T cell immunity against early antigens of Human Papillomavirus type 16

Jong, Annemieke de

T CELL IMMUNITY AGAINST EARLY ANTIGENS OF HUMAN PAPILLOMAVIRUS TYPE 16

ANNEMIEKE DE JONG

T CELL IMMUNITY AGAINST EARLY ANTIGENS OF HUMAN PAPILLOMAVIRUS TYPE 16

ANNEMIEKE DE JONG

T CELL IMMUNITY AGAINST EARLY ANTIGENS OF HUMAN PAPILLOMAVIRUS TYPE 16

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. D.D. Breimer,
hoogleraar in de faculteit der Wiskunde en
Natuurwetenschappen en die der Geneeskunde,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 27 januari 2005
klokke 15.15 uur
door

Annemieke de Jong

geboren te Amsterdam
in 1971

PROMOTIECOMMISSIE

PROMOTORES	Prof. dr. C.J.M. Melief Prof. dr. P. Vermeij
CO-PROMOTOR	Dr. S.H. van der Burg
REFERENT	Prof. dr. M.A. Stanley, University of Cambridge, UK
OVERIGE LEDEN	Prof. dr. C.J.L.M. Meijer, VUMC, Amsterdam Prof. dr. J.H.F. Falkenburg Prof. dr. G.J. Fleuren Dr. R. Offringa Dr. G.G. Kenter

The studies described in this thesis were performed in the departments of Immunohematology and Blood Transfusion, Clinical Pharmacy and Toxicology, Gynecology, and Pathology of Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands.

ISBN 90-9018981-5

Foto's omslag	*Hinomaru*, Miyashita Ryosuke (宮下 良介), with kind permission; Cryoelectron micrograph of HPV particle, T. Baker, with kind permission.
Vormgeving	Sam Gobin, Leiden
Druk	Offsetdrukkerij Nautilus, Leiden

Financial support for the publication of this thesis was provided by BD Biosciences, Sanquin Reagents, J.E. Jurriaanse Stichting, Onderzoeks- en Ontwikkelingskrediet Apotheek.

The mind is like a parachute;
it only works when it's open
– *O. Welles*

aan mijn ouders

CONTENTS

Introduction

Human papillomaviruses (HPV) underlie the carcinogenesis of a substantial fraction of human cancers, including cancer of the uterine cervix. Cervical cancer is the second leading cause of cancer-related deaths among women worldwide, and it is the first malignancy acknowledged by the World Health Organisation to be virally induced in essentially all cases [1-3]. The close association between an infectious agent and cancer provides an important opportunity to target this disease by means of immunotherapeutic strategies. The fact that anogenital HPV infections are extremely common implies that HPV infection itself is not the decisive factor in cancer development. Rather, it is the failure to control HPV infection and the subsequent establishment of persistent infection that predisposes progression towards malignancy [4, 5]. This failure of immunosurveillance is observed only in a minority of infected subjects, whereas most infected individuals fortunately clear the infection without the development of HPV induced lesions. Clues as to how lesions expressing viral antigens can progress in the face of immune defence are likely to be found in the immune response against HPV antigens. Detailed knowledge of this HPV-specific immunity, both in individuals successfully controlling HPV infection and those who have evidently failed to do so, will assist the rational design of therapeutic interventions in the course of HPV induced disease.

Human Papillomaviruses

Human papillomaviruses are small double-stranded DNA viruses, and over a hundred types have been identified and fully or partially sequenced to date [6]. All identified types are strictly epitheliotropic, and depend on the differentiation process of the external epithelium for viral propagation. Papillomaviruses infect the epithelial cells either of the skin or of the anogenital and oropharyngeal mucosa. Based on their oncogenic potential, human papillomaviruses can be divided into low-risk types, causing benign epithelial proliferations, and high-risk types, which can incidentally result in malignant transformation of epithelial cells [7]. HPV type 16 (HPV16) is the most prevalent high-risk type in HPV-associated anogenital malignancies – HPV16 DNA is present in more than 50% of cervical tumors [8] – and forms the focus of the HPV-specific immunity outlined in this thesis.

HPV16 viral particles contain the double-stranded closed circular DNA genome, associated with histone-like proteins and encapsidated by 72 capsomers. The HPV16 genome consists of approximately 7900 base pairs harbouring 8 open reading frames (Figure 1). The genome can be divided into three regions: a non-coding long control region (LCR), an early (E) and a late (L) region. The late genes encode structural proteins, whereas the early genes mainly encode proteins with regulatory functions engaged in genome persistence and DNA replication. The LCR harbors specific enhancer elements, which are responsive to both viral and cellular regulatory factors [9]. The functions of the HPV16 encoded proteins are described in Table 1.

Viral life cycle

The papillomavirus life cycle differs from all other virus families by its tight restriction to the differentiating epithelium. Establishment of infection requires the availability of epidermal or mucosal epithelial cells that are capable of proliferation (basal cells). After infection of the basal stem cell, the virus utilises the host DNA replication machinery to multiply a few of its viral genomes [9]. In the basal cell, HPV genomes are established as episomes at low copy number, which replicate in synchrony with the cellular DNA replication. The viral gene expression is largely suppressed in the basal layer, although the limited expression of specific early viral proteins (e.g. E5,E6,E7) results in enhanced proliferation of the infected cells and their lateral expansion [6, 25]. The restricted expression of viral genes in the basal cells is suggested to be the result of tight control by cellular factors [26, 27].

The episomal HPV genomes persist in the nucleus of the infected stem cells and are distributed during mitosis on to the daughter cells. Therefore, the infected stem cell acts as a reservoir. In con-

Table 1. HPV16 protein function

Protein	Function
E1	DNA helicase. Essential for initiation of viral replication [10]
E2	Interacts withs E1, facilitating the binding of E1 to origin of replication [11] Viral transcription factor [12, 13]
E4	Associates with keratin cytoskeleton of epithelial cells resulting in collapse of cytokeratin network facilitating viral particle release [14]
E5	Interferes with endocytic trafficking, reducing IFNγ-induced HLA class II surface expression [15, 16] Stimulates cell growth by enhancing effect of growth factors [17] Prevents apoptosis following DNA damage [18]
E6	Prevents apoptosis by targeting the degradation of p53 and Bak [19, 20] Activation of telomerase [21]
E7	Prevents cell-growth arrest/differentiation by the release of E2F transcription factor from pRB-E2F complex (E7 binds to pRB and pRB family members), resulting in deregulation of G1/S cell cycle checkpoint [22] Blocks function of cyclin-dependent kinases p21 and p27 [23, 24]
L1	Major capsid protein
L2	Minor capsid protein

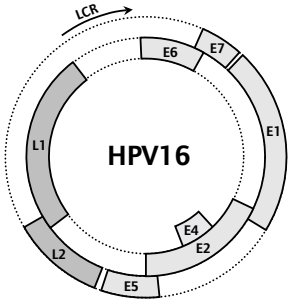

Figure 1. HPV16 genome. Schematic representation of the circular HPV genome. The open reading frames (ORFs) are depicted as shaded fragments and the three circles correspond to the three reading frames in which the sense strand can be translated. The region between L1 and E6 (long control region, LCR) is an important transcriptional regulatory region.

trast to uninfected keratinocytes, which exit the cell cycle as soon as they detach from the basement membrane, HPV infected cells enter into the S phase after reaching the suprabasal layer. This entry into the S phase results in amplification of the viral genomes to thousands of copies per cell [9]. Papillomaviruses herewith possess a mechanism to overcome the block in DNA synthesis that occurs along with the differentiation of the epithelial cells, although this does not result in full replication of the genome of the differentiated host cells [28]. 'Late' viral gene expression is initiated in the upper layers of the epithelium, where the viral particles are assembled and released (Figure 2). The virus actually delays nuclear condensation in differentiating keratinocytes by halting apoptosis until viral replication is completed [29].

The development of cervical cancer

Persistence of high-risk cervical HPV infection is characterized by the formation of dysplastic precursor lesions, which can be classified cytologically (Pap 1-5) or histologically (CIN I-III). The histological features of more advanced dysplastic lesions include a significant extension of actively replicating cells into the upper parts of the epithelium, loss of coordinated epithelial differentiation, and together with this loss of the complete viral life cycle. It has been proposed that CIN lesions constitute a progressive neoplastic disease continuum leading up to invasive cervical carcinoma. However, the majority of low-grade lesions (CIN I/II) will regress spontaneously indicating that the development of CIN and cervical carcinoma is not a one-way process [30, 31]. Furthermore, the disease process can show considerable variation; low-grade lesions can persist for years in some patients, whilst in others immediate progression to high-grade dysplasia (CIN III) is observed [32, 33]. So far, treatment of HPV-induced high-grade lesions and cancer is mostly limited to surgical removal, which has proven successful in those cases in which the lesions are locally confined. Because this treatment modality is symptomatic and lacks an anti-viral component, there is a risk of recurrent disease [34-36].

The role of HPV (onco)proteins in malignant transformation

Persistence of high-risk HPV infection, causing genomic instability is a prerequisite for disease progression and the development of precursor lesions. However, malignant transformation of the infected keratinocytes requires an additional step, involving the integration of HPV DNA into the host genome. During this process of viral integration, a substantial part of the genome is frequently deleted [37, 38]. In the resultant organization of HPV DNA, the viral transcripts spanning the E6 and E7 region are often linked to flanking cellular sequences, and subsequently the transcription

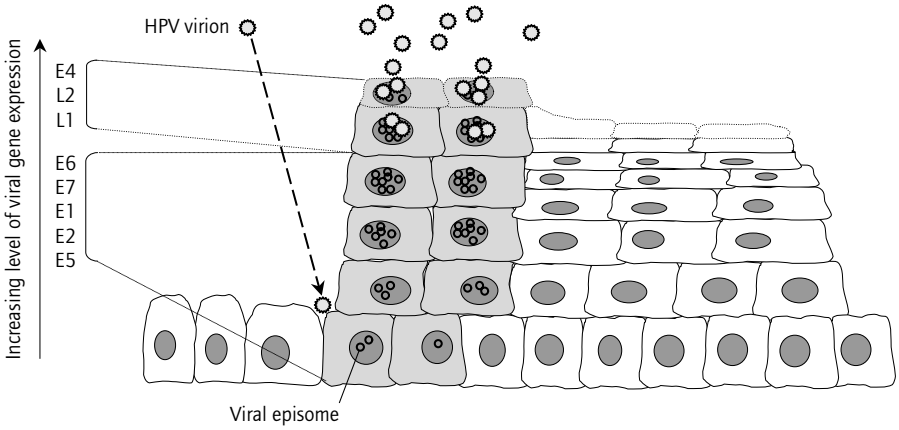

Figure 2. HPV infection and propagation. HPV infection of the cervix often occurs at the so-called squamocolumnar junction or transformation zone. This is where the columnar epithelium of the endocervix is connected to squamous epithelium of the exocervix, and the undifferentiated basal stem cell is readily accessible for HPV infection. After entry of the HPV virion into the basal epithelial (stem)cell, the viral genome is amplified to several copies. The infected cell divides along the basement membrane and then matures vertically. In the suprabasal layers of the epithelium HPV early proteins are abundantly expressed and the viral replication takes place. Several of these proteins (e.g. E6 and E7) interfere with epithelial differentiation. Only in the most superficial layers of the epithelium, E4 and the late viral genes L1 and L2 and the E4 re-expressed, and the HPV DNA is encapsidated and the virions are released at the epithelial surface.

of the oncogenes can be modulated (enhanced) by flanking host-cell promoters [39]. In addition, co-transcribed cellular sequences may result in increased stability of the mRNA encoding the viral oncoproteins [40]. Overall, the integration of high-risk HPV into the host genome results the constitutive expression of the viral oncogenes.

The E2 protein is described to be the major regulator of viral protein expression, and capable of suppressing E6 and E7 oncogene expression [41]. Therefore, the deletion of the E2 gene in the process of viral integration has been held responsible for the unleashed expression of both oncoproteins. This has, however, been contradicted by a recent study in which the suppressive effect of E2 on E6 and E7 expression was observed only for integrated and not for episomal viral DNA [42]. Therefore, the increased oncoprotein expression after viral integration may more likely be explained by factors other than the absence of the controlling function of E2, such as the aforementioned enhanced transcription of the oncogenes by flanking host-cell promoters, and the increased stability of chimeric transcripts. Although the E1 and/or E2 genes are often disrupted and part of the viral genome deleted in HPV16 positive cervical carcinoma, episomal copies of the viral DNA can often still be detected in the tumor cells, resulting in a variable expression of the HPV16 proteins. However, the lack of cellular differentiation at this stage of disease precludes completion of the viral life cycle.

The oncoproteins E6 and E7 proteins play a significant role in the process of malignant transformation. Independently they have the capacity to immortalize various human cell types in vitro; the efficiency of which is increased when the oncoproteins are expressed together [43]. A number of interactions have been reported between high-risk E6/E7 and host-cell proteins (Figure 3). For E6, the most prominent functions originate from its interaction with, followed by the degradation of, p53 and the pro-apoptotic protein Bak. These events result in resistance to apoptosis and an increase in chromosomal instability. In addition, E6 has been shown to associate with myc proteins in vivo, resulting in the cooperative activation of the telomerase reverse transcriptase promoter (references in Table 1).

E7 interacts with the RB protein, which releases the transcription factor E2F from RB inhibition.

The released E2F functions as a transcriptional activator, resulting in deregulated G1/S transition. Moreover, E7 is shown to block the function of the cyclin-dependent kinase inhibitors p21 and p27. High E2F activity might lead to apoptosis in E7-expressing cells, however, E6 in turn prevents this by guiding the degradation of apoptosis-inducing proteins p53 and Bak (references in Table 1). Taken together, the interference of E6 and E7 with cell cycle control, allows the accumulation of mutations in host cell DNA, thereby promoting malignant cell transformation.

Immune responses against HPV

Immune evasion and regulation in the HPV-infected epithelium

Anogenital HPV infections are extremely common, and the cumulative lifetime incidence is estimated to be as high as 80-85% [44]. The fact that most infections are cleared and that low-grade CIN lesions often regress spontaneously, indicates that in the majority of individuals the immune system succeeds in eliminating the virus before malignant disease can develop. Indirect evidence for the major role of the cellular immune response in this process is given by the fact that the prevalence of persistent HPV infections and HPV-positive lesions is greatly increased in immunosuppressed subjects, such as transplant recipients and HIV-positive patients [45-48].

The duration of a transient anogenital HPV infection ranges from 7–14 months [32, 49], indicating that the virus is capable of persisting in the host for some time before elimination. This lag time between HPV infection and clearance is at least partially linked to the evasion of and/or interference with innate immune defences [50]. Lack of innate immune triggering will in turn avoid activation of the adaptive immune system to eradicate virus-infected cells. Several characteristics of the viral infection contribute to the temporary circumvention of immune triggering, including the minimal disturbance of the epithelial architecture initially caused by the virus and the absence of significant keratinocyte lysis and inflammation. Furthermore, the differentiation dependent protein expression limits abundant production of viral proteins to the top epithelial layer, less accessible and visible to

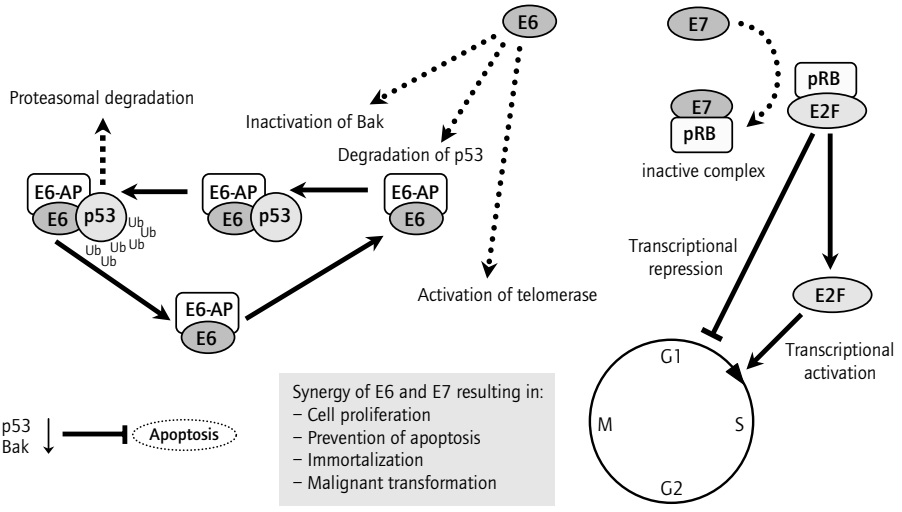

Figure 3. Mechanisms of E6 and E7 interference with cell cycle control. Together with E6-AP, the E6 protein facilitates the ubiquitination and proteasomal degradation of p53. The combination of Bak inactivation and reduced p53 activity results in loss of cell cycle control by the prevention of apoptosis. Both the activation of telomerase by E6 and the release of E2F transcription factor by the binding of E7 to pRB contribute to cell proliferation.

cells of the immune system. Moreover, the uptake of HPV16 virus-like particles (VLP) by Langerhans cells – the antigen presenting cells (APC) present in the epithelium – does not result in activation of these cells [51]. This in contrast to the uptake of VLP by monocyte-derived dendritic cells (DC), which does induce the upregulation of maturation markers on the DC and the secretion of IL-12. Taken together, the overall lack of inflammatory signals involved in papillomavirus infection allows a temporary evasion of immune surveillance.

Besides the avoidance of immune system activation, papillomaviruses also harbour mechanisms to actively interfere with innate immune triggering. The oncoproteins E6 and E7 have been shown to prevent the immunoregulatory effects of the type I interferon pathway by physically interfering with specific components of this pathway, e.g. inhibition of Interferon regulatory factors IRF1 and IRF3 (reviewed in [52]). Furthermore, the selective downregulation of MCP-1 expression by E6 and E7 in epithelial cells may also contribute to the program of HPV immune evasion, as this chemokine is particularly relevant in the setting of viral infection due to its ability to attract monocytes, memory T cells and NK cells in vivo [53]. Interference with the initiation of an adaptive immune response has been suggested by Matthews et al., who demonstrated E6-mediated downregulation of E-cadherin, resulting in Langerhans cell (LC) depletion of HPV16-infected epithelium [54]. This may limit the presentation of viral antigens by LC, thus preventing the initiation of a cell-mediated immune response and promoting survival of the virus.

Although keratinocytes have the capacity to secrete pro-inflammatory cytokines under certain conditions, they can also produce immunoregulatory factors such as TGFβ and IL-10. Both these cytokines play an essential role in the differentiation of Langerhans cells (LC) and their retention in the epidermis [55, 56]. Furthermore, these factors can inhibit the induction of LC maturation by pro-inflammatory factors. It is evident that LC-mediated activation of T cells in the regional lymph nodes requires the pro-inflammatory signals to dominate over the homeostasis of LC as controlled by TGFβ and IL-10. Lack of sufficient pro-inflammatory signals will result in immunological ignorance or tolerance [57]. Several reports describe increased levels of IL-10 in HPV-induced high-grade CIN lesions [58-60], and an increase in this immunoregulatory cytokine may have profound effects both on the resident LC in the skin, influencing their migration pattern and their antigen presenting capacity after migration to draining lymph nodes [61]. Furthermore, local IL-10 secretion in the HPV-induced lesion may negatively affect the functionality of many types of effector cells migrating to the lesions [62]. Giannini et al. suggested that immunosurveillance within the epithelium of the transformation zone – compared to the exocervix – is intrinsically perturbed by the altered expression of chemokines/cytokines (e.g. TNF-α, MIP3α) and by the concomitant diminished density of immature LC. Furthermore, the allo-stimulatory capacity of LC derived from the transformation zone was reduced compared to those from the exocervix, and the function of high-grade CIN-derived LC appeared even further incapacitated [63].

Notwithstanding these considerations, the situation during natural HPV infection appears to generally favor the induction of effective immunity rather than tolerance, as indicated by the fact that the vast majority of active HPV infections are eventually eliminated. However, in susceptible individuals, a period of decreased vigilance of the immune system can result in minor disturbance of the balance between pro- and anti-inflammatory signals and thereby allow the virus sufficient time to establish a status quo in which HPV infected and/or HPV-transformed cells are difficult to eliminate. The fact that – in contrast to low-grade CIN lesions – more advanced cervical lesions rarely show spontaneous regression, underlines the need for the immune system to act during the early pre-malignant phase in order to be effective against the virus.

HPV-positive keratinocytes that have undergone malignant transformation have a pronounced capacity to secrete immunomodulatory cytokines. Cervical carcinoma cells were found to secrete IL-10 [64], VEGF [65], PGE2 [66], TGFβ [67], and IL-6 [68]. In vitro experiments have shown that

activation of dendritic cells (DC) or LC in the presence of these cytokines was shown to result in APC with decreased costimulatory capacity, lacking IL-12 secretion, which are poor stimulators of Th1/CTL immunity and which can even induce T cell tolerance [69-73]. In several cases such APC were shown to rather induce Th2-type immunity [74, 75], which is suggested to be relatively ineffective against solid tumors and which seems to prevail in patients with progressed cancers [64, 76].

Viral latency

Many lines of evidence point towards the existence of a latent phase of papillomavirus infection, which can be defined as the presence of viral DNA in the absence of differentiation-dependent virion production [77]. Indications for viral latency are mainly derived from both the Canine oral papillomavirus (COPV) and Cottontail rabbit papillomavirus (CRPV) model by the detection of viral DNA in post-regression tissue [78] and the induction of viral protein expression in previously infected sites by UV irradiation [79]. In humans, HPV latency has been suggested in the larynx and trachea of patients with recurrent respiratory papillomatosis (RRP). HPV DNA could be detected in biopsies from clinically normal laryngeal and tracheal tissues derived from RRP patients [80]. Indirect evidence for a latent phase in HPV infection is given by the rapid appearance of multiple HPV lesions in immunocompromised patients [77]. Most studies do not allow distinction between true latency and subclinical infection. In the immunocompetent host, HPV infection may be held in a subclinical state by effective cellular immunity, which can then readily evolve under immunosuppressive conditions.

Mechanism of viral clearance

The term clearance is used to indicate the absence of active viral infection; regarding the presumed viral latency phase, this does not indicate a complete eradication of viral DNA. Little is known of the clearance process of HPV infections and HPV-induced lesions in the female genital tract. The regression of genital warts – associated with low-risk HPV infection of the external genital mucosa – is shown to be characterized by an active cell-mediated immune response [81]. Regressing warts contained significantly more T cells and macrophages than did non-regressing controls, and in this regression process, CD4+ T cells predominated in both the wart stroma and the surface epithelium. The great importance of CD4+ T cells in the control of HPV infections, is substantiated by reports showing that low CD4+ T cell count is strongly associated with multiple HPV infections, high viral load, and viral persistence in HIV-infected individuals and by the extensive HPV induced lesions observed in HIV negative patients with idiopathic CD4+ T-lymphocytopenia [47, 82-84].

Although the study of genital wart regression provides information on the cellular immune response, the unpredictable regression/clearance pattern of HPV infections makes it difficult to obtain a complete chronological picture of the regression process. In contrast, the COPV model does provide the opportunity to study the process of wart regression, because the experimentally induced mucosal papillomas undergo a rapid and predictable regression after maturity [85]. This revealed that regressing papillomas had a marked influx of αβ T cells, both CD4+ and CD8+. The peak of CD4+ influx preceded that of CD8+, and CD4+ T cells were clearly predominant in the regressing lesions [86].

Both animal and human studies point at a major role of the adaptive cellular immune response in the clearance of papillomavirus infections, and this suggests the formation of papillomavirus-specific immune memory. In the case of COPV, there is a clear immunity to rechallenge with the virus, after resolution of the primary infection [85]. Regarding human papillomaviruses, indirect evidence for the development of HPV-specific immune memory is given by the fact that the prevalence/incidence of anogenital infections decreases with increasing age in individuals with a similar exposure rate [87]. Furthermore, a second infection with the same HPV type has been shown to result in rapid viral clearance [88].

Cellular immunity against HPV proteins

The confinement of HPV infection to the epithelium renders the Langerhans cell (LC) the professional APC primarily responsible for the induction of T cell immunity against HPV antigens. Importantly, as the HPV infection cycle is specifically adapted to the keratinocyte differentiation program, it is unlikely that HPV infection of LC will result in the expression of the HPV antigens by the LC themselves. Therefore, the priming of T cell immunity against HPV will depend on the uptake and (cross)presentation of HPV antigens by the antigen presenting cells. The efficiency of this process will be affected by the levels to which the viral proteins accumulate in the infected keratinocytes that serve as antigen source and by the release of HPV proteins from apoptotic/(necrotic) keratinocytes.

