, Hans W. Nijman, Ate G.J. van der Zee

From the Department of Gynecology, Erasmus University Medical Center, Rotterdam, the Netherlands:
Manon van Seters, Theo J.M. Helmerhorst

From the Department of Gynecology, Academic Medical Center, Amsterdam, the Netherlands:
Marc van Beurden

From the Department of Anesthesiology, Erasmus University Medical Center, Rotterdam, the Netherlands:
Claudia Heijmans-Antonissen

From the Paterson Institute for Cancer Research, Christie Hospital, Manchester, United Kingdom:
Lucy J.C. Smyth, Emma J. Davidson, Debbie Burt, Peter L. Stern

From the Deutsches Krebsforschungszentrum, Heidelberg, Germany:
Peter Sehr, Michael Pawlita

From the University of Wales College of Medicine, Cardiff, United Kingdom:
Stephen Man, Alison N. Fiander, Amanda Tristram

From Xenova Research Limited, Cambridge, United Kingdom:
Julian K. Hickling

From the Academic Unit of Obstetrics and Gynecology, St. Mary's Hospital, Manchester, United Kingdom:
Henry C. Kitchener