Effective intervention of the cellular immune system in productive HPV infection is likely to benefit from T cell immunity against papillomavirus immediate early antigens, because these antigens are expressed throughout suprabasal layers of the infected epithelium [9]. Although T cells directed against 'late' HPV antigens can definitely play a role in the anti-HPV immune response, these antigens are only expressed when the viral infection cycle is almost complete and the differentiating keratinocyte has moved up in the epithelium [89]. Therefore, cellular immune responses against these antigens will most likely not result in effective viral clearance. Studies addressing T cell immunity against HPV have focused primarily on type 16 (HPV16) because of the high prevalence of this oncogenic type high-grade CIN lesions and cervical carcinoma. More specifically, responses against the E6 and E7 oncoproteins of HPV16 have been extensively studied, due to their constitutive expression in HPV16-positive (pre)malignant lesions. However, during productive viral infection and in low-grade CIN, other early proteins (E1, E2 and E5) are also widely expressed in the (supra)basal epithelium and may also provide targets for the cellular immune system [90, 91].

So far, no clear-cut association has been established between HPV-specific T cell responses and protection against progression of HPV-induced disease. In a cross-sectional analysis by de Gruijl et al. it was shown that strong Th responses against HPV16E7, as determined by cytokine-induced proliferation of a reporter cell line, were associated with persistence and progression of HPV16-positive lesions. However, a longitudinal analysis by the same group showed that HPV16E7-specific Th responses were associated with both clearance and persistence, in which it was suggested that these responses developed as a consequence of increased antigen availability resulting from either clearance of progression of cervical lesions [92, 93]. Our previous cross-sectional analysis of HPV16E7 responses by IFNγ ELISPOT in healthy individuals and patients with HPV16-positive cervical cancer or high-grade CIN showed frequent E7-specific responses in HPV16+ patients, whereas these were rarely detected in healthy controls [94]. In a longitudinal study performed by Kadish et al. proliferative responses against HPV16E7 and/or E6 in CIN patients were found to be associated with clearance of HPV infection and regression of CIN [95]. It must be noted, however, that only a fraction of the CIN patients participating in this study was HPV16 positive, and only the C-terminal part of E7 was used as a readout. Several other studies report highly variable percentages of HPV16E7- and/or E6-specific Th-responses in patients [96, 97].

Like in the case of Th responses, contradictory results have been obtained regarding CTL responses against the HPV16-derived oncoproteins. Lack of E6-specific CTL was found to be an important factor in HPV16 persistence [98], whereas a study by Bontkes et al suggested that CTL against E6 and E7 are generally only found in persistent CIN [99]. In concordance with the latter study, Ressing et al. detected CTL responses against the – previously determined – HLA-A*0201 restricted epitope $HPV16E7_{11\text{-}20}$ in cervical carcinoma and CIN patients (HPV16- and HLA-A*0201-positive) and not in healthy individuals [100]. Youde et al. showed by HLA-A*0201 tetramer staining that – after a short in vitro stimulation – the frequency of CD8+ T cells specific for $E7_{11\text{-}20}$ was significantly higher in HPV16+ CIN III patients than in healthy controls [101].

Many of the inconsistencies observed between the different studies of cellular immunity against the HPV16 oncoproteins may be explained by the patient selection (whether or not included patients

are HPV16 positive) and, more importantly, by the differences in technical approach. Several older studies involve multiple in vitro stimulations, and do therefore not truly reflect the in vivo situation. Furthermore, the choice of antigen – recombinant protein or synthetic peptides spanning only a limited region of the total protein – has most likely influenced the outcome of the analyses.

A limited number of studies has addressed the cellular immunity against other HPV antigens. Bontkes et al. studied the HPV16E2-specific Th-response in CIN patients and observed that Th cell responses against the C-terminal domain of E2 frequently occurred at the time of HPV clearance, although no significant association between E2-specific Th responses and disease outcome was observed [102]. Proliferative responses against the E5 protein of HPV16 were significantly reduced in the patients with high-grade CIN and cervical carcinoma, as compared to patients with low-grade lesions [103]. The responses were exclusive to those with HPV16-positive lesions, but the difference in response frequency lost statistical significance when only the HPV16-positive patients were included in the analysis. Therefore, the observed results require confirmation in a larger HPV16-positive population. T cell responses against the E4 antigen of HPV16 have shown no correlation with infection and/or disease [104]. As for the late antigens L1 and L2, expression of E4 is restricted to the upper differentiated layers of the epithelium [89], and as such T cell responses against this antigen are not likely to contribute significantly to viral clearance. The late antigen HPV16L1 is a major target of the cellular immune system, as L1-specific responses were abundantly detected in the peripheral blood of HPV16+ CIN patients [105, 106]. The analyses were not performed with the entire L1 antigen, but only with a predetermined immunogenic region. Again, these T cell responses were not associated with disease severity, as they were observed both in those with virus clearance and in those with persistence, irrespective of CIN grade. The high degree of L1 conservation between HPV types, brings up the question whether L1 cross-reactive T cells – recognizing the HPV16L1 peptides in vitro – have contributed to the high percentage of responders. Overall, these studies that addressed HPV16-specific cellular immunity indicate that responses against the viral proteins are occasionally induced at one point during HPV16-induced disease, but their role in protection against disease remains unclear.

Humoral immune responses against HPV

Serum IgG antibodies against HPV16 capsid are generally related to persistent HPV16 infection, although a large fraction of patients with HPV16-positive CIN fails to mount a systemic antibody response against the virus [107]. In transient HPV16 infections, type-specific serum IgG antibodies are induced in only a minority of individuals and antibody levels wane over time [108]. Similarly, mucosal IgG is barely induced in transient infections. Mucosal IgA responses on the other hand, were detected more frequently and appeared to reflect recent or ongoing HPV infection [109]. In established HPV infection type-specific antibodies may have a role in limiting the spread of active HPV infection by preventing viral reinoculation after virion release. Their role as a diagnostic marker in the process of HPV-induced disease is limited due to the lack of detectable antibody levels in a large fraction of persistently infected individuals.

HPV vaccination

Prophylactic vaccines

The identification of high-risk papillomaviruses as a necessary cause of the development of malignancy implies that prevention of HPV infection should prevent the development of cancer. A successful example of such prophylaxis is given by Hepatitis B virus (HBV) vaccination, which has dramatically decreased the incidence of HBV infection and thus the subsequent risk of developing liver cancer [110, 111]. Prophylactic HPV vaccines have focused on the induction of type-specific neutralizing antibodies against the viral capsid (L1 and/or L2) capable of blocking the interaction of the virions with

the keratinocytes and thereby preventing subsequent viral entry steps. Several animal models of papillomavirus infection have provided convincing evidence that neutralising antibodies can prevent new infection [112, 113]. In humans, encouraging results have been obtained with an HPV16 virus-like particle (VLP) vaccine. Virus-like particles consist of L1 major capsid protein, which mimics the natural virus by self-assembly into virion conformation. In a double-blind, randomized, placebo-controlled vaccination trial an HPV16 VLP vaccine or placebo was administered to 1533 HPV16-negative women aged 16-23 [114]. Over the follow up period, persistent HPV16 infections – defined as detection of HPV16 DNA on two consecutive 6 monthly visits – and CIN were detected in the placebo group only. A small number of vaccine recipients was HPV16 DNA positive at a single visit, indicating that the vaccine does not establish sterilizing immunity in all cases. However, the fact that persistent infection does not ensue, suggests that the vaccination may reduce the viral load and limit rounds of reinoculation.

The results from the initial VLP-based prophylactic vaccination trial are very promising. However, several important issues remain to be addressed, such as duration of protection – which is required to be several decades – and efficacy in immunocompromised individuals; this in view of the high incidence of HIV in the target population in the developing world. The fact that VLPs offer genotype-specific protection implies that a multivalent vaccine is required, targeting the most common high-risk HPV types in order to achieve maximal protection against HPV-induced malignancy. Follow-up studies on immunized individuals may also shed light on the significance of oncogenic HPV in the development of other cancers (e.g. oral and oesophageal cancer).

Therapeutic vaccines

Once HPV infection has been established it is unlikely that antibodies will contribute substantially to eradication of infected cells. The clearance of HPV-infection or established HPV-induced disease likely requires T cells specific for the viral antigens. The E6 and E7 oncoproteins are the only viral antigens that are constitutively expressed from infection up to malignancy; therefore, all therapeutic vaccines designed to date target these oncoproteins. Low-grade lesions harbour more potential targets, as the complete viral cycle occurs and these lesions are genetically stable. However, the additional contribution of targeting other viral proteins in the treatment of low-grade lesions is largely unexplored. Results from animal studies suggest that targeting of early antigen E1 and E2 can contribute to therapeutic vaccine efficacy. In the COPV model, which may serve as a model for low-grade, low-risk lesions, DNA vaccination with codon-optimized E1 and E2 showed both prophylactic and therapeutic efficacy [115]. The E2 antigen was also shown to be a preferable target in the CRPV model [116]. In addition, targeting of oncoproteins E6 and E7 was effective in this model [117]. However, results obtained in papillomavirus animal models demand caution when extrapolating these to the human situation [89]. Differences in tissue tropism, transmission route and persistence have shown to result in distinct gene expression patterns (also between human HPV types), which in turn affect the accessibility of viral antigens to the immune system. The value of natural host – papillomavirus combinations, however, exceeds that of mouse models for the prediction of therapeutic vaccine efficacy. These models make use of HPV-positive transplantable tumors and ignore the fact that, in humans, tumor development is preceded by a period of persistent HPV infection, in which tolerance to the viral antigens can be induced as a result of continuous exposure of the immune system to these antigens in a non-inflammatory environment. Furthermore, transplantable tumors grow outside the epithelial context in which these tumors normally arise. The importance of the latter is demonstrated in transgenic mouse models expressing HPV16 E7 under control of a keratinocyte-specific promoter. E7-transgenic skin grafted onto non-transgenic littermates was not rejected, even if the mice received an E7-specific immunization that resulted in effective E7-specific immunity capable of rejecting E7-expressing transplantable tumors [118]. This demonstrates the major influence of the epithelial site of antigen expression and of the nature of the keratinocyte and its environment.

At present, several vaccines have been tested in phase I/II clinical trials. They include peptide-based vaccines, fusion proteins, antigen-pulsed DC or recombinant vaccinia viruses (reviewed in [119, 120]). Clinical responses observed in these studies were limited to those patients with premalignant lesions. Modest clinical responses have been observed in patients with high-grade vulvar intraepithelial neoplasia (VIN) upon vaccination with recombinant vaccinia virus encoding the modified oncogenes of HPV16 and 18 or a heterologous prime-boost regimen consisting of three recombinant HPV16L2E6E7 protein vaccinations followed by the recombinant vacciniavirus vaccine [121-123]. It must be noted, however, that these trials were not placebo controlled and therefore the naturally occurring fluctuation of lesion size was not accounted for. Nevertheless, the initial results are encouraging, and VIN is a candidate condition for therapeutic vaccination in view of the multifocality of the disease and limited effective treatment options. In a fraction of patients, systemic T cell responses against E6 and/or E7 associated with IFNγ were detected. However, the results of the immunological analyses lacked a clear-cut correlation with clinical outcome. In a double-blind, randomised, placebo-controlled clinical trial, a plasmid-DNA based vaccine containing defined HPV16/18 E6 and E7 encoding sequences (ZYC101a) showed no significant effect on the resolution (defined as normal or CIN I) in a population of CIN 2/3 patients. Again, no correlation was observed between HPV-specific immune response and clinical response [124].

Initial trials involving synthetic peptide-based vaccines, focused on HLA*A-0201 restricted CTL epitopes of HPV16E7 [125, 126]. In a phase I trial of 15 HPV16-positive cervical carcinoma patients with residual or recurrent disease, vaccination with two HPV16E7 CTL epitopes and a non-related universal Th epitope (PADRE) resulted in the induction of PADRE-specific Th responses but not HPV16E7-specific CTL [127]. In contrast, E7-specific responses were observed in a fraction of high-grade CIN and VIN patients upon vaccination with a similar E7 vaccine. Again, the immunological data did not clearly correspond to the clinical observations [128]. Pre-clinical studies suggest that longer peptides containing both Th- and CTL epitopes are more efficient at eliciting strong CTL responses than minimal epitopes, and therapeutic efficacy with 30 amino acid long peptides has been demonstrated in both murine and CRPV model [117, 129]. The epitope linkage increases the chance of simultaneous presentation of both the MHC class I- and class II-restricted epitopes on the surface of a single APC, thereby facilitating the delivery of cognate T cell help to CTL priming [130]. Furthermore, in contrast to minimal CTL epitopes, the longer peptides require uptake and processing and therefore exclude unfavorable exogenous binding to non-professional APC that lack the proper costimulatory context for CTL induction (M. Bijker, personal communication). Currently we are conducting a phase I/II trial involving vaccination with 32-35 aminoacid long overlapping synthetic peptides spanning HPV16 E6 and E7. The preliminary immunological data reveal the induction of strong IFNγ-associated T cell responses against the viral oncoproteins, predominantly E6, upon vaccination (unpublished data).

Vaccination trials involving cervical cancer patients have so far not demonstrated clinical efficacy. Generally, these trials have been carried out in patients with late-stage disease, who are often immunocompromised due to radio- and/or chemotherapy and ongoing disease. In addition, mutations and deletions in antigen processing and antigen presentation are commonly observed in advanced cervical malignancies [131, 132]. Vaccination of patients with early cervical cancer and those with premalignant lesions will have to prove the true therapeutic value of vaccines. In high-grade lesions and cancer, they are not likely to succeed as a stand-alone therapy, but may function as adjuvant therapy combined with other treatment modalities. The great challenge in therapeutic vaccination strategies will be to overrule tolerance or redirect pre-existing non-protective T cell responses to HPV antigens, which are likely to be encountered in these patients. The generally unfavorable cytokine milieu in the lesions and the genetic instability of high-grade lesions and cancer pose additional hurdles for effective HPV-specific immunotherapy.

Scope of this thesis

Previous studies of HPV-specific immunity have focused primarily on patients with HPV-positive lesions, who have evidently failed to control HPV infection. In this thesis, the emphasis has been shifted towards successful HPV-specific immunity, which is expected to be found in healthy individuals. Issues that have been addressed include the analysis of T cell memory, the associated cytokines and the viral antigens targeted by these T cells. Besides charting successful HPV-specific immunity, we pinpointed several characteristics of HPV-specific immune failure in cervical carcinoma patients. The importance of the latter lies in the fact that the efficacy of immunotherapeutic intervention in this patient group will strongly depend on the nature of the pre-existing HPV-specific immune response.

As a first step, we investigated HPV16-specific immunity in the healthy population, which – given the common nature of HPV16 infections – harbors a large fraction of individuals who have previously experienced HPV16 infection. The cellular immunity against the HPV16 early proteins E2 and E6 frequently observed in healthy subjects (*Chapters 2 and 3*) most likely represents effective HPV16-specific immunity induced by prior HPV16 infection, and we compared this with the immune responses in peripheral blood of patients with HPV16-positive (pre)malignant lesions (*Chapter 4*). Together with the detailed analysis of virus-specific CD4+ T cells derived from patients' tumors and tumor-draining lymph nodes, this provided evidence for the existence of an anti-inflammatory cytokine polarization of the HPV16-specific T cell response in cervical carcinoma patients (*Chapter 5*).

The last two chapters both describe potential future treatment modalities. One involves TA-CIN, a fusion protein vaccine comprising of HPV16 L2 E6 and E7, which was tested in a dose-escalating study in healthy volunteers (*Chapter 6*). The other therapeutic approach is based on the transfer of HPV16-specific T cells in advanced stage cervical cancer. *Chapter 7* describes the first steps in the development of such a therapy, by the enrichment of Th1 type HPV16E6-specific memory T cells from the peripheral blood of healthy blood donors, which could serve as T cell therapy after allogeneic stem cell transplantation. The efficacy of the latter treatment option is – in contrast to active therapeutic vaccination – independent of the presence of unfavorably polarized T cells in cervical cancer patients, because these are deleted in the pre-treatment for transplantation. Finally, the general discussion of this thesis outlines a hypothesis of successful and failing HPV-specific immunity, and provides directions for future immunotherapeutic approaches of HPV-induced disease.

References

1. zur Hausen H. Papillomavirus infections-a major cause of human cancers. *Biochim Biophys Acta,* 1288: F55-78, 1996.
2. Bosch FX, Lorincz A, Munoz N, Meijer CJ, and Shah KV. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol,* 55: 244-265, 2002.
3. Parkin DM. Global cancer statistics in the year 2000. *Lancet Oncol,* 2: 533-543, 2001.
4. Ho GY, Burk RD, Klein S, Kadish AS, Chang CJ, Palan P, Basu J, Tachezy R, Lewis R, and Romney S. Persistent genital human papillomavirus infection as a risk factor for persistent cervical dysplasia. *J Natl Cancer Inst,* 87: 1365-1371, 1995.
5. 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,* 12: 235-236, 2002.
6. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer,* 2: 342-350, 2002.
7. Lowy DR, and Howley PM. Papillomaviruses. *Fields Virology,* 4th Edition: Chapter 66, 2001.
8. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, and Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst,* 87: 796-802, 1995.
9. Howley PM, and Lowy DR. Papillomaviruses and their replication. *Fields Virology,* 4th Edition: Chapter 65, 2001.
10. Seo YS, Muller F, Lusky M, and Hurwitz J. Bovine papilloma virus (BPV)-encoded E1 protein contains multiple activities required for BPV DNA replication. *Proc Natl Acad Sci U S A,* 90: 702-706, 1993.
11. Sakai H, Yasugi T, Benson JD, Dowhanick JJ, and Howley PM. Targeted mutagenesis of the human papillomavirus type 16 E2 transactivation domain reveals separable transcriptional activation and DNA replication functions. *J Virol,* 70: 1602-1611, 1996.
12. Bouvard V, Storey A, Pim D, and Banks L. Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes. *Embo J,* 13: 5451-5459, 1994.
13. Phelps WC, and Howley PM. Transcriptional trans-activation by the human papillomavirus type 16 E2 gene product. *J Virol,* 61: 1630-1638, 1987.
14. Roberts S, Ashmole I, Johnson GD, Kreider JW, and Gallimore PH. Cutaneous and mucosal human papillomavirus E4 proteins form intermediate filament-like structures in epithelial cells. *Virology,* 197: 176-187, 1993.
15. Thomsen P, van Deurs B, Norrild B, and Kayser L. The HPV16 E5 oncogene inhibits endocytic trafficking. *Oncogene,* 19: 6023-6032, 2000.
16. Zhang B, Li P, Wang E, Brahmi Z, Dunn KW, Blum JS, and Roman A. The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-γ. *Virology,* 310: 100-108, 2003.
17. Venuti A, Salani D, Poggiali F, Manni V, and Bagnato A. The E5 oncoprotein of human papillomavirus type 16 enhances endothelin-1-induced keratinocyte growth. *Virology,* 248: 1-5, 1998.
18. Zhang B, Spandau DF, and Roman A. E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. *J Virol,* 76: 220-231, 2002.
19. Werness BA, Levine AJ, and Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science,* 248: 76-79, 1990.
20. Jackson S, Harwood C, Thomas M, Banks L, and Storey A. Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. *Genes Dev,* 14: 3065-3073, 2000.
21. Veldman T, Horikawa I, Barrett JC, and Schlegel R. Transcriptional activation of the telomerase hTERT gene by human papillomavirus type 16 E6 oncoprotein. *J Virol,* 75: 4467-4472, 2001.
22. Dyson N, Howley PM, Munger K, and Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science,* 243: 934-937, 1989.
23. Funk JO, Waga S, Harry JB, Espling E, Stillman B, and Galloway DA. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes Dev,* 11: 2090-2100, 1997.
24. Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW, and Jansen-Durr P. Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene,* 13: 2323-2330, 1996.
25. Crum CP. Contemporary theories of cervical carcinogenesis: the virus, the host, and the stem cell. *Mod Pathol,* 13: 243-251, 2000.
26. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst,* 92: 690-698, 2000.
27. von Knebel Doeberitz M. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *Eur J Cancer,* 38: 2229-2242, 2002.
28. Cheng S, Schmidt-Grimminger DC, Murant T, Broker TR, and Chow LT. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. *Genes Dev,* 9: 2335-2349, 1995.
29. Longworth MS, and Laimins LA. Pathogenesis of Human Papillomaviruses in Differentiating Epithelia. *Microbiol Mol Biol Rev,* 68: 362-372, 2004.
30. Duggan MA, McGregor SE, Stuart GC, Morris S, Chang-Poon V, Schepansky A, and Honore L. The natural history of CIN I lesions. *Eur J Gynaecol Oncol,* 19: 338-344, 1998.
31. Katase K, Teshima H, Hirai Y, and Hasumi K. Natural history of cervical human papillomavirus lesions. *Intervirology,* 38: 192-194, 1995.
32. Ho GY, Bierman R, Beardsley L, Chang CJ, and Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med,* 338: 423-428, 1998.
33. Schiffman M, and Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. *J Natl Cancer Inst* Monogr 14-19, 2003.
34. Chao A, Lin CT, Hsueh S, Chou HH, Chang TC, Chen MY, and Lai CH. Usefulness of human papillomavirus testing in the follow-up of patients with high-grade cervical intraepithelial neoplasia after conization. *Am J Obstet Gynecol,* 190: 1046-1051, 2004.
35. Nagai N, Mukai K, Oshita T, Shiroyama Y, and Ohama K. Human papillomavirus DNA status after loop excision for cervical intraepithelial neoplasia grade III – A prospective study. *Int J Mol Med,* 13: 589-593, 2004.
36. Bodner K, Bodner-Adler B, Wierrani F, Kimberger O, Denk C, and Grunberger W. Is therapeutic conization sufficient to eliminate a high-risk HPV infection of the uterine cervix? A clinicopathological analysis. *Anticancer Res,* 22: 3733-3736, 2002.
37. Chen CM, Shyu MP, Au LC, Chu HW, Cheng WT, and Choo KB. Analysis of deletion of the integrated human papillomavirus 16 sequence in cervical cancer: a rapid multiplex polymerase chain reaction approach. *J Med Virol,* 44: 206-211, 1994.
38. Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, and Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virol,* 61: 962-971, 1987.
39. Wagatsuma M, Hashimoto K, and Matsukura T. Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: amplification of viral sequences together with cellular flanking sequences. *J Virol,* 64: 813-821, 1990.
40. Jeon S, and Lambert PF. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proc Natl Acad Sci U S A,* 92: 1654-1658, 1995.
41. McBride AA, Romanczuk H, and Howley PM. The papillomavirus E2 regulatory proteins. *J Biol Chem,* 266: 18411-18414, 1991.
42. Bechtold V, Beard P, and Raj K. Human papillomavirus type 16 E2 protein has no effect on transcription from episomal viral DNA. *J Virol,* 77: 2021-2028, 2003.
43. Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, and Schiller JT. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *Embo J,* 8: 3905-3910, 1989.
44. Jenkins D, Sherlaw-Johnson C, and Gallivan S. Can papilloma virus testing be used to improve cervical cancer screening? *Int J Cancer,* 65: 768-773, 1996.
45. Penn I. Cancers of the anogenital region in renal transplant recipients. Analysis of 65 cases. *Cancer,* 58: 611-616, 1986.
46. Ho GY, Burk RD, Fleming I, and Klein RS. Risk of genital human papillomavirus infection in women with human immunodeficiency virus-induced immunosuppression. *Int J Cancer,* 56: 788-792., 1994.
47. Palefsky JM and Holly EA. Chapter 6: Immunosuppression and co-infection with HIV. *J Natl Cancer Inst* Monogr 41-46, 2003.
48. Bouwes Bavinck JN, and Berkhout RJ. HPV infections and immunosuppression. *Clin Dermatol,* 15: 427-437, 1997.
49. Woodman CB, Collins S, Winter H, Bailey A, Ellis J, Prior P, Yates M, Rollason TP, and Young LS. Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. *Lancet,* 357: 1831-1836, 2001.
50. Tindle RW. Immune evasion in human papillomavirus-associated cervical cancer. *Nat Rev Cancer,* 2: 59-65, 2002.
51. Fausch SC, Da Silva DM, Rudolf MP, and Kast WM. Human papillomavirus virus-like particles do not activate Langerhans cells: a possible immune escape mechanism used by human papillomaviruses. *J Immunol,* 169: 3242-3249, 2002.
52. Woodworth CD. HPV innate immunity. *Front Biosci,* 7: d2058-2071, 2002.

53. Kleine-Lowinski K, Rheinwald JG, Fichorova RN, Anderson DJ, Basile J, Munger K, Daly CM, Rosl F, and Rollins BJ. Selective suppression of monocyte chemoattractant protein-1 expression by human papillomavirus E6 and E7 oncoproteins in human cervical epithelial and epidermal cells. *Int J Cancer,* 107: 407-415, 2003.
54. Matthews K, Leong CM, Baxter L, Inglis E, Yun K, Backstrom BT, Doorbar J, and Hibma M. Depletion of Langerhans cells in human papillomavirus type 16-infected skin is associated with E6-mediated down regulation of E-cadherin. *J Virol,* 77: 8378-8385, 2003.
55. Wang B, Amerio P, and Sauder DN. Role of cytokines in epidermal Langerhans cell migration. *J Leukoc Biol,* 66: 33-39, 1999.
56. Jakob T, Ring J, and Udey MC. Multistep navigation of Langerhans/dendritic cells in and out of the skin. *J Allergy Clin Immunol,* 108: 688-696, 2001.
57. Offringa R, de Jong A, Toes RE, van der Burg SH, and Melief CJ. Interplay between human papillomaviruses and dendritic cells. *Curr Top Microbiol Immunol,* 276: 215-240, 2003.
58. El-Sherif AM, Seth R, Tighe PJ, and Jenkins D. Quantitative analysis of IL-10 and IFN-γ mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. *J Pathol,* 195: 179-185, 2001.
59. Giannini SL, Al-Saleh W, Piron H, Jacobs N, Doyen J, Boniver J, and Delvenne P. Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. *Clin Exp Immunol,* 113: 183-189, 1998.
60. Mota F, Rayment N, Chong S, Singer A, and Chain B. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. *Clin Exp Immunol,* 116: 33-40, 1999.
61. Muller G, Muller A, Tuting T, Steinbrink K, Saloga J, Szalma C, Knop J, and Enk AH. Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells in vivo. *J Invest Dermatol,* 119: 836-841, 2002.
62. Akdis CA, and Blaser K. Mechanisms of interleukin-10-mediated immune suppression. *Immunology,* 103: 131-136, 2001.
63. Giannini SL, Hubert P, Doyen J, Boniver J, and Delvenne P. Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development of squamous intraepithelial lesions of the cervix. *Int J Cancer,* 97: 654-659, 2002.
64. Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, and Huang SC. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol,* 167: 2972-2978, 2001.
65. Fujimoto J, Sakaguchi H, Hirose R, Ichigo S, and Tamaya T. Expression of vascular endothelial growth factor (VEGF) and its mRNA in uterine cervical cancers. *Br J Cancer,* 80: 827-833, 1999.
66. Sales KJ, Katz AA, Davis M, Hinz S, Soeters RP, Hofmeyr MD, Millar RP, and Jabbour H. N. Cyclooxygenase-2 expression and prostaglandin E(2) synthesis are up-regulated in carcinomas of the cervix: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *J Clin Endocrinol Metab,* 86: 2243-2249, 2001.
67. Comerci JT Jr, Runowicz CD, Flanders KC, De Victoria C, Fields AL, Kadish AS, and Goldberg GL. Altered expression of transforming growth factor-β 1 in cervical neoplasia as an early biomarker in carcinogenesis of the uterine cervix. *Cancer,* 77: 1107-1114, 1996.
68. Takano H, Harigaya K, Ishii G, Sugaya Y, Soeta S, Nunoyama T, Shirasawa H, Shimizu K, Tokita H, Simizu B, Mikata A, and Sekiya S. Interleukin-6 (IL-6) production in carcinoma of the cervix. *Arch Gynecol Obstet,* 258: 25-33, 1996.
69. De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, and Moser M. Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol,* 27: 1229-1235, 1997.
70. Enk AH, Jonuleit H, Saloga J, and Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *Int J Cancer,* 73: 309-316, 1997.
71. Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, Kavanaugh D, and Carbone DP. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nat Med,* 2: 1096-1103, 1996.
72. Kalinski P, Schuitemaker JH, Hilkens CM, and Kapsenberg ML. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J Immunol,* 161: 2804-2809, 1998.
73. Steinbrink K, Wolfl M, Jonuleit H, Knop J, and Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol,* 159: 4772-4780, 1997.
74. King C, Davies J, Mueller R, Lee MS, Krahl T, Yeung B, O'Connor E, and Sarvetnick N. TGF-β1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. *Immunity,* 8: 601-613, 1998.
75. Liu L, Rich BE, Inobe J, Chen W, and Weiner HL. Induction of Th2 cell differentiation in the primary immune response: dendritic cells isolated from adherent cell culture treated with IL-10 prime naive CD4+ T cells to secrete IL-4. *Int Immunol,* 10: 1017-1026, 1998.
76. Tatsumi T, Kierstead LS, Ranieri E, Gesualdo L, Schena FP, Finke JH, Bukowski RM, Mueller-Berghaus J, Kirkwood JM, Kwok WW, and Storkus WJ. Disease-associated bias in T helper type 1 (Th1)/Th2 CD4(+) T cell responses against MAGE-6 in HLA-DRB10401(+) patients with renal cell carcinoma or melanoma. *J Exp Med,* 196: 619-628, 2002.
77. Stubenrauch F, and Laimins LA. Human papillomavirus life cycle: active and latent phases. *Semin Cancer Biol,* 9: 379-386, 1999.
78. Moore RA, Nicholls PK, Santos EB, Gough GW, and Stanley MA. Absence of canine oral papillomavirus DNA following prophylactic L1 particle-mediated immunotherapeutic delivery vaccination. *J Gen Virol,* 83: 2299-2301, 2002.
79. Zhang P, Nouri M, Brandsma JL, Iftner T, and Steinberg BM. Induction of E6/E7 expression in cottontail rabbit papillomavirus latency following UV activation. *Virology,* 263: 388-394, 1999.
80. Abramson AL, Nouri M, Mullooly V, Fisch G, and Steinberg BM. Latent Human Papillomavirus infection is comparable in the larynx and trachea. *J Med Virol,* 72: 473-477, 2004.
81. Coleman N, Birley HD, Renton AM, Hanna NF, Ryait BK, Byrne M, Taylor-Robinson D, and Stanley MA. Immunological events in regressing genital warts. *Am J Clin Pathol,* 102: 768-774, 1994.
82. Tobin E, Rohwedder A, Holland SM, Philips B, and Carlson JA. Recurrent 'sterile' verrucous cyst abscesses and epidermodysplasia verruciformis-like eruption associated with idiopathic CD4 lymphopenia. *Br J Dermatol,* 149: 627-633, 2003.
83. Manchado Lopez P, Ruiz de Morales JM, Ruiz Gonzalez I, and Rodriguez Prieto MA. Cutaneous infections by papillomavirus, herpes zoster and Candida albicans as the only manifestation of idiopathic CD4+ T lymphocytopenia. *Int J Dermatol,* 38: 119-121, 1999.
84. Stetson CL, Rapini RP, Tyring SK, and Kimbrough RC. CD4+ T lymphocytopenia with disseminated HPV. *J Cutan Pathol,* 29: 502-505, 2002.
85. Nicholls PK, and Stanley MA. Canine papillomavirus-A centenary review. *J Comp Pathol,* 120: 219-233, 1999.
86. Nicholls PK, Moore PF, Anderson DM, Moore RA, Parry NR, Gough GW, and Stanley MA. Regression of canine oral papillomas is associated with infiltration of CD4+ and CD8+ lymphocytes. *Virology,* 283: 31-39, 2001.
87. Canadas MP, Bosch FX, Junquera ML, Ejarque M, Font R, Ordonez E, and de Sanjose S. Concordance of prevalence of human papillomavirus DNA in anogenital and oral infections in a high-risk population. *J Clin Microbiol,* 42: 1330-1332, 2004.
88. Nakagawa M, Viscidi R, Deshmukh I, Costa MD, Palefsky JM, Farhat S, and Moscicki A. B. Time course of humoral and cell-mediated immune responses to human papillomavirus type 16 in infected women. *Clin Diagn Lab Immunol,* 9: 877-882., 2002.
89. Peh WL, Middleton K, Christensen N, Nicholls P, Egawa K, Sotlar K, Brandsma J, Percival A, Lewis J, Liu WJ, and Doorbar J. Life cycle heterogeneity in animal models of human papillomavirus-associated disease. *J Virol,* 76: 10401-10416, 2002.
90. Mayer TJ, and Meyers C. Temporal and spatial expression of the E5a protein during the differentiation-dependent life cycle of human papillomavirus type 31b. *Virology,* 248: 208-217, 1998.
91. Ozbun MA, and Meyers C. Human papillomavirus type 31b E1 and E2 transcript expression correlates with vegetative viral genome amplification. *Virology,* 248: 218-230, 1998.
92. de Gruijl TD, Bontkes HJ, Stukart MJ, Walboomers JMM, Remmink AJ, Verheijen RHM, Helmerhorst TJM, Meijer CJLM, and Scheper RJT. 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,* 77: 2183-2191, 1996.
93. de Gruijl TD, Bontkes HJ, Walboomers JM, Stukart MJ, Doekhie FS, Remmink AJ, Helmerhorst TJ, Verheijen RH, Duggan-Keen MF, Stern PL, Meijer CJ, and Scheper RJ. 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,* 58: 1700-1706, 1998.
94. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief C J, and Offringa R. 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,* 91: 612-618, 2001.
95. Kadish AS, Ho GY, Burk RD, Wang Y, Romney SL, Ledwidge R, and Angeletti RH. Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia. *J Natl Cancer Inst,* 89: 1285-1293, 1997.
96. Tsukui T, Hildesheim A, Schiffman MH, Lucci J 3rd, Contois D, Lawler P, Rush BB, Lorincz AT, Corrigan A, Burk RD, Qu W, Marshall MA, Mann D, Carrington M, Clerici M, Shearer GM, Carbone DP, Scott DR, Houghten RA, and Berzofsky JA. Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. *Cancer Res,* 56: 3967-3974, 1996.

97. Luxton JC, Rowe AJ, Cridland JC, Coletart T, Wilson P, and Shepherd PS. 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,* 77: 1585-1593, 1996.
98. Nakagawa M, Stites DP, Patel S, Farhat S, Scott M, Hills NK, Palefsky JM, and Moscicki AB. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. *J Infect Dis,* 182: 595-598, 2000.
99. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, Verheijen RH, Stukart MJ, Meijer CJ, Scheper RJ, Stacey SN, Duggan-Keen MF, Stern PL, Man S, Borysiewicz LK, and Walboomers JM. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. *Int J Cancer,* 88: 92-98, 2000.
100. Ressing ME, van Driel WJ, Celis E, Sette A, Brandt MP, Hartman M, Anholts JD, Schreuder GM, ter Harmsel WB, Fleuren GJ, Trimbos BJ, Kast WM, and Melief CJ. 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,* 56: 582-588, 1996.
101. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, and Man S. 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,* 60: 365-371, 2000.
102. Bontkes HJ, de Gruijl TD, Bijl A, Verheijen RH, Meijer CJ, Scheper RJ, Stern PL, Burns JE, Maitland NJ, and Walboomers JM Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia. *J Gen Virol,* 80: 2453-2459, 1999.
103. Gill DK, Bible JM, Biswas C, Kell B, Best JM, Punchard NA, and Cason J. Proliferative T cell responses to human papillomavirus type 16 E5 are decreased amongst women with high-grade neoplasia. *J Gen Virol,* 79 (Pt 8): 1971-1976, 1998.
104. Nakagawa M, Stites DP, Farhat S, Judd A, Moscicki AB, Canchola AJ, Hilton JF, and Palefsky JM. T cell proliferative response to human papillomavirus type 16 peptides: relationship to cervical intraepithelial neoplasia. *Clin Diagn Lab Immunol,* 3: 205-210, 1996.
105. de Gruijl TD, Bontkes HJ, Walboomers JM, Coursaget P, Stukart MJ, Dupuy C, Kueter E, Verheijen RH, Helmerhorst TJ, Duggan-Keen MF, Stern PL, Meijer CJ, and Scheper RJ. 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,* 80 (Pt 2): 399-408, 1999.
106. Luxton JC, Rose RC, Coletart T, Wilson P, and Shepherd PS. Serological and T-helper cell responses to human papillomavirus type 16 L1 in women with cervical dysplasia or cervical carcinoma and in healthy controls. *J Gen Virol,* 78: 917-923, 1997.
107. de Gruijl TD, Bontkes HJ, Walboomers JM, Schiller JT, Stukart MJ, Groot BS, Chabaud MM, Remmink AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ, and Scheper RJ. Immunoglobulin G responses against human papillomavirus type 16 virus-like particles in a prospective nonintervention cohort study of women with cervical intraepithelial neoplasia. *J Natl Cancer Inst,* 89: 630-638, 1997.
108. Sasagawa T, Yamazaki H, Dong YZ, Satake S, Tateno M, and Inoue M. 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,* 75: 529-535, 1998.
109. Sasagawa T, Rose RC, Azar KK, Sakai A, and Inoue M. Mucosal immunoglobulin-A and -G responses to oncogenic human papilloma virus capsids. *Int J Cancer,* 104: 328-335, 2003.
110. Kao JH, and Chen DS. Global control of hepatitis B virus infection. *Lancet Infect Dis,* 2: 395-403, 2002.
111. Stuver SO. Towards global control of liver cancer? *Semin Cancer Biol,* 8: 299-306, 1998.
112. Breitburd F, Kirnbauer R, Hubbert NL, Nonnenmacher B, Trin-Dinh-Desmarquet C, Orth G, Schiller JT, and Lowy DR. Immunization with virus-like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol,* 69: 3959-3963, 1995.
113. Ghim S, Newsome J, Bell J, Sundberg JP, Schlegel R, and Jenson AB. Spontaneously regressing oral papillomas induce systemic antibodies that neutralize canine oral papillomavirus. *Exp Mol Pathol,* 68: 147-151, 2000.
114. Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM, and Jansen KUA. Controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med,* 347: 1645-1651, 2002.
115. Moore RA, Walcott S, White KL, Anderson DM, Jain S, Lloyd A, Topley P, Thomsen L, Gough GW, and Stanley MA. Therapeutic immunisation with COPV early genes by epithelial DNA delivery. *Virology,* 314: 630-635, 2003.
116. Brandsma JL, Shlyankevich M, Zhang L, Slade MD, Goodwin EC, Peh W, and Deisseroth AB. Vaccination of rabbits with an adenovirus vector expressing the papillomavirus E2 protein leads to clearance of papillomas and infection. *J Virol,* 78: 116-123, 2004.
117. Vambutas A, De Voti J, Nouri M, Drijfhout JW, Lipford GB, Bonagura VR, van der Burg SH, and Melief CJM. 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 preclinical Cottontail Rabbit Papillomavirus model. *submitted,* 2004.
118. Frazer IH, De Kluyver R, Leggatt GR, Guo HY, Dunn L, White O, Harris C, Liem A, and Lambert P. 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,* 167: 6180-6187, 2001.
119. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. *Nat Rev Immunol,* 4: 46-54, 2004.
120. Galloway DA. Papillomavirus vaccines in clinical trials. *Lancet Infect Dis,* 3: 469-475, 2003.
121. Baldwin PJ, van der Burg SH, Boswell CM, Offringa R, Hickling JK, Dobson J, Roberts J S, Latimer JA, Moseley RP, Coleman N, Stanley MA, and Sterling JC. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. *Clin Cancer Res,* 9: 5205-5213, 2003.
122. Davidson EJ, Boswell CM, Sehr P, Pawlita M, Tomlinson AE, McVey RJ, Dobson J, Roberts JS, Hickling J, Kitchener HC, and Stern PL. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. *Cancer Res,* 63: 6032-6041, 2003.
123. Smyth LJ, Van Poelgeest MI, Davidson EJ, Kwappenberg KM, Burt D, Sehr P, Pawlita M, Man S, Hickling JK, Fiander AN, Tristram A, Kitchener HC, Offringa R, Stern PL, and Van Der Burg SH. 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,* 10: 2954-2961, 2004.
124. Garcia F, Petry KU, Muderspach L, Gold MA, Braly P, Crum CP, Magill M, Silverman M, Urban RG, Hedley ML, and Beach KJ. ZYC101a for treatment of high-grade cervical intraepithelial neoplasia: a randomized controlled trial. *Obstet Gynecol,* 103: 317-326, 2004.
125. Kast WM, Brandt RM, Drijfhout JW, and Melief CJ. Human leukocyte antigen-A2.1 restricted candidate cytotoxic T lymphocyte epitopes of human papillomavirus type 16 E6 and E7 proteins identified by using the processing-defective human cell line T2. *J Immunother,* 14: 115-120, 1993.
126. Ressing ME, Sette A, Brandt RM, Ruppert J, Wentworth PA, Hartman M, Oseroff C, Grey HM, Melief CJ, and Kast WM. Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. *J Immunol,* 154: 5934-5943, 1995.
127. Ressing ME, van Driel WJ, Brandt RM, Kenter GG, de Jong JH, Bauknecht T, Fleuren GJ, Hoogerhout P, Offringa R, Sette A, Celis E, Grey H, Trimbos BJ, Kast WM, and Melief CJ. 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,* 23: 255-266, 2000.
128. Muderspach L, Wilczynski S, Roman L, Bade L, Felix J, Small LA, Kast WM, Fascio G, Marty V, and Weber J. A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. *Clin Cancer Res,* 6: 3406-3416, 2000.
129. Zwaveling S, Ferreira Mota SC, Nouta J, Johnson M, Lipford GB, Offringa R, van der Burg SH, and Melief CJ. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol,* 169: 350-358, 2002.
130. Melief CJ, Van Der Burg SH, Toes RE, Ossendorp F, and Offringa R. Effective therapeutic anticancer vaccines based on precision guiding of cytolytic T lymphocytes. *Immunol Rev,* 188: 177-182, 2002.
131. Fowler NL, and Frazer IH. Mutations in TAP genes are common in cervical carcinomas. *Gynecol Oncol,* 92: 914-921, 2004.
132. Ritz U, Momburg F, Pilch H, Huber C, Maeurer MJ, and Seliger B. Deficient expression of components of the MHC class I antigen processing machinery in human cervical carcinoma. *Int J Oncol,* 19: 1211-1220, 2001.

Annemieke de Jong, Sjoerd van der Burg, Kitty Kwappenberg, Jeanette van der Hulst, Kees Franken, Annemieke Geluk, Krista van Meijgaarden, Jan Wouter Drijfhout, Gemma Kenter, Pieter Vermeij, Cornelis Melief, Rienk Offringa

Cancer Res 62: 472-479, 2002

2

Frequent detection of HPV16 E2-specific T-helper immunity in healthy subjects

Abstract · The incidence of genital human papillomavirus (HPV) infections is high in young sexually active individuals. Most infections are cleared within 1 year after infection. The targets for the cellular immune response in this process of viral clearance remain to be identified, but the expression pattern of the E2 protein in early infection and low-grade CIN renders this early protein a candidate antigen. We therefore studied the HPV16 E2-specific T cell responses in more detail. Very strong proliferative responses against one or more peptide-epitopes derived from this antigen can be found in PBMC cultures of approximately half of the healthy donors. Further analysis revealed that at least a majority of these responses represent reactivity by memory CD4+ Th1-type cells capable of secreting IFNγ upon antigenic stimulation. Interestingly, all E2-peptides against which strong responses were detected are clustered in the key functional domains of the E2-protein, which are conserved to considerable extent between HPV types. This suggests that HPV16 E2-specific Th memory may be installed through encounter with HPV types other than HPV16. Indeed, one HPV16 E2-specific Th-clone was found to cross-react against homologuous peptides from other HPV types, but three other Th-clones failed to show similar cross-reactivity. Part of the HPV16 E2-specific Th memory may therefore relate to previous encounter of other HPV types, whereas the majority of the immune repertoire concerned is most likely established through infection with HPV16 itself. Our data are the first to reveal that the T cell repertoire of healthy donors can contain particularly high frequencies of E2-specific memory Th-cells, and suggest that boosting of this immunity can be employed for preventive and therapeutic vaccination against HPV-induced lesions.

Introduction

Genital infection with Human Papillomavirus (HPV) is one of the most common sexually transmitted diseases [1-3]. So-called high-risk HPV types (e.g. type 16) are causally related to the development of cervical intraepithelial neoplasia (CIN) and cervical carcinoma [4]. Fortunately, in a majority of immunocompetent individuals the HPV infection is asymptomatic, while most premalignant cervical lesions, in analogy to warts, are found to regress spontaneously [5-7]. This regression is likely to be mediated by cellular immune responses, since regressing genital warts are predominantly infiltrated with CD4+ T cells and macrophages, consistent with a delayed type hypersensitivity reaction [8, 9]. The role of immune surveillance in controlling infection by various HPV types is further indicated by the increased incidence of HPV infections, HPV-associated warts, CIN lesions and cervical carcinoma in immunocompromised subjects [10, 11]. Moreover, cervical neoplastic lesions frequently display deficiencies in the expression of HLA class I [12-14].

The investigation of anti-HPV immunity in humans has so far primarily focused on responses against the E6 and E7 oncoproteins of HPV16/18, as expression of these proteins is tightly associated with HPV-induced dysplasia. Notably, these responses do not necessarily correlate with spontaneous regression [15-19]. This suggests that the observed responses are in many cases not capable of eliminating the HPV-infected cells. In fact, although clearance of HPV infections as well as spontaneous regression of especially low-grade CIN lesions is frequently observed, more progressed lesions are rarely cleared. Importantly, the E6 and E7 oncoproteins are primarily expressed at high levels in more advanced lesions. During most stages of the productive infection cycle of HPV as well as in low-grade lesions the expression levels of these proteins are kept in check by the E2 protein and, as a consequence, are modest. The E2 protein is the major regulator of viral DNA replication and gene expression [20] and this antigen is uniformly and abundantly expressed in cells containing episomal HPV genomes. The highest E2 expression is found in koilocytes in low-grade cervical intra-epithelial neoplasia (CIN), whereas with progression of the lesions to invasive carcinoma, E2 expression is lost [21, 22]. This corresponds with the observation that in high-grade CIN and cervical carcinoma the E2 gene is frequently disrupted due to linearization of the viral genome, which is required for integration of viral DNA into the cellular genome.

In terms of immune intervention, the E6 and E7 oncoproteins are the major targets in high-grade CIN and cervical carcinoma, as these proteins are constitutively expressed and are required for maintenance of the transformed state [20]. However, in the context of productive infection and low-grade CIN the E2 protein constitutes an attractive target for the immune system, as it is highly expressed in these settings, thereby exhibiting an expression pattern complementary to that of E6 and E7. In the cottontail rabbit papillomavirus (CRPV) model, which is the major animal model for cancers associated with papillomavirus infection, proliferative responses against the E2 protein were associated with spontaneously regressing papillomas [23]. Moreover, E2-specific immunization was shown to enhance regression of CRPV-induced papillomas [24]. Finally, a follow-up study in HPV16-positive women diagnosed with CIN revealed that T-helper (Th-) responses against the C-terminal domain of the E2 protein were frequently observed at the time of viral clearance, suggesting that these responses may contribute to viral clearance [25].

The proposed role of the E2-specific T cell response in control of HPV infection and in regression of HPV-induced lesions, as well as the high incidence of genital (sub-clinical) HPV infection [3, 7], suggests that a pool of E2-specific memory T cells may be present in a sizeable fraction of the healthy population. We performed a detailed analysis of the E2-specific Th-response in healthy individuals. Our data reveal that the E2 protein exhibits several highly immunogenic regions that contain naturally processed Th-epitopes. Moreover, the analysis of the CD45RO+ memory fraction of PBMC revealed the presence of E2-specific memory Th-responses in a major fraction of healthy individuals.

Materials and Methods

Lymphocytes · Peripheral blood mononuclear cells (PBMC) and serum for proliferation assays were obtained from HLA-typed anonymous healthy blood donors after informed consent. Furthermore, for ELISPOT assays, PBMC were isolated from buffycoats of anonymous healthy blood donors after informed consent. Because these donors are anonymous, no data on medical history are available. Importantly, donors with a known recent history of infection, including abnormal pap-smear were, as part of normal regulations, discouraged to donate blood.

Antigens · A set of peptides spanning the whole HPV16 E2 protein consisting of 23 overlapping peptides, 22 of which have a length of 30 amino acids and one of which ($E2_{331-365}$) has a length of 35 amino acids, was used. These peptides share an overlap of 15 amino acids. For epitope fine-mapping of HPV16 E2 specific Th-clones, peptides with a length of 15 and 20 amino acids were used. For determining the cross-reactivity of HPV16 E2-specific Th-clones the following peptides derived from different HPV types were used, with a length varying between 25-30 amino acids. Amino acid mismatches are underlined; amino acids with similar physico-chemical properties, but not identical are indicated in bold: K**P**Y**K**ELY**SSM**SSTWHWTSDNKNSK**N** (HPV33), FKKHKGLYCNVSST-WHWTSN**D**TNQ**Q** (HPV26), K**Y**KQLYEQVSSTWHWTCT**D**GKHKN (HPV31), **R**K**Y**ADH**YSEI**SSTWHWT-GCN (HPV45), QRD**K**FL**T**TVKIPNT**V**TVSKG**Y**MSI (HPV35), QRDDFLNTVKIPNT**VS**VSTG**Y**M**T**I (HPV31), QRQQFLDVVKIPPTI**S**HKLGFMS**L** (HPV6B), QRSQFL**A**LVKIPKTIKHSLGML**T**I (HPV7), QRNNFL**T**TVKIP-**QS**ITSTLGIMS**L** (HPV26). The peptides spanning the Influenza Matrix protein of A/PR/8/34, which were used as control peptides in ELISPOT assays, consisted of 16 30-mer peptides overlapping by 15 amino acids. Peptides were synthesized by solid phase technique on an automated multiple peptide synthesizer (Abimed AMS 422, Langenfeld, Germany), and analyzed by reverse phase HPLC. The lyophilized peptides were dissolved in 50 µl of DMSO, diluted in PBS to a final concentration of 2.5 mg/ml. The HPV16 E2 C-terminal ($E2_{280-365}$) protein and HPV16 E7 protein were produced according to previously described procedures [26]. Memory Response Mix (MRM), consisting of a mixture of tetanus toxoid (0.75 LF/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 to confirm the capacity of PBMC to proliferate and produce cytokine in response to common recall antigens.

HLA-DR peptide binding assay · Binding of peptides to HLA-DR was measured as reported previously [27]. Briefly, as a source of DR molecules B-LCL homozygous for DR were used: LG2.1 (DRB*0101, DR1), IWB (DRB1*0201, DR2), HAR (DRB*0301, DR3), and BSM (DRB*0401, DR4). DR molecules were purified by affinity chromatography and the purity confirmed by SDS-PAGE. The analysis of peptide binding to purified DR molecules was performed using N-terminally fluorescence-labeled standard peptides. As standard peptide in the binding assays $HA_{308-319}$ (PKYVKQNTLKLAT, DR1 and DR2), hsp65 3-13 (KTIAYDEEARR, DR3) or $HA_{308-319}$ Y→F (PKFVKQNTLKLAT) was used.

Short-term T cell proliferation assay · Immunogenicity of individual HPV16 E2 peptides was determined by short term proliferation assays of healthy donor PBMC with HPV16 E2 peptides, according to previously described procedures [27]. Briefly, freshly isolated PBMC were seeded at a density of 1.5×10^5 cells/well in a 96-well U-bottom plate (Costar, Cambridge, MA) in 200 µl of IMDM (Iscove's Modified Dulbecco's Medium, Bio Whittaker, Verviers, Belgium) supplemented with 10% autologous serum. HPV16 E2 peptides were added at a concentration of 10 µg/ml. Medium alone was taken along as negative control; phytohemagglutinine (PHA, 0.5 µg/ml) served as a positive control. For each peptide 8 parallel micro-cultures were initiated; each donor was tested twice. Peptide-specific proliferation was measured at day 6 by [^{3}H]-thymidine incorporation. Peptides were scored positive, when in both assays, the proliferation of >50% of the test wells exceeded the mean prolif-

eration + 3 × SD of the control wells, and the stimulation index (SI) of the positive test wells over medium control wells was higher than 3.

Generation and analysis of long-term HPV16 E2 specific Th-cultures · Long-term HPV16 E2-specific T cell cultures and Th-clones were established according to previously described procedures [27]. Briefly, PBMC from healthy HLA-typed donors were stimulated in vitro with the following HPV16 E2 peptides ($E2_{271-300}$ + $E2_{286-315}$; $E2_{301-330}$ + $E2_{316-345}$; $E2_{331-365}$). 15×10^6 PBMC were seeded in 25 cm^2 culture flasks (Nalge Nunc, USA) in 6 ml IMDM supplemented with 10% autologous serum. Peptides were added at a concentration of 5 µg/ml. At day 7, 15×10^6 PBMC were added, together with fresh medium and peptides. At day 14 and 21 viable T cells were harvested from the cultures, counted and restimulated with an equal amount of autologous irradiated PBMC and peptide (5 µg/ml). T cell growth factor (Biotest, Dreieich, Germany) was added 2 days after restimulation at a final concentration of 10%. The T cell cultures were tested for peptide recognition by proliferation assay at day 28. Peptide-specific T cell cultures were cloned by limiting dilution and T cell clones were subsequently tested for the recognition of E2-peptide and -protein-pulsed APC.

Specificity of the Th-clones was analyzed as described previously [27]. Notably, in proliferation assays in which Th-clones were tested for protein recognition, autologous monocytes were used as APC. For measurement of proliferation, cultures were pulsed with 0.5 µCi [^{3}H]-thymidine (5 µCi/mM, Amersham, UK) per well for 18 hours. Plates were harvested with a Micro cell Harvester (Skatron, Norway). Filters were packed in plastic covers containing 10 ml of scintillation fluid and subsequently counted on a 1205 Betaplate counter (Wallac, Turku, Finland). HLA class II blocking experiments were performed using murine monoclonal antibodies: anti-DQ SPV. L3, anti-DR B8.11.2, and anti-DP B7/21. Supernatants of the proliferation assays were harvested 24 hrs after incubation and analyzed for the presence of IFNγ by ELISA [27].

Detection of memory Th-cells by ELISPOT · Memory cells (CD45RO+) were isolated freshly from buffycoats by MACS after incubation with CD45RO microbeads (cat. no. 460-01, Miltenyi Biotec, Germany). The purity of the obtained CD45RO+ fraction was > 95% as determined by flowcytometry after surface staining for CD45RO and CD45RA (CD45RA-FITC, cat. no. 347723, CD45RO-PE, cat. no. 347967, Becton Dickinson Biosciences, USA). CD45RO+ cells were seeded at a density of 10^6 cells/well in a 24-wells plate (Costar) in 1 ml of IMDM supplemented with 10% FCS. 10^6 irradiated, autologous cells were added to each well as APC. The responder cells were incubated with either medium alone, pools of HPV16 E2 peptides at 5 µg/ml/peptide, MRM 1:50 dilution or pools of Influenza Matrix peptides (positive controls) and cultured for 11 days in order to improve the detection of antigen-specific cells [28]. The cells were then harvested, washed and seeded in 4 replicate wells at a density of 5×10^4 cells/well of a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with a IFNγ catching antibody. Per well, 10^5 irradiated, autologous PBMC were added as APC together with 5 µg/ml peptide. ELISPOT analysis was further performed according to the instructions of the manufacturer (Mabtech AB, Natcha, Sweden). Analysis of the number of spots was done with a fully automated computer-assisted-video-imaging analysis system (Carl Zeiss Vision).

Intracellular cytokine staining (ICS) of memory T cells · Autologous monocytes were isolated from PBMC by adherence to a flat-bottom 48-wells plate during 2 hours in X-vivo15 medium (Bio Whittaker, Verviers, Belgium) at 37°C, and then used as APC. CD45RO+ cells were stimulated for 11 days with peptide, then harvested, washed and suspended in IMDM + 0.1% BSA at a concentration of 1.5×10^6 cells/ml. 200 µl of cell suspension was added to the monocytes + 200 µl of 10 µg/ml HPV16 E2 peptide (stimulated) or 200 µl of medium (non-stimulated control). After 1 hour of incubation at 37°C, 800 µl of IMDM + 10% FCS + 12.5 µg/ml Brefeldin A (Sigma) was added and cells were incubated for another 5 hours. The cells were then harvested, transferred into a V-bottom 96-wells plate, washed twice with ice-cold PBS and fixed with 50 µl paraformaldehyde 4% for 4 minutes on ice. Following fixation, the cells were washed once with cold PBS and once with PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1%.

This was followed by an incubation in 50 µl PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1%/FCS 10% for 10 minutes on ice. Cells were washed twice with PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1% and supernatant was removed before 25 µl of PBS/NaAz 0.2%/BSA 0.5%/Saponin 0.1% containing 1 µl FITC-labelled mouse-anti-human IFNγ (0.5 mg/ml, BD Pharmingen, cat.no. 554551) 2 µl PE-labelled anti-CD4 (BD Bioscience, cat.no. 345769), and 2 µl PerCP-labelled anti-CD8 (BD Bioscience, cat.no. 347314) was added. Following 30 minutes of incubation at 4°C, the cells were washed, suspended in 100 µl 1% paraformaldehyde and analyzed by flowcytometry.

Homology search in protein database · The search for sequence homology of the overlapping HPV16 E2 peptides in a protein database (SwissProt) was performed using standard Basic Local Alignment Tool (BLAST: www.ncbi.nlm.gov/blast/blastcgi). Statistical significance threshold (EXPECT) was 10 [29]. Reported matches revealing >70% aminoacid homology with HPV16 E2 peptides were included.

Statistical analysis · Statistical analysis of the E2-specific responses as measured by ELISPOT were tested by the unpaired (two-tailed) T-test with Welch correction using GraphPad InStat (GraphPad Software Inc.).

RESULTS

High reactivity of healthy donor PBMC against HPV16 E2-derived peptides

We examined the proliferative responses of healthy donor PBMC against HPV16 E2 protein by using an array of overlapping 30-mer peptides covering the entire E2 sequence. Incubation of freshly isolated PBMC of 8 HLA-typed donors with each of the 23 E2-derived 30-mer peptides showed that 4 out of 8 donors reacted to 2 or more of the peptides. The observed E2 peptide-specific proliferative responses were remarkably strong (Table 1). In all cases more than 75% of the parallel microcultures reacted against the stimulating peptide. For instance, in two independent experiments we found the peptide-specific proliferation of donor # 8 against peptides $E2_{31\text{-}60}$, $E2_{46\text{-}75}$, $E2_{91\text{-}120}$, $E2_{151\text{-}180}$, $E2_{271\text{-}300}$ and $E2_{286\text{-}315}$ to exceeded background proliferation in 75-94% of all eight parallel microcultures tested (Figure 1). This points at the presence of a very high frequency of E2-specific T cells in the PBMC isolates. In particular, PBMC of donors # 3, 5 and 8 displayed strong responses with a broad specificity. Please note that the 30-mer peptides have a 15 amino acid overlap with their neighboring peptides. Consequently, responses against adjacent peptides (e.g. donor # 3, $E2_{31\text{-}60}$ and $E2_{46\text{-}75}$) most likely involve the same epitope, whereas responses against non-adjacent peptides are directed against distinct epitopes. Not only the frequency of responding cultures but also the magnitude of the proliferative responses were remarkably high. The peptide-specific proliferation of several cultures from donors #3, 5 and 8 exceeded background with mean stimulation indices ranging from 9.2–16.5 and, as such, are comparable to responses found against the tetanus toxoid antigen in several of the donors (#3, 4, 5 and 7; SI ranging from 13.3–25; see Table 1). These stimulation indices clearly exceed the threshold ($SI \geq 3$) that is commonly used for the detection of memory T cell responses [30, 31]. Note that responses against tetanus toxoid are considerably higher in some of the other donors ($SI \geq 50$ in donors # 2, 6 and 8), but that these strong values most likely represent very broad responses against multiple epitopes comprised by an entire antigen, rather than against a single 30-mer E2-peptide. Taken together, our data indicate that the T cell repertoire of healthy donors can contain particularly high frequencies of T cells specific for the HPV16 E2 antigen resulting in vigorous proliferative responses, and suggest that these responses may reflect T cell memory.

HPV16 E2-specific Th cultures recognize naturally processed epitopes.

The proliferation data pointed at the existence of multiple immunogenic Th-epitopes within HPV16 E2. We performed a more detailed analysis of the nature and specificity of such responses, thereby

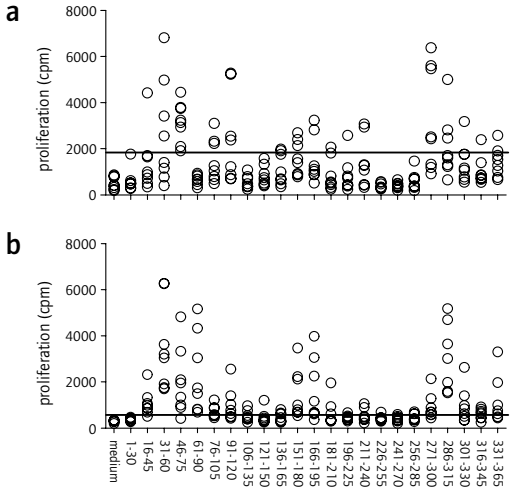

Figure 1. The E2 protein contains highly immunogenic sequences. Freshly isolated PBMC derived from healthy blood donors were stimulated with the indicated HPV16 E2 peptides (10 μg/ml) for 6 days after which proliferation was measured by [^{3}H]-thymidine incorporation. **a** + **b** represent two independent experiments in which donor 8-derived PBMC showed strikingly strong proliferative responses against multiple E2 peptides. Circles represent proliferation of the individual microcultures. The line indicates the cut-off value of mean + 3 × SD of medium control.

focusing on the C-terminal region, which our data (Table 1) revealed to contain several highly immunogenic peptides. The overlapping peptides comprised in this C-terminal region ($E2_{271\text{-}300}$; $E2_{286\text{-}315}$; $E2_{301\text{-}330}$; $E2_{316\text{-}345}$; $E2_{331\text{-}365}$) were tested for their capacity to bind to HLA-DR molecules. Each of the five peptides showed intermediate to strong binding to two or more of the common HLA-DR molecules tested (Table 2), which supports the notion that these peptides can indeed represent class II MHC-restricted Th-epitopes. Subsequently, long-term E2-specific Th cultures were generated through stimulation of PBMC from HLA-typed healthy blood donors at weekly intervals with either peptides $E2_{271\text{-}300}$ and $E2_{286\text{-}315}$, peptides $E2_{301\text{-}330}$ and $E2_{316\text{-}345}$, or with peptide $E2_{331\text{-}365}$. PBMC from 2 donors showed strong peptide-specific proliferative responses against one or more of the stimulating peptides (data not shown). Through cloning via limiting dilution of these cultures, we succeeded in the isolation of stable T cell clones, uniformly displaying a CD4+CD8− phenotype, against four distinct peptide-epitopes. Two of these Th-clones were established from HLA-DR15(2)-, -DQ6(1)-PBMC stimulated with $E2_{301\text{-}330}$ and $E2_{316\text{-}345}$. Although both Th-clones recognized $E2_{301\text{-}330}$, in depth analysis of the specificity of these Th-clones revealed that they recognized distinct, yet overlapping, sequences, restricted by different class II HLA molecules. One of the clones recognized peptide $E2_{316\text{-}330}$ in the context of HLA-DR15(2), whereas the other clone was specific for peptide $E2_{311\text{-}325}$ in the context of HLA-DQ6(1) (Figure 2a,b). Fine-mapping of the peptides recognized by the two other Th-clones revealed a similar relationship, in that one recognized peptide $E2_{346\text{-}355}$ in the context of DR15(2), whereas the other reacted against the $E2_{351\text{-}365}$ peptide in a DR1-restricted manner, both present in $E2_{331\text{-}365}$ (Figure 2a,b and not shown). Notably, these data are in correspondence with the peptide binding data in Table 2, in that longer variants of these DR15(2) and DR1-restricted epitopes were indeed found to bind to the restricting HLA molecules (binding assay not available for HLA-DQ6).

Further evidence that the four E2 peptides identified represent physiologically relevant Th epitopes is provided by the fact that the E2-specific Th-clones did not only respond against peptide-loaded APC, but also specifically responded against APC that were pulsed with the E2 protein (Figure 2a-d, upper panels). Because in the latter case presentation of the peptide epitopes depends on

Table 1. Immunogenicity of HPV16 E2-derived peptides.

	Donor 1[a] DR4, 7	Donor 2 DR4, 6	Donor 3 DR1, 3	Donor 4 DR2, 4	Donor 5 DR2, 7	Donor 6 DR3, 5	Donor 7 DR1, 4	Donor 8 DR2, 6
$E2_{1-30}$[b]	·	·	·	·	·	·	·	·
$E2_{16-45}$	·	·	·	·	·	·	·	·
$E2_{31-60}$	·	·	81% (8,8)[c]	·	·	·	·	88% (8,6)
$E2_{46-75}$	·	·	88% (5,5)	·	81% (6,8)	·	·	94% (6,5)
$E2_{61-90}$	·	·	·	·	·	·	·	·
$E2_{76-105}$	·	·	·	·	·	·	·	·
$E2_{91-120}$	·	·	·	·	94% (4,8)	·	·	75% (4,0)
$E2_{106-135}$	·	·	·	·	·	·	·	·
$E2_{121-150}$	·	·	·	·	·	·	·	·
$E2_{136-165}$	·	·	·	·	·	·	·	·
$E2_{151-180}$	·	·	81% (6,4)	·	·	·	·	81% (4,1)
$E2_{166-195}$	·	·	94% (16,5)	·	81% (10,6)	·	·	·
$E2_{181-210}$	·	·	·	·	·	·	·	·
$E2_{196-225}$	·	·	·	·	·	·	·	·
$E2_{211-240}$	·	·	·	·	·	·	·	·
$E2_{226-255}$	·	·	·	·	·	·	·	·
$E2_{241-270}$	·	·	·	·	·	·	·	·
$E2_{256-285}$	·	·	·	·	·	·	·	·
$E2_{271-300}$	·	·	·	·	·	·	·	88% (4,8)
$E2_{286-315}$	·	·	88% (16,2)	·	·	·	·	94% (9,2)
$E2_{301-330}$	·	·	·	·	·	·	·	·
$E2_{316-345}$	·	·	·	·	·	·	94% (4,7)	·
$E2_{331-365}$	·	·	100% (11,4)	·	·	·	75% (4,1)	·
TT	100% (29,1)	100% (56,5)	100% (25)	100% (16,4)	100% (23,5)	100% (139)	100% (13,3)	100% (59)

Indicated are the percentages of positive wells and (mean stimulation index) for positive scoring peptides. Tetanus toxoid (TT) at a concentration of 1 LF/ml was used as a positive control.

[a] PBMC from 8 healthy blood donors were tested against HPV16 E2 peptides in short-term proliferation assays.

[b] HPV16 E2 peptides are indicated by the first and last amino acid.

[c] Peptides were scored positive, when the proliferation of ≥ 50% of the test wells exceeded the mean proliferation + 3 × SD of the control wells, and the mean stimulation index (SI) of all test wells over medium control wells was more than 3 in two independent assays.

uptake and processing of the E2 antigen, and not merely on exogenous loading of class II molecules at the APC cell surface, these data provide definite proof that the four E2 peptides recognized by our Th-clones correspond to naturally processed epitopes. Finally, all four Th-clones produced IFNγ upon antigenic stimulation, which is indicative of a Th-type 1 cytokine profile. Taken together our data show that the T cell repertoire of healthy individuals harbors IFNγ-secreting E2-specific CD4+ Th-cells (Figure 2a-d, lower panels).

Table 2. Binding affinity of HPV16 E2-derived peptides to different HLA-DR types.

Sequence	HPV16 E2	DR1	DR2	DR3	DR4
FNSSHKGRINCNSNTTPIVHLKGDANTLKC	271-300[a]	22[b]	65	37	>70
TPIVHLKGDANTLKCLRYRFKKHCTLYTAV	286-315	8	68	20	>70
LRYRFKKHCTLYTAVSSTWHWTGHNVKHKS	301-330	6	8	>70	10
SSTWHWTGHNVKHKSAIVTLTYDSEWQRDQ	316-345	6	55	30	>70
AIVTLTYDSEWQRDQFLSQVKIPKTITVSTGFMSI	331-365	8	20	10	15

[a] Sequence, first and last amino acid number of the peptide in the HPV16 E2 protein are indicated.

[b] Binding affinity of each peptide is expressed as the IC_{50} value: this is the peptide concentration (μM) at which binding of the standard fluorescence labeled peptide is reduced to 50% of its maximal value. >70 respresents undetectable binding.

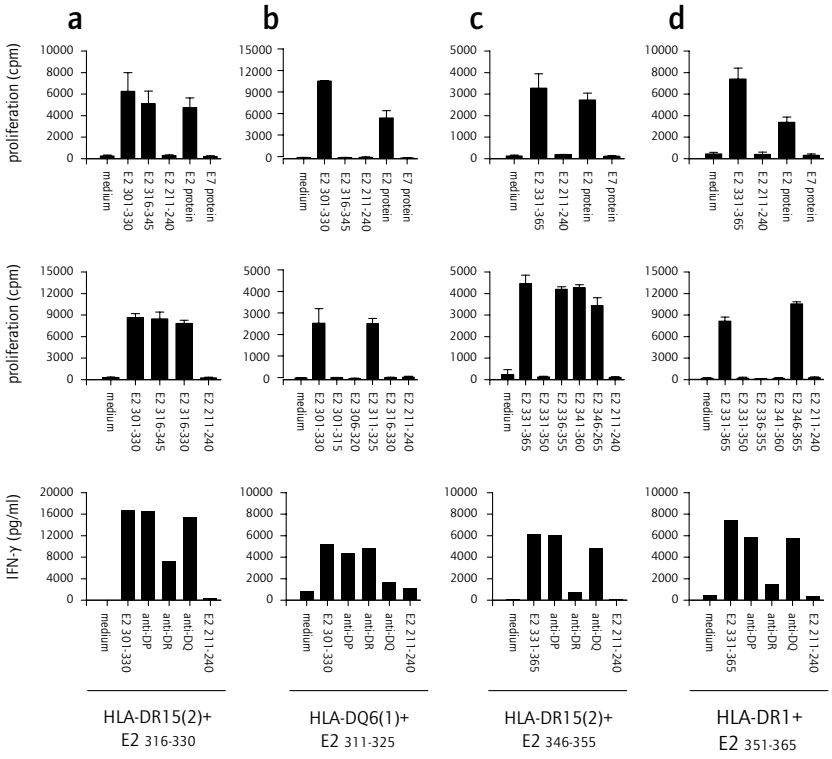

Figure 2. The C-terminal domain of HPV16 E2 contains naturally processed Th-epitopes. PBMC of HLA-DR and -DQ homozygous healthy blood donors (respectively DR15(2), DQ6(1) and DR1, DQ1) were stimulated at weekly intervals with peptides $E2_{301-330}$+$E2_{316-345}$ or peptide $E2_{331-365}$. Four stable Th-clones (A-D) derived from these polyclonal cultures were tested in 3-day proliferation assay, or stimulated for 24 hrs to measure the production of IFNγ by ELISA. Th-clones recognized not only peptide-loaded (5 μg/ml) APC but also E2 protein-loaded APC (10 μg/ml) indicating that the recognized peptides represent naturally processed Th-epitopes (upper panels). Fine-mapping of the minimal epitope (middle panels) and HLA-class II restriction using HLA-DR, -DQ and -DP blocking antibodies (lower panels) of these Th-clones is shown.

Detection of HPV16 E2 specific memory Th-cells in healthy individuals

The strikingly frequent detection of HPV16 E2-specific Th immunity in healthy individuals, as described in the first paragraph, prompted us to analyze whether the underlying T cell repertoire would represent immunological memory as a result of previous encounter with antigen, or whether it would primarily consist of particularly abundant naïve T cell precursors specific for this antigen. In view of the high incidence of, generally transient, genital HPV infections in young sexually active individuals [2, 3, 6, 32], as well as the prominent expression of E2 during HPV infection [22], it is conceivable that T cell memory against E2 could be found in healthy subjects. We examined the nature of the HPV16 E2-specific immunity detected by us through analysis of the E2-specific reactivity of the CD45RO+ fraction of healthy donor PBMC, which contains antigen-experienced T cells but is devoid of their naïve counterparts. Because we found the T cell repertoire of healthy donors to contain IFNγ-producing Th-cells (Figure 2), we measured the antigen-specific T cell responses through IFNγ ELISPOT. We first analyzed the reactivity of CD45RO+ T cells of two healthy donors while focusing our attention on a selection of E2 peptides that on basis of our previous experiments (Table 1) appear to be localized in the most immunogenic regions of HPV16 E2. Interestingly, these CD45RO+ PBMC were found to respond against multiple E2 peptides (Figure 3a), supporting the notion that

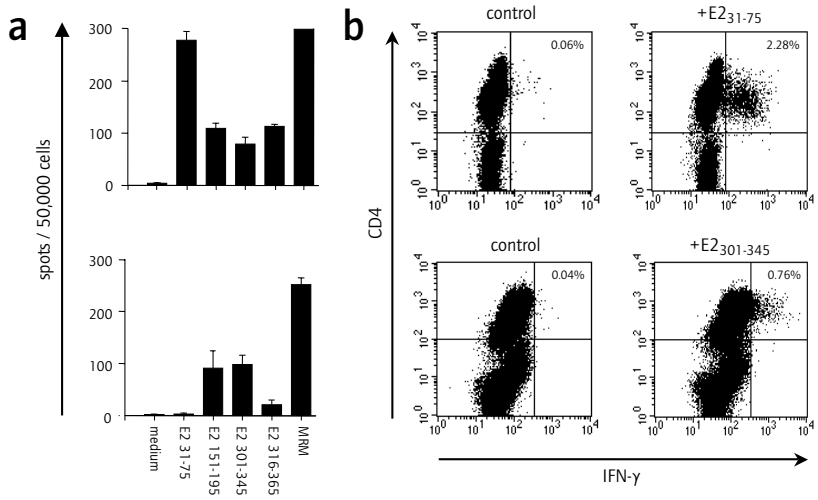

Figure 3. HPV16 E2-specific Th-cells are contained within the CD45RO+ memory fraction of healthy donor-derived PBMC. CD45RO+ PBMC of two different donors (upper and lower panels of **a** and **b**) were isolated by MACS magnetic bead isolation and stimulated with the indicated pools of two peptides (e.g. E2 31-75: $E2_{31\text{-}60}$ and $E2_{46\text{-}75}$). The number of antigen-specific T cells was analysed by IFNγ ELISPOT at day 11 (**a**). The mean number of spots + SD per 50,000 PBMC are shown; bars ± SD. A mix of three recall antigens (MRM) was used as a positive control. HPV16 E2-specific T cells are contained in the CD4+ T cell subset (**b**). In an independent experiment, CD45RO+ PBMC of the same subjects were stimulated with indicated peptide pools and the percentage of IFNγ-producing cells were analyzed by intracellular cytokine staining. The percentage of CD45RO+ PBMC producing IFNγ in response to peptide stimulation (right panels) versus medium control (left panels) is indicated.

healthy subjects can display HPV16E2-specific T cell memory. We confirmed that the responding IFNγ-producing cells belonged to the CD4+ Th-cell subset by employing IFNγ intracellular cytokine staining instead of ELISPOT as a readout (Figure 3b). A broader survey of E2-specific reactivity against the full array of peptides, using CD45RO+ PBMC from 8 additional healthy donors, revealed that 4 of these PBMC isolates responded against one or more of the HPV16 E2 peptides (Figure 4 and not shown). Taken together, our data reveal the presence of CD45RO+ memory-type, IFNγ-secreting Th-cells reactive against HPV16 E2 peptides in approximately half of the healthy donors tested. Notably, the incidence by which these responses are detected is very similar to that of the strong ($SI \geq 3$) proliferative responses found in total PBMC (Table 1), implicating that also these responses are likely to represent reactivity by memory T cells rather than by in vitro primed naïve T cells.

Cross-reactivity of HPV16 E2 specific Th-clones with peptide sequences of other HPV types

Due to the common nature of HPV infections, a majority of the human population is likely to encounter multiple HPV types [3, 33]. Furthermore, the protein sequences of the viral gene products are conserved to considerable extent between HPV types. It is therefore possible that at least a fraction of the T cell repertoire induced by a previous encounter with a given type of HPV could cross-react, and therefore cross-protect, during subsequent infection with other HPV types. Alignment of the sequence of the HPV16 E2 protein with that of various other HPV types revealed that it is most prominently conserved with that of other high-risk types (Figure 5c). Although this conservation is somewhat less conspicuous when the HPV16 E2 sequence is compared to that of low risk or common types (Figure 5a,b), it is evident that in all cases maximal conservation is confined to certain regions within the E2 sequence. In particular, three areas of HPV16E2 share homology with E2 of other HPV types: the N-terminal portions $E2_{31\text{-}120}$ and $E2_{151\text{-}195}$ and the C-terminal portion $E2_{271\text{-}365}$. These

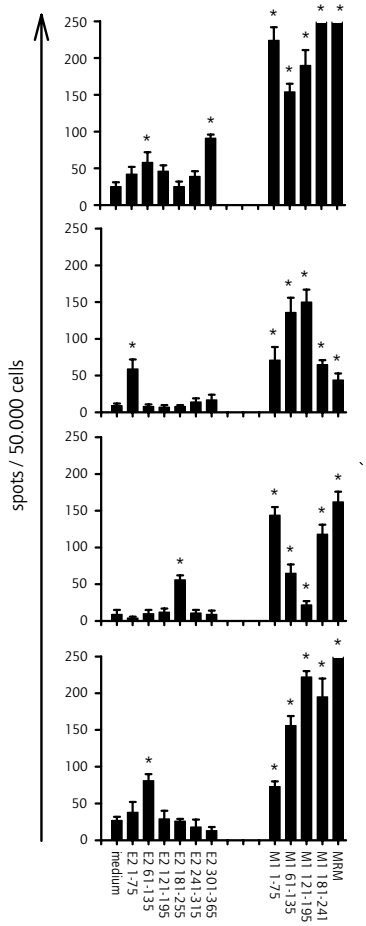

Figure 4. HPV16 E2-specific memory Th-responses are frequently detected in healthy individuals. The CD45RO+ PBMC fraction of 8 healthy blood donors were incubated with the full array of HPV16 E2 peptides divided in six pools of 4 peptides (e.g. E2 1-75: $E2_{1-30}$, $E2_{16-45}$, $E2_{31-60}$ and $E2_{46-75}$). The number of antigen-specific T cells was analyzed by IFNγ ELISPOT at day 11. In 4 out of 8 blood donors HPV16 E2-specific responses were detected at day 11. The mean number of spots + SD per 50,000 PBMC are shown; bars ± SD. Responses that significantly exceeded the medium control, as determined by a student t-test, are indicated with an asterisk (*). Pools of Influenza Matrix 1 peptides and a mix of recall antigens (MRM) were used as positive controls.

regions co-localize with the major functional domains of E2, in that the N-terminal domain harbors the transcriptional activation functions of this protein whereas the C-terminal portion mediates its sequence-specific DNA-binding properties. The intervening sequences ranging from residues 210 to 270 constitute the so-called hinge-region connecting the two key functional domains, which is poorly conserved between HPV types and also shows considerable intratypic variation for HPV16 [34-36]. Interestingly, our analyses of E2-specific responses in short-term proliferation assays have revealed that the most immunogenic peptides are clustered in the two conserved domains of the HPV16 E2 sequence (see Table 1 and summary in Figure 5d). In view of these considerations, we tested whether our established Th-clones, raised against epitopes derived from the C-terminal part of the HPV16 E2 sequence (Figure 2), would be capable of cross-reacting with E2 peptides of a number of other HPV types that shared maximal homology with HPV16 with respect to this particu-

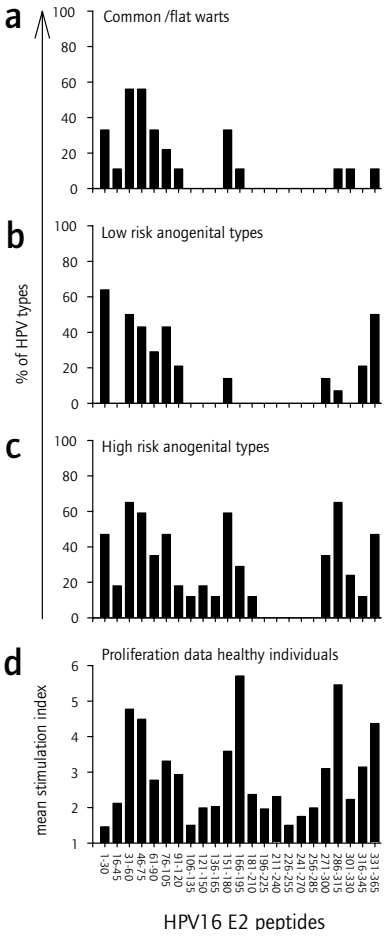

Figure 5. Highly immunogenic regions, as determined in the T cell proliferation assays are contained in the most conserved regions of HPV16 E2. The sequences of the HPV16 E2 30-mer peptides used in our immunological experiments were compared with the corresponding sequences of other HPV types using BLAST (see materials and methods). For each of these HPV16 E2 peptides, the percentage of HPV types sharing >70% amino acid homology (identical or with similar physico-chemical properties) with HPV16 is depicted. Distinction is made between HPV types 2,3,4,10,26,27,28,29,49 associated with common/flat warts (**a**), low-risk anogenital HPV types 6,11,40,42,43,44,54,55,61,62,64,67,71,74 (**b**), and high-risk anogenital HPV types 16,18,31,33,35,39,45,51,52,52,56,58,66,69,68,34,53,59 (**c**). The bottom panel of this figure (**d**) constitutes a summary of the proliferation assay data as obtained with the overlapping set of HPV16 E2 30-mer peptides (Table 1 and Figure 1). Indicated are the mean stimulation indices (SI) of all the assays (8 donors, tested twice). The mean SI is defined as the mean of the test well values divided by the mean of the control wells.

lar E2 sequence. Indeed, the DQ6-restricted $E2_{311-325}$-specific Th-clone showed strong recognition of the corresponding peptides of HPV types 26, 31, 33 and 45 (Figure 6a). The amino acid homology within this epitope varies from 73 to 87% (identical or amino acids with similar physico-chemical properties). Our other Th-clones did not reveal considerable cross-reactivity for highly homologous E2 peptides of other HPV types (Figure 6b and not shown). These data suggest that part of the HPV16 E2-reactive Th memory detected in our assays may relate to encounter of HPV types other than HPV16, but that the majority of this immune repertoire was most likely established through encounter with HPV16 itself.

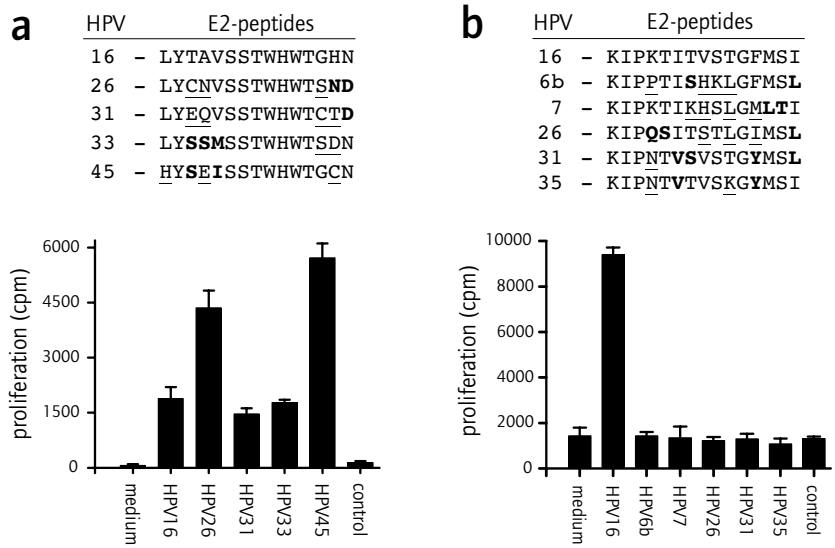

Figure 6. Cross-reactivity of HPV16 E2-specific Th-clones against corresponding peptide sequences derived from different HPV types. All four Th-clones were tested for cross-reactivity with peptides derived from other HPV types sharing maximal homology with HPV16 with respect to their cognate epitope. The DQ6-restricted $E2_{311-325}$-specific Th-clone showed strong recognition of the homologous peptides derived from HPV types 26, 31, 33 and 45 (**a**), whereas the other three Th-clones, including the DR1-restricted $E2_{351-365}$-specific Th-clone (**b**), recognized only the HPV16 E2-derived peptide. In the amino acid sequence of homologous peptides the amino acid mismatches (underlined) and non-identical amino acids with similar physico-chemical properties (bold) compared to the HPV16 sequence are indicated. The irrelevant non-related peptide $E2_{211-240}$ was used as negative control.

Discussion

Through analysis of the proliferative responses in PBMC cultures from healthy subjects against the HPV16 E2 antigen we have demonstrated that this protein contains highly immunogenic peptide sequences to which strong T cell reactivity is detected. Subsequent testing of the CD45RO+ memory fraction of healthy donor PBMC revealed the presence of HPV16 E2-specific IFNγ-secreting CD4+ T cells in approximately half of these donors. Taken together, our data demonstrate that the T cell repertoire of a majority of the healthy subjects contains particularly high frequencies of Th-cells reactive against the HPV16 E2 antigen and that this repertoire has been established through previous encounter with HPV16 and/or other HPV types.

By employing the short-term proliferation assay we have previously monitored the reactivity of healthy donor PBMC against a series of HPV16 E7-derived peptides [19]. In this former study we scored all responses positive that were above background (mean + 2 × SD). We choose these non-stringent criteria because we did not expect HPV16 E7-specific T cell memory in healthy subjects. Indeed, we did not record any HPV16 E7-specific responses in healthy donor PBMC with a stimulation index of more than 3, the cutoff level commonly used for identification of memory type T cell responses [30, 31]. In contrast, proliferation assays with HPV16 E2, as described in the present study, revealed overwhelmingly strong responses. Because half of the healthy donor PBMC showed responses with a magnitude comparable to that with recall antigens (SI ≥ 3), we decided to focus our study on these very strong responses. In agreement with the magnitude of these responses, we now demonstrate that the T cell repertoire of approximately half of healthy donors comprises CD45RO+ memory-type T cells against HPV16 E2. Although the low numbers of individuals tested precludes

definitive conclusions, the T cell reactivity detected by proliferation assay and ELISPOT revealed a similar peptide-reaction pattern.

Importantly, we have not found any previous report concerning the presence of memory T cell responses against E2 or any of the other non-structural HPV16 proteins in healthy individuals. In fact, HPV16 infected individuals diagnosed with CIN were particularly found to display Th-responses against the C-terminal domain of HPV16 E2 at the time of viral clearance, whereas no E2-specific Th-responses were detected in the healthy control group [25]. However, the methodology applied for analysis of these responses is essentially different. The IL-2 bio-assay used in the study by Bontkes et al. measures the total response of all cultured cells and therefore lacks the sensitivity of single cell cytokine analysis applied in the present study. Furthermore, the pre-selection of the CD45RO+ memory PBMC pool directly ex-vivo (Figures 3 and 4) allows enrichment of HPV16 E2-specific memory T cells, as well as re-activation of so-called central memory T cells by extended culturing of these cells in vitro without the risk of in vitro priming of naive T cells. By employing this highly sensitive method, we now reveal the presence of E2-specific Th-cells in healthy individuals. It is interesting to note that HPV16 E2-specific IgG and IgA responses have also been detected in healthy individuals [37, 38]. Since antibody isotype switching is Th-dependent, these observations constitute an independent confirmation of the presence of E2-specific Th memory in healthy subjects as reported in the present study.

In pre-clinical animal models, in particular the CRPV model, it was shown that the E2-specific immune response plays a role in spontaneous papilloma regression [23] and, furthermore, that regression could be enhanced by E2-directed vaccination [24, 39]. Also in humans, there are strong indications that the E2-specific immune response is associated with HPV clearance [25]. The strong immunogenicity of the E2 protein, together with the presence of E2-specific Th-immunity in a large proportion of the human population, could be exploited in immunotherapy and prevention of HPV16-positive CIN lesions. Boosting of pre-existing E2-specific Th-memory, as established by previous encounters with HPV16 or other HPV-types, can provide the powerful, pathogen-specific T cell help that is required for optimal induction of CTL responses against various HPV16 antigens including E2, E6 and E7 [40, 41].

Acknowledgements · The authors are greatly indebted to all healthy blood donors and to Willemien Benckhuijsen for synthesis of peptides. We thank Drs. T. Mutis and J.P. Medema for helpful discussions and critical reading.

References

1. Burk RD, Kelly P, Feldman J, Bromberg J, Vermund SH, DeHovitz JA, and Landesman, SH. Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. *Sex Transm Dis,* 23: 333-341, 1996.
2. Karlsson R, Jonsson M, Edlund K, Evander M, Gustavsson A, Boden E, Rylander E, and Wadell G. Lifetime number of partners as the only independent risk factor for human papillomavirus infection: a population-based study. *Sex Transm Dis,* 22: 119-127, 1995.
3. Koutsky L. Epidemiology of genital human papillomavirus infection. *Am J Med,* 102: 3-8, 1997.
4. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, and Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst,* 87: 796-802, 1995.
5. Duggan MA, McGregor SE, Stuart GC, Morris S, Chang-Poon V, Schepansky A, and Honore L. The natural history of CIN I lesions. *Eur J Gynaecol Oncol,* 19: 338-344, 1998.
6. Evander M, Edlund K, Gustafsson A, Jonsson M, Karlsson R, Rylander E, and Wadell, G. Human papillomavirus infection is transient in young women: a population-based cohort study. *J Infect Dis,* 171: 1026-1030, 1995.
7. Ho GY, Bierman R, Beardsley L, Chang CJ, and Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med,* 338: 423-428, 1998.
8. Aiba S, Rokugo M, and Tagami H. Immunohistologic analysis of the phenomenon of spontaneous regression of numerous flat warts. *Cancer,* 58: 1246-1251, 1986.
9. Coleman N, Birley HD, Renton AM, Hanna NF, Ryait BK, Byrne M, Taylor-Robinson D, and Stanley MA. Immunological events in regressing genital warts. *Am J Clin Pathol,* 102: 768-774, 1994.
10. Benton C, Shahidullah H, and Hunter JAA. Human papillomavirus in the immunosupressed. *Papillomavirus Rep,* 3: 23-26, 1992.
11. Serraino D, Carrieri P, Pradier C, Bidoli E, Dorrucci M, Ghetti E, Schiesari A, Zucconi R, Pezzotti P, Dellamonica P, Franceschi S, and Rezza G. Risk of invasive cervical cancer among women with, or at risk for, HIV infection. *Int J Cancer,* 82: 334-337, 1999.
12. Garrido F, Ruiz-Cabello F, Cabrera T, Perez-Villar J, Lopez-Botet M, Duggan-Keen M, and Stern PL. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today,* 18: 89-95, 1997.
13. Hilders CG, Houbiers JG, van Ravenswaay Claasen HH, Veldhuizen RW, and Fleuren, G J. Association between HLA-expression and infiltration of immune cells in cervical carcinoma. *Lab Invest,* 69: 651-659, 1993.
14. Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, and Stern PL. Natural history of HLA expression during tumour development. *Immunol Today,* 14: 491-499, 1993.
15. Hopfl R, Heim K, Christensen N, Zumbach K, Wieland U, Volgger B, Widschwendter A, Haimbuchner S, Muller-Holzner E, Pawlita M, Pfister H, and Fritsch P. Spontaneous regression of CIN and delayed-type hypersensitivity to HPV-16 oncoprotein E7. *Lancet,* 356: 1985-1986, 2000.
16. de Gruijl T, Bontkes HJ, Walboomers JMM, Stukart MJ, Doekhie FS, Remmink AJ, Helmerhorst TJM, Verheijen RHM, Duggan-Keen MF, Stern PL, Meijer CJLM, and Scheper RJ. 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,* 58: 1700-1706, 1998.
17. de Gruijl TD, Bontkes HJ, Stukart MJ, Walboomers JM, Remmink AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ, and Scheper RJ. 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,* 77: 2183-2191, 1996.
18. Luxton JC, Rowe AJ, Cridland JC, Coletart T, Wilson P, and Shepherd PS. 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,* 77: 1585-1593, 1996.
19. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief CJ, and Offringa R. 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,* 91: 612-618, 2001.
20. zur Hausen H. Papillomavirus infections – a major cause of human cancers. *Biochim et Biophys Acta,* 1288: F55-F78, 1996.
21. Stevenson M, Hudson LC, Burns JE, Stewart RL, Wells M, and Maitland NJ. Inverse relationship between the expression of the human papillomavirus type 16 transcription factor E2 and virus DNA copy number during the progression of cervical intraepithelial neoplasia. *J Gen Virol,* 81: 1825-1832, 2000.
22. Maitland NJ, Conway S, Wilkinson NS, Ramsdale J, Morris JR, Sanders CM, Burns JE, Stern PL, and Wells M. Expression patterns of the human papillomavirus type 16 transcription factor E2 in low- and high-grade cervical intraepithelial neoplasia. *J Pathol,* 186: 275-280, 1998.
23. Selvakumar R, Ahmed R, and Wettstein FO. Tumor regression is associated with a specific immune response to the E2 protein of cottontail rabbit papillomavirus. *Virology,* 208: 298-302, 1995.
24. Selvakumar R, Borenstein LA, Lin YL, Ahmed R, and Wettstein FO. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. *J Virol,* 69: 602-605, 1995.
25. Bontkes HJ, de Gruijl TD, Bijl A, Verheijen RH, Meijer CJ, Scheper RJ, Stern PL, Burns, JE, Maitland NJ, and Walboomers JM. Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia. *J Gen Virol,* 80: 2453-2459, 1999.
26. Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den Hartigh J, Ottenhoff, TH, and Drijfhout JW. Purification of His-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr Purif,* 18: 95-99, 2000.
27. van der Burg SH, Kwappenberg KM, Geluk A, van der Kruk M, Pontesilli O, Hovenkamp E, Franken KL, van Meijgaarden KE, Drijfhout JW, Ottenhoff TH, Melief CJ, and Offringa R. 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,* 162: 152-160, 1999.
28. McCutcheon M, Wehner N, Wensky A, Kushner M, Doan S, Hsiao L, Calabresi P, Ha T, Tran TV, Tate KM, Winkelhake J, and Spack EGA sensitive ELISPOT assay to detect low-frequency human T lymphocytes. *J Immunol Methods,* 210: 149-166, 1997.
29. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res,* 25: 3389-3402, 1997.
30. Bermas BL, and Hill JA. Proliferative responses to recall antigens are associated with pregnancy outcome in women with a history of recurrent spontaneous abortion. *J Clin Invest,* 100: 1330-1334, 1997.
31. Fryauff DJ, Church LW, Richards AL, Widjaja H, Mouzin E, Ratiwayanto S, Hadiputranto H, Sutamihardja MA, Richie TL, Subianto B, Tjitra E, and Hoffman SL. Lymphocyte response to tetanus toxoid among Indonesian men immunized with tetanus-diphtheria during extended chloroquine or primaquine prophylaxis. *J Infect Dis,* 176: 1644-1648, 1997.
32. Ho GY, Burk RD, Fleming I, and Klein RS. Risk of genital human papillomavirus infection in women with human immunodeficiency virus-induced immunosuppression. *Int J Cancer,* 56: 788-792, 1994.
33. Thomas KK, Hughes JP, Kuypers JM, Kiviat NB, Lee SK, Adam DE, and Koutsky LA. Concurrent and sequential acquisition of different genital human papillomavirus types. *J Infect Dis,* 182: 1097-1102, 2000.
34. Giri I, and Yaniv M. Structural and mutational analysis of E2 transactivating proteins of papillomaviruses reveals three distinct functional domains. *EMBO J,* 7: 2823-2829, 1988.
35. Eriksson A, Herron JR, Yamada T, and Wheeler CM. Human papillomavirus type 16 variant lineages characterized by nucleotide sequence analysis of the E5 coding segment and the E2 hinge region. *J Gen Virol,* 80: 595-600, 1999.
36. Terry G, Ho L, and Cuzick J. Analysis of E2 amino acid variants of human papillomavirus types 16 and 18 and their associations with lesion grade and HLA DR/DQ type. *Int J Cancer,* 73: 651-655, 1997.
37. Dillner J. Mapping of linear epitopes of human papillomavirus type 16: the E1, E2, E4, E5, E6 and E7 open reading frames. *Int J Cancer,* 46: 703-711, 1990.
38. Rocha-Zavaleta L, Jordan D, Pepper S, Corbitt G, Clarke F, Maitland NJ, Sanders CM, Arrand JR, Stern PL, and Stacey SN. Differences in serological IgA responses to recombinant baculovirus- derived human papillomavirus E2 protein in the natural history of cervical neoplasia. *Br J Cancer,* 75: 1144-1150, 1997.
39. Han R, Cladel NM, Reed CA, Peng X, and Christensen ND. 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,* 73: 7039-7043, 1999.
40. Ossendorp F, Mengede E, Camps M, Filius R, and Melief CJM. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med,* 187: 1-10, 1998.
41. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, and Melief CJ. T cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature,* 393: 480-483, 1998.

Marij Welters, **Annemieke de Jong**, Susan van den Eeden, Jeanette van der Hulst, Kitty Kwappenberg, Sabrin Hassane, Kees Franken, Jan Wouter Drijfhout, Gert Jan Fleuren, Gemma Kenter, Cornelis Melief, Rienk Offringa, Sjoerd van der Burg

Cancer Res 63, 636-641, 2003

3

Frequent display of HPV16 E6-specific memory T-helper cells in the healthy population as witness of previous viral encounter

Abstract · Genital HPV infection is common and the majority of infected individuals successfully deal with this virus. Clearance of HPV is presumably mediated by T cells but HPV16 specific T cell memory was usually detected in patients with progressive disease and not in healthy subjects, suggesting that HPV-immunity comes too late. We now show the presence of HPV16 E6-specific memory T-helper responses in a major fraction (12/20) of healthy individuals by application of the IFNγ-ELISPOT assay. Although nearly all E6-peptides were recognized, the majority of the responders targeted peptide sequences of the C-terminal half ($E6_{81-158}$) of HPV16 E6. In a direct comparison, the presence of HPV16 E6-specific T cells coincided with HPV16 E2-specific T cell reactivity in healthy individuals, whereas hardly any HPV16 E7-specific Th-immunity was found. This indicates that the induction of T cell reactivity against HPV16 E7 is sub-optimal during infection when compared to that against HPV16 E2- and E6. In conclusion, the presence of HPV16 E6-specific Th-memory in the healthy population demonstrates that HPV infection leads to T cell immunity against immediate early proteins expressed during infection. Because this HPV16 E6-specific T cell immunity was frequently detected in healthy subjects, our data suggest that the observed IFNγ-producing proliferating T cells circulating in the peripheral blood play a role in protection against persistent HPV infection and associated development of malignancies.

Introduction

Genital infection of both men and women with oncogenic human papillomavirus (HPV) types is quite common [1-3], however, only a minor fraction of infected subjects develop progressing epithelial lesions or cancer [4-6]. Most pre-malignant lesions regress spontaneously. This is likely mediated by cellular immune responses because regressing (genital) warts are infiltrated with CD4+ T cells, CD8+ T cells and macrophages [7, 8]. The role of immune surveillance in controlling infection by various HPV types is further indicated by the increased incidence of HPV infections, HPV-associated warts, cervical intra-epithelial neoplasia (CIN) lesions and cervical carcinoma in immunocompromised subjects [9-11]. This notion is sustained by the observations that papillomavirus-specific T cell immunity can protect against malignant transformation of papillomas in rabbits [12].

Examination of HPV-specific T cell immunity in patients with HPV+ cervical lesions has revealed the occasional presence of cytotoxic T-lymphocyte (CTL) responses against the E6 and E7 oncoproteins [13-18]. Because an overt correlation between the presence of such CTL immunity and regression or progression of the disease has so far not been found, the functional significance of these responses is at present a matter of debate. Most likely, not only CTL numbers, but also their activation state needs to be taken into account. Accumulating evidence pertinently shows that proper activation of CTL requires delivery by professional antigen presenting cells (APC) of strong co-stimulatory signals in addition to the antigenic signal. Especially in the case of anti-viral immunity in chronic infections, this co-stimulatory action of APC requires virus-specific T-helper (Th) cell activity [19-23]. We previously found that E7-specific Th immunity was frequently observed in HPV16+ patients, but only rarely in healthy subjects [24]. In contrast, over 50% of the healthy subjects tested showed very strong memory-type Th responses against one or more epitopes of HPV16 E2. It is conceivable that this Th memory is a witness of previous encounter with HPV16 or possibly other HPV-types encoding cross-reactive epitopes [25]. Apparently, the T cell immune system deals with the E2 and E7 antigens in a completely different manner. Because high expression of E6/E7 and E2 is mutually exclusive during infection and subsequent tumor development, these findings prompted us to now also monitor Th-immunity against HPV16 E6 in patients and healthy subjects. Similar to what was found for E2, memory Th-immunity against E6 was frequently and abundantly observed in healthy subjects, suggesting a role for these Th-cells in protection against re-infection by high-risk HPV and as such the HPV-mediated induction of anogenital lesions and cervical cancer.

Materials and Methods

Subjects and Controls · PBMC of anonymous healthy blood bank donors (D) were obtained after informed consent. Because these donors are anonymous only a general age and gender distribution can be given. The currently studied healthy individuals displayed a median age of 44 years old (range 25–68) and comprised of 40% women and 60% males. No additional data is available, nor was 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. The study of the subjects (P) with cervical carcinoma in this paper was nested in the "CIRCLE study" that investigates cellular immunity against HPV16 infected cervical lesions. Women presenting with histological proven cervical carcinoma at the department of gynecology of the Leiden University Medical Center (LUMC) were, after informed consent, enrolled in this study. The study design was approved by the Medical Ethical Committee of the LUMC. Blood was drawn at day of treatment prior to surgery. Subjects with stage IB-IIA were treated by a radical hysterectomy. The age of the patients ranged between 29 and 76 years old and these women were

typed for HPV16 using HPV16-specific primers on DNA isolated from paraffin-embedded sections of biopsies or surgical resection specimens [26].

Antigens · A set of peptides spanning the whole HPV16 E2 protein, consisting of 22 30-mer peptides with 15 amino acids of overlap and the C-terminal peptide with a length of 35 amino acids, were used for the T cell proliferation assays. They are indicated by the first and last amino acid in the protein (e.g. $E2_{1\text{-}30}$, residues 1-30, and the last peptide $E2_{331\text{-}365}$). For the ELISPOT assays a large set of 22-residue long HPV16 E6 overlapping (12 residues) peptides were used to obtain a detailed insight in the immunogenic regions of the E6 protein. In addition, 8 HPV16 E6-derived 32-mer overlapping (14 residues) peptides and 4 HPV16 E7 35-mer overlapping (14 residues) peptides were used for the T cell cultures and proliferation assays. These long HPV16 E2, E6 and E7 peptides were used in the proliferation assay to lower the number of PBMC required, as we wanted to use pools of maximal two peptides. Pools of four 30-mer peptides, with 15 amino acids overlap, spanning the influenza matrix 1 protein of A/PR/8/34 (M1) were used as positive control in the ELISPOT assay. The peptides were synthesized and dissolved as described previously [27]. Tetanus toxoid (TT, 1 LF/ml; National Institute of Public Health and the Environment, Bilthoven, The Netherlands) was used as positive control in the short-term T cell proliferation assays. Recombinant HPV16 E6 protein and HPV16 E7 protein (the latter protein served as control protein in proliferation assays) were produced in recombinant E. coli transformed with pET-19b-HPV16 E6 and pET-19b-HPV16 E7 [24].

Analysis of antigen-specific Th-cells by IFNγ-ELISPOT · The presence of HPV16 E6-specific Th-cells was analyzed by ELISPOT as previously described ADDIN ENRfu [24]. Briefly, peripheral blood mononuclear cells (PBMC) were seeded at a density of 2×10^6 cells/well of a 24-well plate (Costar, Cambridge, MA) in 1 ml of Iscove's medium (Bio-Whittaker, Verviers, Belgium) enriched with 10% fetal calf serum (FCS; Greiner, Mannheim, Germany), in the presence or absence of 5 μg/ml of indicated E6-derived 22-mer peptide. As a positive control, PBMC were cultured in the presence of indicated pools of influenza A/PR/8/34 M1 protein-derived peptides, consisting of four overlapping 30 amino acid long peptides in each pool, because previous analyses showed that all healthy individuals displayed reactivity against one or more of these M1 peptide pools [24]. Following four days of incubation at 37°C PBMC were harvested, washed and seeded in six replicate wells at a density of 10^5 cells/well on a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ catching antibody (Mabtech AB, Nacha, Sweden). Then, the ELISPOT plates were incubated for 18 hours at 37°C to allow catching of the antigen-specific produced IFNγ. The ELISPOT assay was further performed according to the instructions of the manufacturer (Mabtech). The number of spots was analyzed with a fully automated computer assisted video imaging analysis system (Carl Zeiss Vision). Specific spots were calculated by subtracting the mean number of spots plus $2 \times$ SD of the medium only control from the mean number of spots of experimental wells. Antigen-specific T cell frequencies were considered to be increased compared to non-responders when T cell frequencies were $\geq 1/10^4$ PBMC [24].

Short-term T cell proliferation assay · Healthy donor-derived PBMC were incubated with 12 pools of HPV16 E2-derived 30-mer peptides, four pools of E6 32-mer peptides and two pools of E7 35-mer peptides (each pool containing two overlapping peptides), according to procedures described previously [25, 27]. Briefly, freshly isolated PBMC were seeded at a density of 1.5×10^5 cells/well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 200 μl of Iscove's medium (Bio-Whittaker) supplemented with 10% autologous serum. In previous experiments, we observed that proliferative responses to HPV16 E2 peptides were relatively reduced with the use of (heat-inactivated) human AB serum (Sigma) as compared to autologous serum. HPV16 E2-, E6- and E7-derived peptides were added at a concentration of 10 μg/ml. Medium alone was taken along as a negative control and TT served as a positive control. For each peptide pool, 8 parallel microcultures were incubated. Peptide-specific proliferation was measured at day 6 by [^{3}H]-thymidine incorporation. Cultures were scored

positive, when the proliferation of ≥ 75% of the test wells exceeded the mean proliferation plus 3×SD of the medium only control wells, and the stimulation index (SI) of the mean test wells over mean medium control wells was ≥ 3.

Long-term HPV16 E6-specific T cell cultures · PBMC derived from healthy blood donors were incubated with 8 overlapping 32-mer peptides spanning the entire HPV16 E6 protein at a concentration of 1 μg/ml in Iscove's medium (Bio-Whittaker) supplemented with 10% re-calcified autologous plasma. One day after stimulation, T cell growth factor (TCGF; Biotest, Dreieich, Germany) was added at a final concentration of 5%. At day 10, the cell culture was harvested and incubated with autologous monocytes and E6 peptides (1 μg/ml) at a 10:1 ratio for 16 hours, after which T cells producing IFNγ in response to the E6 peptides were enriched by means of a cytokine secretion assay (Miltenyi, Biotech, Bergisch Gladbach, Germany). This assay is based on a bi-specific antibody recognizing 1) the IFNγ molecule and 2) the CD45 molecule. The captured IFNγ is detected by a PE-labeled secondary antibody, and subsequent magnetic labeling allows enrichment of the antigen-specific cells. The isolated cells were cultured for 10-14 days with irradiated autologous PBMC at 1:10 ratio and 10% TCGF without any further addition of antigen. The polyclonal T cell culture was tested for recognition of E6 peptides (5 μg/ml) and protein (10 μg/ml) in a 3-day proliferation assay. Autologous adherent monocytes were used as APC, and proliferation was measured by [^{3}H]-thymidine incorporation.

Results

E6-specific IFNγ-secreting Th-cells in HPV16 positive cervical cancer patients

In addition to E7, the E6 oncoprotein is essential for maintenance of the transformed state in cervical carcinoma. Both proteins are thus interesting targets for anti-tumor T cell immunity. We therefore extended our previous analysis of HPV16 E7-specific Th-immunity in HPV16+ cervical cancer patients [24] to the E6 antigen. Hence, PBMC of 11 HPV16+ cervical cancer patients were stimulated with a set of overlapping 22-mer peptides covering the complete HPV16 E6 protein, after which the number of IFNγ-producing E6-specific Th-cells was determined by ELISPOT. In 3 of the 11 HPV16+ patients (P4, P5, and P8), HPV16 E6-specific Th-frequencies ≥1/10,000 PBMC were demonstrated. Based on our experience, responses of such a magnitude are clearly positive whereas responses lower than this frequency may fall within background reactivity [24]. Patient P4 responded to peptide $E6_{31\text{-}52}$ and $E6_{131\text{-}152}$ indicating the presence of memory Th-cells specific for two distinct epitopes. The other two patients (P5 and P8) responded to only one E6 peptide, suggesting that in both cases a single E6-epitope was recognized (Table 1). Overall, the frequency and magnitude of Th1 type response against E6 detected in patients is comparable to that previously found for E7 [24].

Frequent memory Th-reactivity against HPV16 E6 in healthy blood donors

We subsequently analyzed whether these E6-specific responses are also detectable in healthy donors, because this population undoubtedly includes individuals who have effectively dealt with HPV16 infection [3, 6, 28]. Therefore, we assessed the presence of HPV16 E6-specific Th-cells in the blood of a group of 20 healthy individuals. Interestingly, 12 out of 20 healthy blood donors showed HPV16 E6-specific Th type 1 responses upon stimulation with one or more of the HPV16 E6 22-mer peptides (Table 1). Analysis of the reactivity pattern revealed that most responses detected were directed against peptide sequences of the C-terminal half ($E6_{81\text{-}158}$) of HPV16 E6 (Table 1). The magnitude of the E6-specific responses was very similar to that found in patients. However, the fraction of healthy donors showing strong IFNγ Th responses against E6 peptides appears higher than that of the patients (60% versus 27%). Furthermore, some of the healthy subjects reacted even against four or more E6-peptides (range 1–7) whereas the three patients responded to one or two peptides

Table 1. HPV16 E6-specific T-helper responses as measured by IFNγ-ELISPOT.

	HPV16 E6[a]															Influenza M1			
	1-22	1-32	1-42	1-52	41-62	51-72	61-82	71-92	81-102	91-112	101-122	111-132	121-142	131-152	137-158	P1	P2	P3	P4
P1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	10	·
P3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P4	·	·	·	28[b]	·	·	·	·	·	·	·	·	·	13	·	·	·	·	·
P5	·	·	·	·	·	·	·	·	·	·	·	·	·	22	·	·	·	62	30
P6	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P7	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P8	·	·	·	·	·	11	·	·	·	·	·	·	·	·	·	31	·	13	·
P9	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
P10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	15	·
P11	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
D1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	16	·	·
D2	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	26	12	·
D3	·	·	·	·	·	·	·	·	·	·	·	·	·	18	·	30	·	19	·
D4	·	·	·	10	·	·	·	·	·	·	·	·	·	·	·	22	15	14	10
D5	·	·	·	·	·	·	·	·	·	10	·	·	·	·	·	·	10	12	37
D6	·	34	·	·	·	·	·	·	11	14	·	12	10	·	·	·	33	41	·
D7	·	·	·	·	·	·	·	·	·	14	·	·	·	·	14	·	21	57	28
D8	·	·	·	·	·	·	·	·	12	14	·	·	·	·	·	27	38	34	16
D9	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	22	10	14	·
D10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	13	23	19	11
D11	·	·	·	·	·	·	·	·		·	·	15	10	27	17	33	28	24	52
D12	·	·	·	·	·	·	·	·	·	·	·	·	14	15	·	·	10	12	·
D13	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	21
D14	·	·	·	10	·	·	·	20	12	·	28	18	37	40	·	12	42	28	45
D15	18	16	·	15	·	·	·	·	12	21	·	11	·	·	·	52	68	64	·
D16	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	39	66	49	48
D17	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	66
D18	·	·	·	·	·	·	·	·	·	·	·	11	·	·	·	·	·	10	·
D19	·	·	·	·	·	·	·	·	·	·	33	38	·	16	13	16	19	27	21
D20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	10	38

[a] PBMC were stimulated with the indicated HPV16 E6 22-mer peptides and subsequent IFNγ-production by T-helper cells was measured by ELISPOT. Influenza M1-derived peptide pools were used to compare HPV-specific T cell frequencies with T cell reactivity to a common recall antigen.

[b] Number of HPV16 E6-specific memory T-helper lymphocytes per 100,000 PBMC in HPV16+ subjects with cervical cancer (P) or healthy blood donors (D) is given. Specific T-helper cell frequencies ≥ 1/10,000 are depicted.

only. It must be noted, however, that also the incidence and breadth of the response against the influenza M1 antigen is higher in healthy subjects (Table 1), indicating an overall reduced immunity in patients.

To further characterize the observed HPV16 E6-specific IFNγ-responses in healthy subjects, the E6-induced proliferation capacity of PBMC was analyzed. Freshly isolated PBMC of 5 healthy donors were stimulated with a pool of eight of the long 32-mer overlapping HPV16 E6-peptides for 10 days and the responding T cells were isolated based on the E6-specific IFNγ-production using cytokine secretion assay and magnetic cell sorting (MACS). Flow cytometric analysis of the T cells specifically responding to E6 revealed that these T cells resided in the CD4+ fraction only (Figure 1). Following a 14-day period of non-specific expansion, the resulting T cell cultures were tested for their proliferative capacity to both E6 peptides and E6 protein, as the latter will indicate their capacity to respond to naturally processed and presented antigen. From 3 of these 5 cultures we were able to isolate IFNγ-producing HPV16 E6-specific Th-cells able to proliferate not only upon stimulation with peptide but also to E6 protein pulsed APC (Figure 1). Two subjects (D21 and D23) predominantly responded to $E6_{73\text{-}109}$ and $E6_{127\text{-}158}$ whereas the third donor (D22) displayed a broader reaction pattern (Figure 1). The area covered by $E6_{73\text{-}109}$ and $E6_{127\text{-}158}$ was also frequently recognized when the E6-response was analyzed by ELISPOT (Table 1). Taken together, our data demonstrate that the memory T cell repertoire of the majority of healthy individuals contains proliferative and IFNγ-producing CD4+ Th-cells capable of reacting against the HPV16-derived E6 oncoprotein.

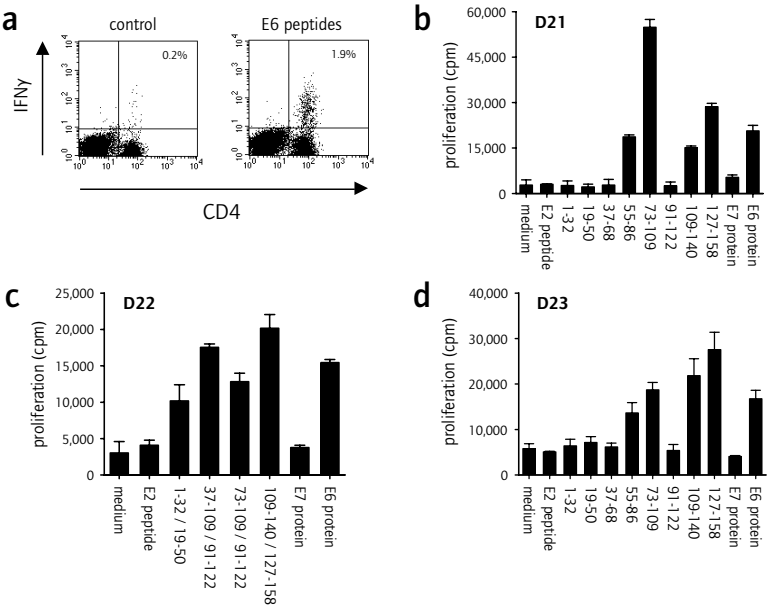

Figure 1 HPV16 E6-peptide specific INFγ-producing Th-cells are able to proliferate against E6 protein-pulsed APC. Freshly isolated PBMC of donors (D) D21-D25 were stimulated with a pool of 8 of the long 32-mer overlapping HPV16 E6-peptides (1 μg/ml/peptide) for 10 days, and T cells producing IFNγ in response to restimulation with E6-peptides were isolated by IFNγ-secretion assay and MACS. **a.** Flow cytometric analysis showed E6-specific IFNγ production in the CD4+ T cell subset only. One representative example (D21) is shown. **b-d.** Following a 14-day period of non-specific expansion T cell cultures were tested for their capacity to proliferate upon stimulation with 5 μg/ml of each single E6 peptide (or pools of two peptides) indicated by the number of their first and last amino acid or the negative control peptide from HPV16 E2 ($E2_{1-30}$). In addition, T cell proliferation, upon stimulation with 10 μg/ml HPV16 E6 protein or the negative control HPV16 E7 protein is shown. Subjects D24 and D25 did not specifically respond after the expansion period and are therefore not shown.

Healthy individuals display coinciding Th-responses against HPV16 E2 and E6, but not against E7. We have recently demonstrated frequent HPV16 E2-specific Th-responses in healthy individuals [25], whereas Th-immunity against the E7 oncoprotein was rarely found in these subjects [24]. The present study revealed that the HPV16 E6 protein is also frequently targeted by Th-cells in healthy subjects. To define the relationship between the E2-, E6- and E7-specific Th response in healthy individuals, the presence of Th reactivity against these three antigens was analyzed in parallel. Fresh PBMC samples derived from 12 healthy blood donors were stimulated with 18 pools of two overlapping 30-35mer peptides that together cover the entire sequences of HPV16 E2, E6 and E7, and T cell proliferation was evaluated after 6 days of culture as described previously [25]. A stimulation index (SI) exceeding 3 was applied as a threshold, as this is commonly used for the detection of memory T cell responses [29, 30]. The incidence and specificity of the proliferative responses against HPV16 E2 and E6 were comparable to our previous ELISPOT experiments (Table 1) [25]. One donor (D33; Figure 2) displayed responses against all 3 antigens and 3 donors (D27 (Figure 2), D30 and D37) showed specific proliferation against E2 and E6 (Table 2). In three other cases (D26, D29, D34) responses were only found against E2, whereas one PBMC sample (D31) only responded to E6 peptides. Most importantly, a vast majority (8/12) of the healthy subjects displayed memory type Th responses against HPV16 E2 and/or E6 in their blood, supporting the notion that encounter with high-risk HPV types, including HPV16, is very common [1-3]. Although in certain cases this event may result in the prevalence of Th memory against either E2 or E6, rather than against both antigens, such immunity against the E7 antigen is only rarely installed (Table 2, Figure 2).

Table 2. Short-term proliferation assay of PBMC from healthy blood donors.

	E2													E6					E7		TT
	1-45	31-75	61-105	91-135	121-165	151-195	181-225	211-255	241-285	271-315	301-345	331-365	1-50	37-86	73-122	109-158	1-56	43-98	
D26[a]	·	75%[b] (8.7)	·	·	·	·	·	·	·	88% (5.7)	88% (11.5)	·	·	·	·	·	·	·	100% (23.9)
D27	·	88% (16.5)	100% (22.9)	·	·	·	·	·	·	·	100% (25.5)	·	75% (4.8)	75% (8.5)	75% (20.9)	·	·	·	100% (140.0)
D28	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	100% (35.0)
D29	·	·	100% (4.7)	·	·	·	·	·	·	·	88% (6.3)	·	·	·	·	·	·	·	100% (15.9)
D30	·	·	·	·	·	·	·	·	·	·	88% (13.1)	·	·	·	88% (7.5)	·	·	·	100% (125.6)
D31	·	·	·	·	·	·	·	·	·	·	·	·	·	·	75% (4.8)	·	·	·	100% (17.1)
D32	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	100% (25.7)
D33	·	88% (5.4)	100% (6.0)	75% (3.5)	·	·	·	·	·	·	100% (8.8)	75% (3.4)	·	88% (4.2)	100% (4.6)	·	·	100% (4.6)	100% (16.1)
D34	·	75% (4.8)	·	·	·	·	·	·	·	75% (4.4)	100% (8.2)	·	·	·	·	·	·	·	100% (76.1)
D35	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	100% (22.1)
D36	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	100% (56.5)
D37	·	75% (8.9)	·	·	·	·	·	·	·	75% (4.3)	·	·	·	·	100% (9.5)	·	·	·	100% (20.7)

[a] PBMC from 12 healthy blood donors (D) were tested against HPV16 E2, E6 and E7 peptide pools, consisting of 2 subsequently overlapping peptides per pool, in a short-term proliferation assay. Tetanus Toxoid (TT) at a concentration of 1 LF/ml was used as a positive control.

[b] Indicated are the percentages of positive wells and (mean stimulation index) of all wells for positive scoring peptides. Peptides were scored positive, when the proliferation of ≥ 75% of the 8 test wells exceeded the mean proliferation plus 3 × SD of the medium only control wells, and the mean stimulation index (SI) of all test wells over control wells was ≥ 3.

Discussion

Data obtained in the few studies that scrutinized HPV-specific immunity in HPV16 positive patients suggested that HPV16 E6-specific T cell immunity might play a role in the protection against progressive HPV16 infection [14, 31]. Successful control of viral infection by the cellular immune system induces T cell memory and therefore E6-specific T cells are expected to be present in those individuals who have effectively dealt with infection. Based on the cumulative 24-month incidence of infection with HPV16 of 7% [6], a substantial fraction of the healthy population is expected to harbor such T cell memory. Indeed, we could detect HPV16 E6-specific memory Th-immunity in more than half (17/32) of the healthy subjects by both IFNγ-ELISPOT and short-term proliferation assays. Isolated E6 peptide-specific Th-cells from such healthy subjects specifically reacted upon stimulation with HPV16 E6-protein showing their capacity to respond not only to peptide but also to naturally processed HPV16 E6 protein.

Because E6 protein sequences are conserved to considerable extent between HPV types, it is conceivable that infection with a given HPV type induces T cells, which are also able to react with peptides of HPV16. Amino acid sequence analysis revealed that especially the genital high-risk HPV types displayed strong homology with HPV16 E6 (data not shown). Therefore, part of the memory Th responses measured in this study might be a reflection of previous infections with high-risk HPV types other than HPV16. Furthermore, the relatively high incidence and prevalence of (sub-clinical) infection with high-risk HPV [3, 6, 28] implies that some of these responses reflect current HPV infection.

In PBMC from patients diagnosed with HPV16+ cervical carcinoma E6-specific Th type 1-immunity was detected in about 30% (3 out of 11) of the patients tested. As such, the number of E6-specific responders does not differ from that of HPV16+ patients with E7-specific Th type 1 reactivity (6 out of 16; about 40%) ([24], and unpublished observations). Based on the frequency of E6-responses in

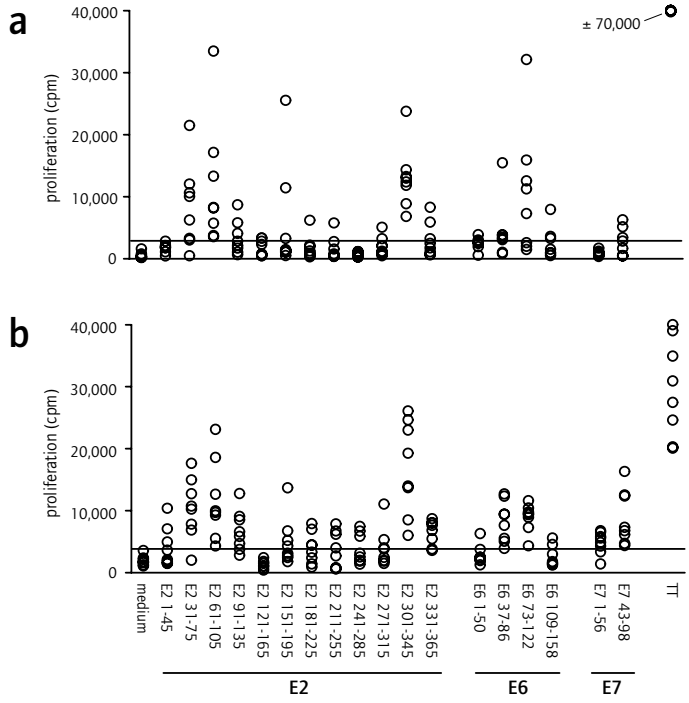

Figure 2. Proliferative Th-responses against HPV16 E2, E6 and E7 of two representative donors. Freshly isolated PBMC derived from healthy blood donors were stimulated with the indicated HPV16 peptides (10 μg/ml) for 6 days, after which proliferation was measured by [^{3}H]-thymidine incorporation. Tetanus toxoid (1 LF/ml) served as a positive control. The open circles represent proliferation in each individual microculture, and the line indicates the cut-off value defined by mean + 3 × SD of the medium control. PBMC of healthy blood donor D27 (**a**) show significant responses against both E2 and E6, and donor D33 (**b**) displays reactivity against all three antigens.

healthy individuals, the HPV16 E6 specific Th-cells in patients are likely to represent the remnants of a failing immune response, whereas Th-reactivity to E7 may reflect de novo responses following cross-priming of E7 derived from a progressive tumor. Interestingly, a direct comparison of Th responses against HPV16 E6 and E7 in healthy individuals reveals a different trend of immune activity. Only one out of 12 healthy blood donors displayed E7-specific Th-immunity, whereas 5 out of these 12 healthy subjects reacted upon stimulation with HPV16 E6 peptides. Moreover, we demonstrated that E6-specific Th responses in general coincided with responses against HPV16 E2. In addition, if one takes into account that probably not all of these anonymous healthy blood donors have encountered HPV16, whereas the patients are all HPV16+, the percentage of healthy subjects responding to HPV16 E6 after successful clearance of the HPV infection is likely to be underestimated. Failure of boosting the E6-specific Th1 type immunity may thus be related to progressive infection, which was also suggested by the work of Kadish et al. [31]. To address this, a more comprehensive study of HPV16-specific immunity in relation to disease and HPV infection status is required.

Collectively, our data suggest that the induction of Th-cell reactivity against the HPV16 E7 protein is sub-optimal when compared to that against HPV16 E2 and E6. Recently it was shown that CD4+ T cell reactivity against the plantar wart causing low-risk HPV type 1 was predominantly directed against the E4 protein (50%) and to a lesser extent against E6 (20%). Th-cell reactivity against HPV1 E7, like our results with HPV16 E7, could not be demonstrated [32]. Presently, it is not clear why

Th-reactivity is preferentially induced against the HPV16 E6 protein and not against E7. Due to the nature of HPV infection, induction of T cell immunity is exclusively dependent on cross-presentation by epithelial resident dendritic cells (DC), the Langerhans cell [33]. Possibly, the localization of the antigen in the infected cell may determine the accessibility of these antigens for Langerhans cells. Low-risk and high-risk E7 proteins are expressed in the nucleus of the cell [34] and Th responses against this antigen are hardly detected in healthy subjects [24, 32]. The expression of HPV1 E4 protein is predominantly cytoplasmic and a large fraction of healthy subjects mount an HPV1 E4-specific Th response [32]. Interestingly, the expression of low-risk HPV E6 protein is also restricted to the nucleus but E6 of high-risk HPV is expressed in the nucleus and the cytoplasm [34]. This difference in localization seems to be reflected by the low Th-response rate against HPV type 1 E6 protein and the frequent induction of HPV16 E6-specific Th-reactivity in healthy subjects. Moreover, the similar cytoplasmic and nuclear expression of HPV16 E2 induces Th-memory in healthy individuals [25, 35].

In conclusion, the high rate of spontaneous clearance of high-risk HPV infections suggests that in general, the Langerhans cell-driven antigen presentation machinery is capable of inducing an effective immune defense against HPV. Our study is the first to demonstrate the presence of HPV16 E6-specific memory Th-immunity in the healthy population and shows that HPV infection indeed leads to T cell immunity against the (immediate) early proteins E2 and E6 expressed during infection. Because the population, in which E2- and E6-specific T cell immunity frequently is found, consists of healthy subjects, the observed IFNγ-producing T cells circulating in the peripheral blood may play a role in protection against newly and/or persistent HPV infections and as such the development of cancer.

Acknowledgements · The authors are greatly indebted to all healthy blood donors and patients participating in the CIRCLE study. Also we would like to thank W. Benckhuijsen for synthesis of the peptides, G. Ranke for obtaining patient material and S. Uljee for HPV typing.

References

1. Burk RD, Kelly P, Feldman J, Bromberg J, Vermund SH, DeHovitz JA, and Landesman SH. Declining prevalence of cervicovaginal human papillomavirus infection with age is independent of other risk factors. *Sex Transm Dis,* 23: 333-341, 1996.
2. Karlsson R, Jonsson M, Edlund K, Evander M, Gustavsson A, Boden E, Rylander E, and Wadell G. Lifetime number of partners as the only independent risk factor for human papillomavirus infection: a population-based study. *Sex Transm Dis,* 22: 119-127, 1995.
3. Koutsky L. Epidemiology of genital human papillomavirus infection. *Am J Med, 102:* (5A) 3-8, 1997.
4. Duggan MA, McGregor SE, Stuart GC, Morris S, Chang-Poon V, Schepansky A, and Honore L. The natural history of CIN I lesions. *Eur J Gynaecol Oncol,* 19: 338-44, 1998.
5. Evander M, Edlund K, Gustafsson A, Jonsson M, Karlsson R, Rylander E, and Wadell G. Human papillomavirus infection is transient in young women: a population-based cohort study. *J Infect Dis,* 171: 1026-1030, 1995.
6. Ho GY, Bierman R, Beardsley L, Chang CJ, and Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med,* 338: 423-428, 1998.
7. Aiba S, Rokugo M, and Tagami H. Immunohistologic analysis of the phenomenon of spontaneous regression of numerous flat warts. *Cancer,* 58: 1246-1251, 1986.
8. Coleman N, Birley HD, Renton AM, Hanna NF, Ryait BK, Byrne M, Taylor-Robinson D, and Stanley M. A. Immunological events in regressing genital warts. *Am J Clin Pathol,* 102: 768-774, 1994.
9. Benton C, Shahidullah H, and Hunter JAA. Human papillomavirus in the immunosupressed. *Papillomavirus Rep,* 3: 23-26, 1992.
10. Serraino D, Carrieri P, Pradier C, Bidoli E, Dorrucci M, Ghetti E, Schiesari A, Zucconi R, Pezzotti P, Dellamonica P, Franceschi S, and Rezza G. Risk of invasive cervical cancer among women with, or at risk for, HIV infection. *Int J Cancer,* 82: 334-337, 1999.
11. Palefsky JM, Minkoff H, Kalish LA, Levine A, Sacks HS, Garcia P, Young M, Melnick S, Miotti P, and Burk R. Cervicovaginal human papillomavirus infection in human immunodeficiency virus-1 (HIV)-positive and high-risk HIV-negative women. *J Natl Cancer Inst,* 91: 226-236, 1999.
12. Selvakumar R, Ahmed R, and Wettstein FO. Tumor regression is associated with a specific immune response to the E2 protein of cottontail rabbit papillomavirus. *Virology,* 208: 298-302, 1995.
13. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, Verheijen RH, Stukart MJ, Meijer CJ, Scheper RJ, Stacey SN, Duggan-Keen MF, Stern PL, Man S, Borysiewicz LK, and Walboomers JM. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. *Int J Cancer,* 88: 92-98, 2000.
14. Nakagawa M, Stites DP, Patel S, Farhat S, Scott M, Hills NK, Palefsky JM, and Moscicki AB. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. *J Infect Dis,* 182: 595-598, 2000.
15. Nakagawa M, Stites DP, Farhat S, Sisler JR, Moss B, Kong F, Moscicki A, and Palefsky JM. Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia. *J Infect Dis,* 175: 927-931, 1997.
16. Ressing ME, van Driel WJ, Celis E, Sette A, Brandt RMP, Hartman M, Anholts JDH, Schreuder GMT, ter Harmsel WB, Fleuren GJ, Trimbos BJ, Kast WM, and Melief CJM. 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,* 56: 582-588, 1996.
17. Evans EM, Man S, Evans AS, and Borysiewicz LK. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. *Cancer Res,* 57: 2943-2950, 1997.
18. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, and Man S. 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,* 60: 365-371, 2000.
19. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, and Riddell SR. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T cell clones from the donor. *N Engl J Med,* 333: 1038-1044, 1995.
20. Cardin RD, Brooks JW, Sarawar SR, and Doherty PC. Progressive loss of CD8+ T cell-mediated control of a g-Herpesvirus in the absence of CD4+T cells. *J Exp Med,* 184: 863-871, 1996.
21. Heslop HE, Ng CY, Li C, Smith CA, Loftin SK, Krance RA, Brenner MK, and Rooney CM. Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med,* 2: 551-555, 1996.
22. Matloubian M, Concepcion RJ, and Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T cell responses during chronic viral infection. *J Virol,* 68: 8056-8063, 1994.
23. Melief CJM, Toes REM, Medema JP, Van der Burg SH, Ossendorp F, and Offringa R. Strategies for Immunotherapy of Cancer. *Adv Immunol,* 75: 235-281, 2000.
24. van der Burg, SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief CJ, and Offringa R. 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,* 91: 612-618, 2001.
25. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, and Offringa R. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res,* 62: 472-479, 2002.
26. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, and Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. *Am J Pathol,* 135: 703-709, 1989.
27. van der Burg SH, Kwappenberg KM, Geluk A, van der Kruk M, Pontesilli O, Hovenkamp E, Franken KL, van Meijgaarden KE, Drijfhout JW, Ottenhoff TH, Melief CJ, and Offringa R. 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,* 162: 152-160, 1999.
28. Lambropoulos AF, Agorastos T, Frangoulides E, Karahaliou R, Bontis J, and Dozi-Vassiliades I. Detection of human papillomavirus using the polymerase chain reaction and typing for HPV16 and 18 in the cervical smears of Greek women. *J Med Virol,* 43: 228-230, 1994.
29. Bermas BL, and Hill JA. Proliferative responses to recall antigens are associated with pregnancy outcome in women with a history of recurrent spontaneous abortion. *J Clin Invest,* 100: 1330-1334, 1997.
30. Fryauff DJ, Church LW, Richards AL, Widjaja H, Mouzin E, Ratiwayanto S, Hadiputranto, H, Sutamihardja MA, Richie TL, Subianto B, Tjitra E, and Hoffman SL. Lymphocyte response to tetanus toxoid among Indonesian men immunized with tetanus-diphtheria during extended chloroquine or primaquine prophylaxis. *J Infect Dis,* 176: 1644-1648, 1997.
31. Kadish AS, Ho GY, Burk RD, Wang Y, Romney SL, Ledwidge R, and Angeletti RH. Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia. *J Natl Cancer Inst,* 89: 1285-1293, 1997.
32. Steele JC, Roberts S, Rookes SM, and Gallimore PH. Detection of CD4(+)- and CD8(+)-T-Cell Responses to Human Papillomavirus Type 1 Antigens Expressed at Various Stages of the Virus Life Cycle by Using an Enzyme-Linked Immunospot Assay of γ Interferon Release. *J Virol,* 76: 6027-6036, 2002.
33. Offringa R, de Jong A, Toes REM, van der Burg SH, and Melief CJM. Interplay between human papillomaviruses and dendritic cells. *Curr Top Microbiol Immunonol,* 276, 215-240, 2002.
34. Guccione E, Massimi P, Bernat A, and Banks L. Comparative analysis of the intracellular location of the high- and low-risk human papillomavirus oncoproteins. *Virology,* 293: 20-25, 2002.
35. Maitland NJ, Conway S, Wilkinson NS, Ramsdale J, Morris JR, Sanders CM, Burns JE, Stern PL, and Wells M. Expression patterns of the human papillomavirus type 16 transcription factor E2 in low- and high-grade cervical intraepithelial neoplasia. *J Pathol*, 186: 275-280, 1998.

Annemieke de Jong, Mariëtte van Poelgeest,
Jeanette van der Hulst, Jan Wouter Drijfhout,
Gert Jan Fleuren, Cornelis Melief,
Gemma Kenter, Rienk Offringa,
Sjoerd van der Burg

Cancer Res 64:5449-5455, 2004

4

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

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 pro-inflammatory 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 Th2 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 analysing 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+ T-helper (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 have 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 histological 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 CINIII patients (n=13) ranged from 29–42 years (median 31 years) We preferred to use CINIII 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 mean-

ing of CIN II lesions in particular. In general, the majority of CINIII lesions are incipient precancers that are destined to persist [2]. PBMC and serum was 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 out 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 were 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. $E2_{1\text{-}45}$ 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, as 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 derived 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 1.5×10^5 cells/well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 150 µl of Iscove's medium (BioWhittaker) 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 micro cultures 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 $3 \times$ 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-labelled 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 50µl of this supernatant was used for cytokine analysis. The cytometric bead array was performed according to manufacturers instruction. Cut-off 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× 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 analysed in the short-term proliferation assays, minus those of 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 previously been collected 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) ELISAs were performed according to manufacturers instruction.

Statistical analysis · Statistical analysis of the HPV16-specific proliferative responses associated with cytokine production was performed using a 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 that other cytokines are also involved. We therefore analysed the cytokine profiles of the HPV16-specific T cells in a cohort of 20 young sexually active healthy women. All women had a normal

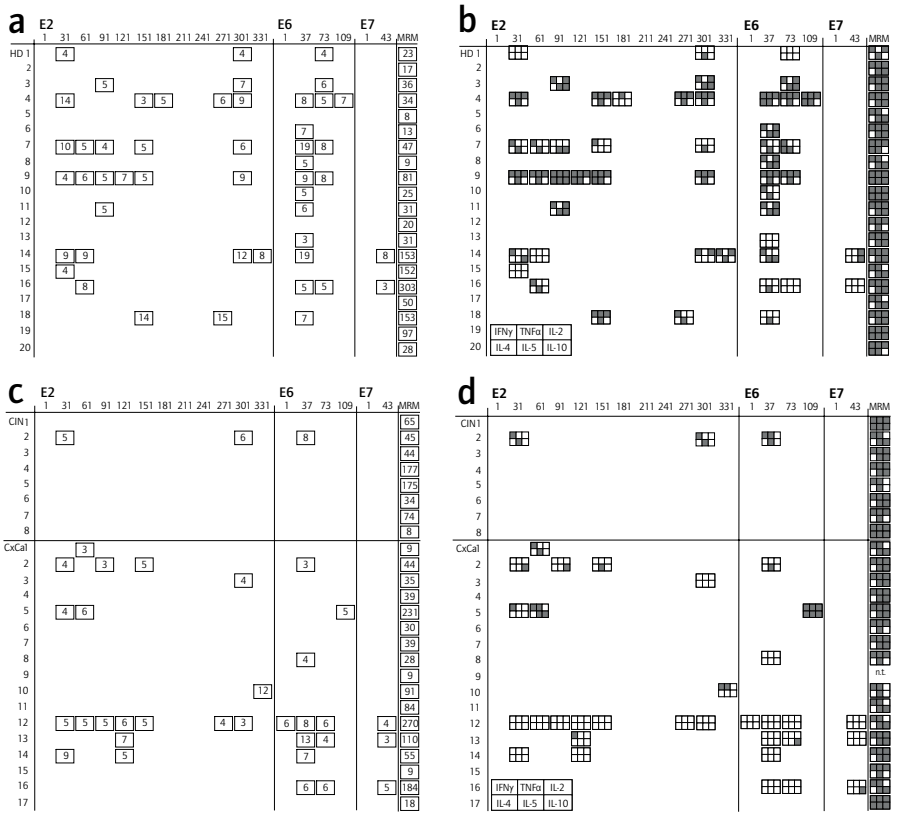

Figure 1. a. Freshly isolated PBMC from 20 healthy female individuals (HD) were tested in short-term proliferation assays using a complete set of HPV16 E2, E6 and E7-derived peptide pools (indicated by the first amino acid of the region in the protein covered by the peptides). Responses were scored positive when the proliferation (cpm) of ≥ 6 out of 8 test wells exceeded the mean proliferation + 3 × 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 analysed 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 representing antigen-specific cytokine production. Cut-off 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 × the concentration of the medium control. **c.** 8 CINIII 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

cytology and were proven HPV16 negative by PCR and VLP ELISA at the time of analysis, therefore 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 performed 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 show proliferative responses against E2 (10/20), and an even larger fraction shows responses against the E6-derived peptides (13/20). E7-specific responses were detected in only a minority of subjects (2/20) (Figure 1a). CFSE labelling 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).

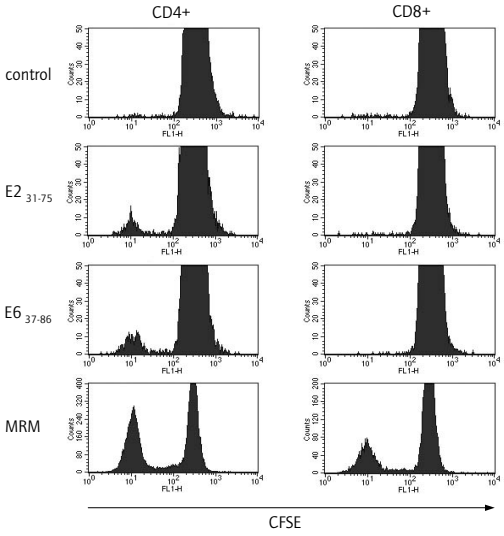

Figure 2. Detection of HPV16-specific T cell proliferation in CD4+ fraction of PBMC. Freshly isolated PBMC derived from 4 healthy subjects were labeled with CFSE, and incubated with the most immunogenic pools of peptides derived from E2 and E6 ($E2_{31\text{-}75}$ and $E6_{37\text{-}86}$ respectively) 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 flowcytometric analysis.

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 analysed 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 T-helper 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 CINIII and 17 subjects with cervical carcinoma (CxCa), were analysed for E2, E6 and E7 reactivity by short-term proliferation assay. In only 1 out of 8 CINIII 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. CxCa 9 not tested against E2), and less frequent responses against the E7 antigen (3/17). The fraction 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 pic-

ture (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 to 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: $p \leq 0.001$, 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. In healthy individuals HPV16-specific IL-10 production was accompanied by IFNγ and/or IL-5 in 11 out of 12 responses, whereas in only 2 out of 6 responses in cervical carcinoma patients ($p < 0.05$; Fisher's

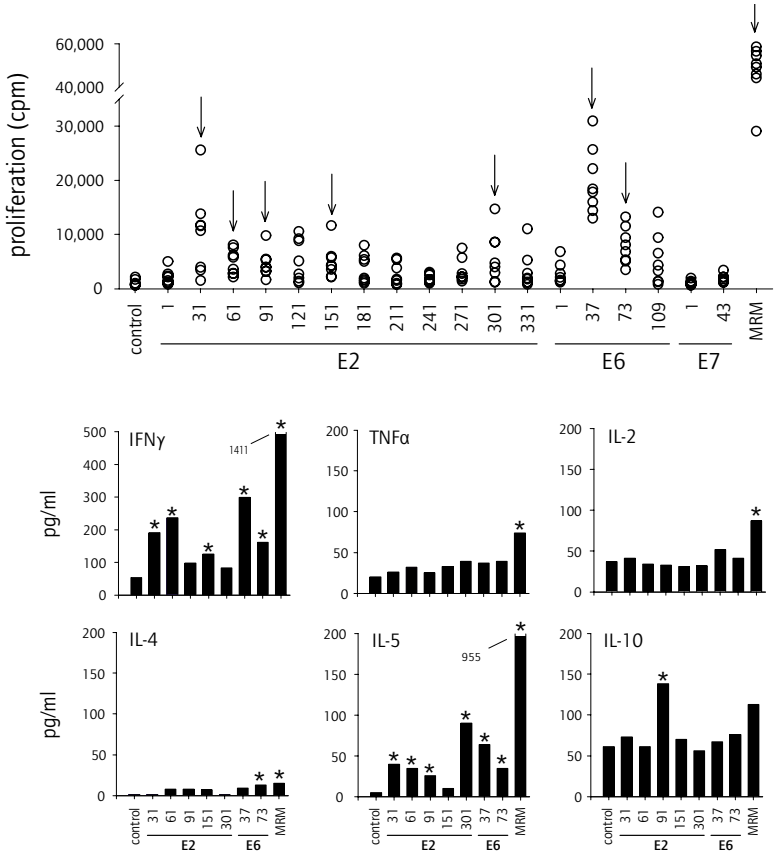

Figure 3. HPV16-specific T cell responses in healthy individuals are predominantly associated with IFNγ and IL-5. Strong proliferative Th-responses against the HPV16 E2 and E6-derived peptides were observed in healthy female-derived PBMC. One representative example (H7) displaying such proliferative responses is depicted in the upper panel. Each open circle represents the proliferation of an individual microculture; arrows indicating positive responses of which the supernatants 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 (*).

Exact Test). The low number of responses in CINIII 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 responses 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, that approximately half of the HPV16+ cervical carcinoma patients had failed to mount any detectable HPV16-specific Th-response. Thus, failure of immune defence 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.

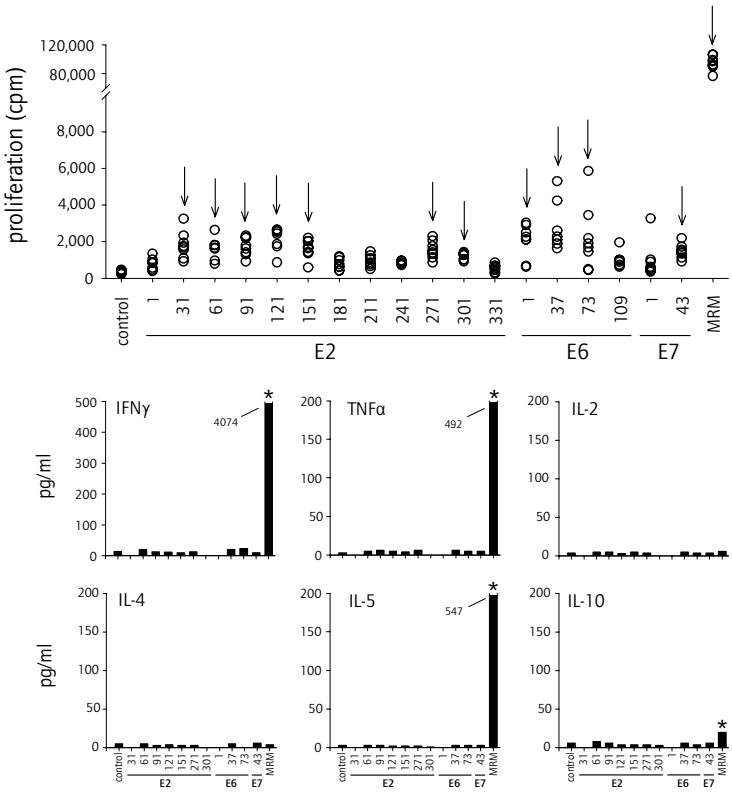

Figure 4. Absence of detectable cytokine levels, despite HPV16-specific proliferation in cervical cancer patients. Approximately half of the HPV16-positive cervical cancer patients (CxCa) revealed HPV16-specific proliferation. One representative example is shown (CxCa12, upper panel). HPV16-specific proliferative responses were generally not associated with detectable cytokine production (lower panels), where as the recall response (MRM) was associated with high levels of IFNγ, TNFα and IL-5. For explanatory notes, see figure 3.

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 towards 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, while 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 analysed 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 to IFNγ, suggesting the loss of Th1 in favour 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 to 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 as well as affected the proliferative capacity of these T cells.

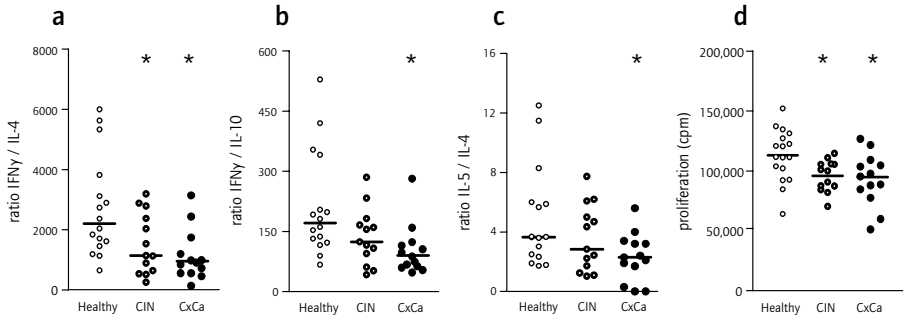

Figure 5. Patients with HPV16+ cervical disease display altered cytokine profile of peripheral T cells. Thawed PBMC derived from healthy female individuals (n=17), CINIII patients (n=13), and cervical carcinoma patients (n=13) were stimulated with PHA (0,5 µg/ml) for 48 hours after which the proliferation was measured by [^{3}H]-thymidine incorporation, and the supernatants were analysed 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 unfavourable 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 towards Th2 type. Instead, the HPV-specific T cell response in patients features an overall defect in inflammatory cytokine production.

Compared to 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 along 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 count in HIV-infected individuals are associated with multiple HPV infections, higher viral load, viral persistence and cervical dysplasia [12]. Without surgical intervention, the majority of established high-grade CIN lesions will evolve towards 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 pro-inflammatory 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 anti-tumor 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 pre-existing 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 favour of

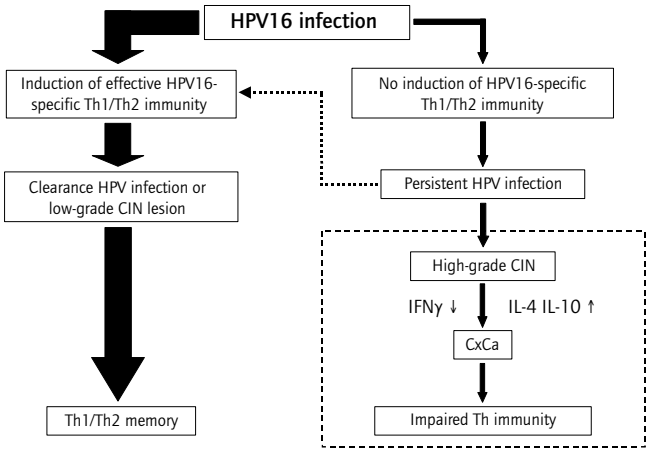

Figure 6. Proposed model for the association between HPV16-specific CD4+ T cell immunity and the development of HPV16-induced disease. Thick arrows represent the fate of the majority of HPV16 infected individuals; the thin arrows the fate of the minority. The dashed arrow indicates the option that persistently 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.

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 to 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 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 side 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 is 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 pro-inflammatory 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 (LUND University, Sweden) for reanalysis of serum samples for anti-HPV16 IgG antibodies.

References

1. Ho GY, Bierman R, Beardsley L, Chang CJ, and Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med*, 338: 423-428, 1998.
2. Schiffman M, and Kjaer SK. Chapter 2: Natural history of anogenital human papillomavirus infection and neoplasia. *J Natl Cancer Inst*, Monogr 14-19, 2003.
3. Sellors JW, Karwalajtys TL, Kaczorowski J, Mahony JB, Lytwyn A, Chong S, Sparrow J, and Lorincz A. Incidence, clearance and predictors of human papillomavirus infection in women. *CMAJ*, 168: 421-425, 2003.
4. Evander M, Edlund K, Gustafsson A, Jonsson M, Karlsson R, Rylander E, and Wadell G. Human papillomavirus infection is transient in young women: a population-based cohort study. *J Infect Dis*, 171: 1026-1030, 1995.
5. Ronco LV, Karpova AY, Vidal M, and Howley PM. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes Dev*, 12: 2061-2072, 1998.
6. Park JS, Kim EJ, Kwon HJ, Hwang ES, Namkoong SE, and Um SJ. 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*, 275: 6764-6769, 2000.
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*, 74: 4174-4182, 2000.
8. Nees M, Geoghegan JM, Hyman T, Frank S, Miller L, and Woodworth CD. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. *J Virol*, 75: 4283-4296, 2001.
9. Fausch SC, Da Silva DM, Rudolf MP, and Kast WM. Human papillomavirus virus-like particles do not activate Langerhans cells: a possible immune escape mechanism used by human papillomaviruses. *J Immunol*, 169: 3242-3249, 2002.
10. Frazer IH, Thomas R, Zhou J, Leggatt GR, Dunn L, McMillan N, Tindle RW, Filgueira L, Manders P, Barnard P, and Sharkey M. Potential strategies utilised by papillomavirus to evade host immunity. *Immunol Rev*, 168: 131-142, 1999.
11. Coleman N, Birley HD, Renton AM, Hanna NF, Ryait BK, Byrne M, Taylor-Robinson D, and Stanley MA. Immunological events in regressing genital warts. *Am J Clin Pathol*, 102: 768-774, 1994.
12. Palefsky JM, and Holly EA. Chapter 6: Immunosuppression and coinfection with HIV. *J Natl Cancer Inst* Monogr 41-46, 2003.
13. Bouwes Bavinck JN, and Berkhout RJ. HPV infections and immunosuppression. *Clin Dermatol*, 15: 427-437, 1997.
14. El-Sherif AM, Seth R, Tighe PJ, and Jenkins D. Quantitative analysis of IL-10 and IFN-γ mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. *J Pathol*, 195: 179-185, 2001.
15. Mota F, Rayment N, Chong S, Singer A, and Chain B. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. *Clin Exp Immunol*, 116: 33-40, 1999.
16. Giannini SL, Al-Saleh W, Piron H, Jacobs N, Doyen J, Boniver J, and Delvenne P. Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. *Clin Exp Immunol*, 113: 183-189, 1998.
17. Clerici M, Merola M, Ferrario E, Trabattoni D, Villa ML, Stefanon B, Venzon DJ, Shearer GM, De Palo G, and Clerici E. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. *J Natl Cancer Inst*, 89: 245-250, 1997.
18. de Gruijl TD, Bontkes HJ, Stukart MJ, Walboomers JMM, Remmink AJ, Verheijen RHM, Helmerhorst TJM, Meijer CJLM, and Scheper RJ. 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*, 77: 2183-2191, 1996.
19. Luxton JC, Rowe AJ, Cridland JC, Coletart T, Wilson P, and Shepherd PS. 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*, 77: 1585-1593, 1996.
20. Tsukui T, Hildesheim A, Schiffman MH, Lucci J 3rd, Contois D, Lawler P, Rush BB, Lorincz AT, Corrigan A, Burk RD, Qu W, Marshall MA, Mann D, Carrington M, Clerici M, Shearer GM, Carbone DP, Scott DR, Houghten RA, and Berzofsky JA. Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. *Cancer Res*, 56: 3967-3974, 1996.
21. van Der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief C J, and Offringa R. 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*, 91: 612-618, 2001.
22. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, and Offringa R. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res*, 62: 472-479, 2002.
23. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, Franken KL, Drijfhout JW, Fleuren GJ, Kenter G, Melief CJ, Offringa R, and van der Burg, SH. 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*, 63: 636-641., 2003.
24. Bosch FX, and de Sanjose S. Chapter 1: Human papillomavirus and cervical cancer-burden and assessment of causality. *J Natl Cancer Inst* Monogr 3-13, 2003.
25. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, and Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. *Am J Pathol*, 135: 703-709, 1989.
26. van der Burg S, Kwappenberg KMC, Geluk A, van der Kruk M, Pontesilli O, Hovenkamp E, Franken KLMC, van Meijgaarden KE, Drijfhout JW, Ottenhof THM, Melief CJM, and Offringa R. 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*, 162: 152-160, 1999.
27. Kirnbauer R, Hubbert NL, Wheeler CM, Becker TM, Lowy DR, and Schiller JT. 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*, 86: 494-499, 1994.
28. Sasagawa T, Rose RC, Azar KK, Sakai A, and Inoue M. Mucosal immunoglobulin-A and -G responses to oncogenic human papilloma virus capsids. *Int J Cancer*, 104: 328-335, 2003.
29. van der Burg SH, Menon AG, Redeker A, Franken KL, Drijfhout JW, Tollenaar RA, Hartgrink HH, van de Velde CJ, Kuppen PJ, Melief CJ, and Offringa R. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. *Int J Cancer*, 107: 425-433, 2003.
30. Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, and Huang SC. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol*, 167: 2972-2978, 2001.
31. Bonagura VR, Hatam L, DeVoti J, Zeng F, and Steinberg BM. Recurrent respiratory papillomatosis: altered CD8(+) T cell subsets and T(H)1/T(H)2 cytokine imbalance. *Clin Immunol*, 93: 302-311, 1999.
32. Nakagawa M, Stites DP, Farhat S, Judd A, Moscicki AB, Canchola AJ, Hilton JF, and Palefsky JM. T cell proliferative response to human papillomavirus type 16 peptides: relationship to cervical intraepithelial neoplasia. *Clin Diagn Lab Immunol*, 3: 205-210, 1996.
33. Maitland NJ, Conway S, Wilkinson NS, Ramsdale J, Morris JR, Sanders CM, Burns JE, Stern PL, and Wells M. Expression patterns of the human papillomavirus type 16 transcription factor E2 in low- and high-grade cervical intraepithelial neoplasia. *J Pathol*, 186: 275-280, 1998.
34. Badaracco G, Venuti A, Sedati A, and Marcante ML. HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. *J Med Virol*, 67: 574-582, 2002.
35. Jonuleit H, Schmitt E, Steinbrink K, and Enk A. H. Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol*, 22: 394-400, 2001.
36. Mahnke K, Schmitt E, Bonifaz L, Enk AH, and Jonuleit H. Immature, but not inactive: the tolerogenic function of immature dendritic cells. *Immunol Cell Biol*, 80: 477-483, 2002.
37. Sombroek CC, Stam AG, Masterson AJ, Lougheed SM, Schakel MJ, Meijer CJ, Pinedo HM, van den Eertwegh AJ, Scheper RJ, and de Gruijl TD. Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. *J Immunol*, 168: 4333-4343, 2002.

Annemieke de Jong, Sjoerd van der Burg,
Jeanette van der Hulst, Cornelis Melief,
Gemma Kenter, Rienk Offringa

5

IL-10 secretion characterizes HPV-specific CD4+ T cell repertoire in cervical tumor and tumor-draining lymph nodes

Abstract · The development of HPV16-positive cervical cancer is associated with failure to mount an effective immune response against the viral antigens. To gain insight in the local immune response against HPV16, we performed a detailed analysis of the tumor draining lymph node of a patient with HPV16-positive cervical cancer. Lymph node-derived HPV16E6-specific CD4+ T cell clones generally lacked a clear Th1 or Th2 cytokine profile and predominantly secreted both IL-10 and IFNγ. Interestingly, when the E6 antigen was presented suboptimally, most clones secreted IL-10 but not IFNγ. The secretion of IFNγ occurred only under optimal conditions involving high concentrations of peptide presented by mature dendritic cells (DC). By contrast, influenza M1-specific CD4+ T cells derived from the same tumor-draining lymph node showed predominant secretion of IFNγ, irrespective of the type of antigen presenting cell (APC) and peptide concentration. This difference in HPV16E6 – and influenza M1-specific T cell cytokine profile suggests an impaired HPV-specific T cell response in this patient. The optimal antigen presenting conditions required to induce HPV16-specific IFNγ secretion are not likely to be encountered in the vicinity of the tumor. Therefore, IL-10 secretion is expected to dominate the HPV16-specific CD4+ T cell response in vivo. The fact that HPV-specific IL-10-secreting CD4+ T cells were also present in tumor infiltrating lymphocyte populations of the majority of cervical cancer patients, suggests these T cells may be a common feature of HPV-induced disease. HPV-specific IL-10 secretion may contribute to the lack of an effective anti-tumor response at the level of T cell priming in the draining lymph node, and may hamper effector function at the site of the tumor.

Introduction

Papillomaviruses harbor several mechanisms to avoid innate immune triggering [1], which explains the lagtime that is generally observed between HPV infection and clearance [2]. However, in the case of persistent HPV infection and HPV-induced malignancies, it is highly unlikely that the failure to mount an effective HPV-specific immune response is merely a result of ignorance caused by the avoidance of immune triggering. In line with this, we detected proliferative responses against the HPV16E6 oncoprotein in peripheral blood mononuclear cells (PBMC) of approximately half of HPV16-positive cervical cancer patients [3]. However, the supernatants of these cell cultures generally lacked detectable levels of Th1 and Th2 cytokines, while IL-10 was occasionally present. In PBMC of the other half of patients detectable HPV16-specific responses were lacking altogether. Thus, an improperly polarized or even actively suppressed HPV-specific immune response offers a more plausible explanation for the apparent immune failure in HPV-induced disease.

HPV-specific T cells are likely primed in lymph nodes draining the cervix, by migrating Langerhans cells (LC) that have taken up antigen in the HPV-positive lesion. The non-inflammatory environment of HPV-positive lesions, with a local increase in IL-10 levels [4], may adversely affect the capacity of LC to induce a potent anti-tumor immune response. IL-10 is implicated both in the modulation of APC function, and in the subsequent induction of regulatory T cells [5, 6]. Consequently, the action of this cytokine may explain the impaired HPV16-specific T cell response detected in the peripheral blood of cervical cancer patients. To gain more insight in the characteristics of the HPV-specific T cell responses in cervical cancer patients at the site of T cell priming, we embarked on the detailed analysis of HPV16 oncoprotein-specific T cells present in tumor-draining lymph nodes. We found these draining lymph nodes to harbor E6-specific CD4+ T cells that secrete IL-10 upon triggering with antigen. Similarly, HPV-specific IL-10-secreting T cells were detected in populations of tumor infiltrating lymphocytes (TIL). These observations offer a possible explanation for the functional deficiency of the HPV-specific T cell response in patients with HPV-positive cervical disease.

Materials and Methods

Lymph nodes, tumors and blood samples · The analyses of cervical tumors and tumor-draining lymph nodes were nested in the "CIRCLE" study that investigates cellular immunity against HPV in patients with HPV-positive (pre)malignant lesions. The study design was approved by the Medical Ethical Committee of the LUMC. Blood was drawn immediately prior to surgery. Tumor and lymph node material described in this study was obtained after informed consent from patients with cervical carcinoma (FIGO stage 1B/IIA, IIB) during radical hysterectomy. From the same patients PBMC and sera were obtained from blood that was drawn prior to surgery. Typing for HPV16 was performed using HPV16-specific primers on DNA isolated from surgical resection specimens [7]. The lymph nodes were derived from the pelvic region and contained tumor cells, indicative of metastatic cancer. To isolate the lymph node mononuclear cells (LNMC) from the tissue, the lymph node material was cut into pieces of approximately 5mm^3 and incubated at 37°C for one hour after addition of collagenase IV 200 IU/ml (Sigma) and DNAse 50 µg/ml (Sigma). A single cell suspension was obtained using a cell strainer (BD Falcon), and dead cells and erythrocytes were removed by ficoll density gradient. Tumor infiltrating lymphocytes (TIL) were obtained by culturing minced tumor tissue in IMDM (Cambrex) supplemented with 10% AB serum (Sigma), 1% T cell growth factor (TCGF, Zeptometrix, USA) and 5 ng/ml IL-15 (PeproTech, USA) for 2-3 weeks.

Antigens · For the short-term proliferation assays of lymph node- and peripheral blood-derived mononuclear cells, a set of peptides spanning HPV16 E2, E6, and E7 proteins was used. The HPV16E2

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 8 HPV16E6 peptides were 32 amino acids long and the 4 E7 peptides had a length of 35 amino acids, all with an overlap of 14 amino acid residues. In the short-term proliferation assays pools of 2 peptides per pool were used, indicated by the first and last amino acid of the region in the protein covered by the peptides. For the generation of influenza M1-specific T cell clones, 16 30-mer peptides with an overlap of 15 amino acids were used, spanning the influenza matrix 1 protein of A/PR/8/34. For the analysis of the tumor infiltrating lymphocyte cultures, an additional set of HPV18 E6 and E7 overlapping peptides was used. Peptides were synthesized by solid phase technique on an automated multiple peptide synthesizer (Abimed AMS 422, Langenfeld, Germany), and analyzed by reverse phase HPLC. The lyophilized peptides were dissolved in 50µl of DMSO, diluted in PBS to a final concentration of 2.5 mg/ml. Memory Response Mix (MRM), consisting of a mixture of tetanus toxoid (0.75 LF/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 to confirm the capacity of PBMC and LNMC to proliferate and produce cytokine in response to common recall antigens. Recombinant HPV16 E6 protein and HPV16 E7 protein were produced in recombinant E. coli transformed with pET-19b-HPV16 E6 and pET-19b-HPV16 E7 as described previously [8].

Short-term proliferation assays · Freshly isolated PBMC respectively LNMC were seeded at a density of 1.5×10^5 cells/well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 150 µl of IMDM (Cambrex) supplemented with 10% autologous serum. HPV16 E2-, E6- and E7-derived peptide pools were added at a concentration of 10 µg/ml/peptide (2 peptides per pool). 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 micro cultures were incubated. 50 µl of supernatant from the microcultures was taken at day 5 after incubation and stored at −20°C until cytokine analysis. Antigen-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 $3 \times$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.

Generation of HPV16E6- and influenza M1-specific T cell clones from LNMC · 5×10^6 LNMC were seeded in 25 cm^2 culture flasks (Nalge Nunc, USA) in 3 ml IMDM supplemented with 10% heat-inactivated AB serum (Sigma), 1% TCGF (Zeptometrix, USA) and 5 ng/ml IL-15 (PeproTech, USA). HPV16E6 or influenza M1 peptides were added at a concentration of 2.5 µg/ml. The specificity of the T cell cultures was tested at day 10, after which the cell culture was cloned by limiting dilution. T cell cloning was performed according to a protocol adapted from Evans et al. [9], replacing IL-2 for 5% TCGF and 2 µg/ml IL-15, and adding 0.2 µg/ml phytohemagglutinin (Murex Diagnostics, UK) for TCR triggering.

Proliferation assays for polyclonal lymph node T cell cultures, T cell clones and TIL cultures · Tumor infiltrating lymphocytes (TIL) and polyclonal lymph node T cell cultures were tested for the recognition of peptide- (5 µg/ml) and protein (10 µg/ml)-pulsed autologous monocytes in a 3-day proliferation assay. Autologous monocytes were obtained from PBMC by adherence as described previously [10]. Briefly, 2 days before performing the proliferation assay, 50 µl of PBMC suspension (4×10^6/ml in X-vivo15 medium, Cambrex) was seeded in a 96-wells plate. After 2 hours of incubation at 37°C the non-adherent cells were washed away and the adherent cells were cultured in X-vivo15 medium containing GM-CSF (500 IU/ml). The T cell cultures (25,000 or 50,000/well) were tested in triplicate. At 48 hours, supernatants of the microcultures were harvested and stored at −20°C until cytokine analysis. Antigen-specific proliferation was measured by [^{3}H]-thymidine incorporation. The initial screening of the T cell clones for specificity was performed by a 3-day proliferation assay; autologous irradiated peptide-pulsed (pool of E6 respectively M1 peptides, 5 µg/ml/peptide) PBMC

were used as APC (10,000/well). In this initial screening the clones were not harvested and counted, but the wells were split and an equal amounts of clone were seeded in the wells for analysis in duplicate. 48 hours after incubation, supernatant was harvested en stored at −20°C until cytokine analysis. In detail analysis of the cytokine profiles of T cell clones was performed with irradiated HLA-matched LPS-matured dendritic cells as APC pulsed overnight with increasing peptide concentrations (0, 0.1, 1, 10, 100 µg/ml). Supernatant was harvested 6, 18, and 48 hours after incubation, and stored at −20°C until cytokine analysis.

Cytokine analysis of supernatants · IFNγ was measured by ELISA as described previously [11]. IL-4 and IL-10 (CLB, Amsterdam) and IL-5 (Pharmingen) ELISAs were performed according to manufacturers instruction. Cytometric Bead Array (Becton Dickinson) was used for the cytokine analysis of the supernatants of the short-term proliferation assays and analysis of the polyclonal lymph node cultures. Supernatants from the individual microcultures were pooled per peptide pool and 50 µl of this supernatant was used for cytokine analysis. The cytometric bead array was performed according to manufacturers instruction. Cut-off 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× the concentration of the medium control [12].

Analysis of IFNγ and IL-10 by cytokine secretion assay · For the single-cell analysis of the T cell clones for the secretion of IL-10 and IFNγ the cytokine secretion assay (Miltenyi biotech, Bergisch Gladbach, Germany) was used, with FITC-labeled anti-IFNγ and PE-labeled anti-IL-10. For the assays two different APC – autologous EBV-transformed B-cells (B-LCL) and HLA-matched LPS-matured dendritic cells (DC) – were used at a 1:5 ratio (APC:T cell). APC (5×10^4) were incubated overnight with increasing concentrations of peptide, after which the APC were washed and T cell clone (2.5×10^5) was added. For the E6-specific T cell clones, the cognate epitopes were determined, and a single peptide was used for these assays. The use of a pool of peptides versus a single peptide did not show different results in cytokine profiles, and for the M1-specific T cells clones a pool of overlapping M1 peptides was used. The optimal timepoint for the cytokine analysis was 3-4 hrs after stimulation, as determined by a cytokine kinetics experiment. After the incubation of the T cell clone with the peptide-pulsed APC, the cytokine secretion assay was performed according to manufacturers instruction. Cells were counterstained with anti-CD4 APC (Becton Dickinson 1:50), and propidium iodide (40 µg/ml) was added prior to flowcytometric analysis. After detection of HPV reactivity in TIL cultures without prior stimulation with HPV antigen, these cultures were stimulated once (with autologous irradiated PBMC pulsed with 10 µg/ml cognate pool peptide, 5% TCGF and 5 ng/ml IL-15), and analyzed for cytokine profiles 14 days later. Monocytes cultured for 2 days with GM-CSF (500 IU/ml) were used as APC. The IL-10/IFNγ secretion in response to stimulation with peptide-pulsed (10 µg/ml) GM-CSF monocytes was measured by cytokine secretion assay according to manufacturers' instruction. Cells were counterstained with anti-CD4 APC (BD 1:50), and propidium iodide (40 µg/ml) was added prior to flowcytometric analysis.

Results

Analysis of HPV16E6-specific CD4+ T cells derived from a tumor-draining lymph node

Patients with HPV16-positive cervical tumors often lack detectable proliferative T cell responses in the peripheral blood against HPV16 early antigens [3]. The underlying reason for this apparent absence of HPV16-specific proliferation is not known, and can theoretically vary from a low frequency of HPV16-specific T cells in the peripheral blood, to active in vitro suppression of the HPV16-specific T cell response. In the present study we had the opportunity to perform a detailed analysis of the HPV16-specific T cell response, not only in the peripheral T cell compartment, but also in the tumor-

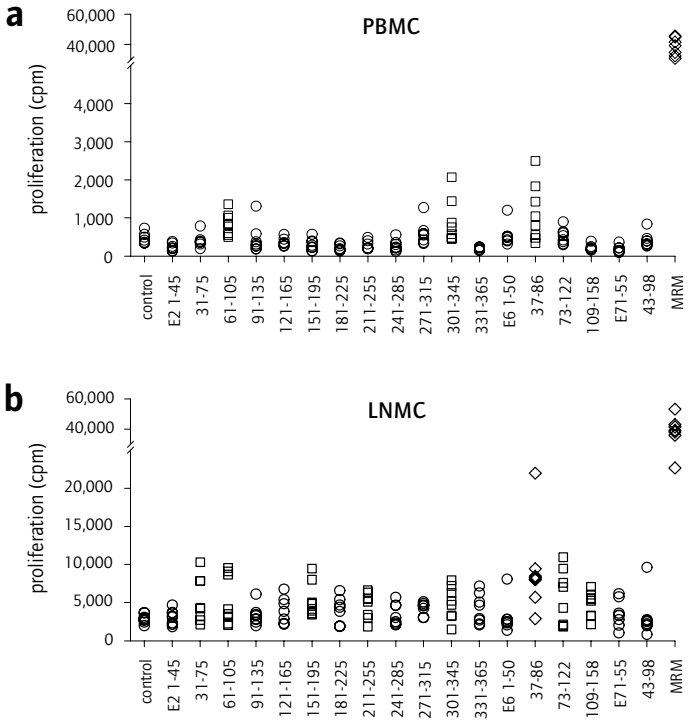

Figure 1. HPV16-specific proliferative responses in PBMC and LNMC of an HPV16+ cervical carcinoma patient. Freshly isolated mononuclear cells derived from (**a**) peripheral blood (PBMC) and (**b**) lymph node (LNMC) were stimulated with pools of HPV16 E2, E6, and E7 peptides (10 μg/ml) for 6 days after which proliferation was measured by [^{3}H]-thymidine incorporation. The peptide pools, consisting of 2 peptides per pool, are indicated by the first and last amino acid of the region of the protein covered by the peptides. MRM, consisting of a mix of common recall antigens was used as a positive control. The open symbols represent proliferation of the individual microcultures. Squares represent responses with SI > 1.5, and diamonds represent responses with a SI ≥ 3, and at least 6 out of 8 microcultures with a proliferation higher than mean + 3 × SD of medium control (defined as memory response).

draining lymph node, which is the site where HPV16-specific T cells encounter their cognate antigen. We obtained lymph node material from a patient diagnosed with FIGO IIB HPV16+ cervical cancer, who showed modest proliferative responses in the peripheral blood against HPV16 $E2_{61\text{-}105}$, $E2_{301\text{-}345}$, and $E6_{37\text{-}86}$ (Figure 1a, stimulation index >1.5). None of these responses in PBMC exceeded the cut-off level for the detection of memory T cell responses, which was defined in our previous studies as ≥ 75% of the test wells above mean plus 3 × SD of the medium control, and a stimulation index ≥ 3 [13]. Therefore, this patient fell into the category of HPV16-positive subjects that lack significant HPV16-specific memory T cell responses in the peripheral blood [3]. Mononuclear cells derived from the tumor-draining lymph node (LNMC) analyzed in a short-term proliferation assay showed a similar pattern as seen in the PBMC, in that responses were observed against HPV16 $E2_{61\text{-}105}$, $E2_{301\text{-}345}$, and $E6_{37\text{-}86}$. Immunity detected in LNMC appeared broader than that in PBMC, as it also covered peptides outside the aforementioned regions in E2 and E6 (Figure 1b). According to the defined criteria for memory T cell responses (see above), the LNMC showed a significant response against $E6_{37\text{-}86}$ (Figure 1b). In correspondence with earlier analyses of HPV16-specific responses in cervical cancer patients [3], the proliferative responses in both PBMC and LNMC were not associated with detectable levels of cytokine secretion in the culture supernatants (data not shown).

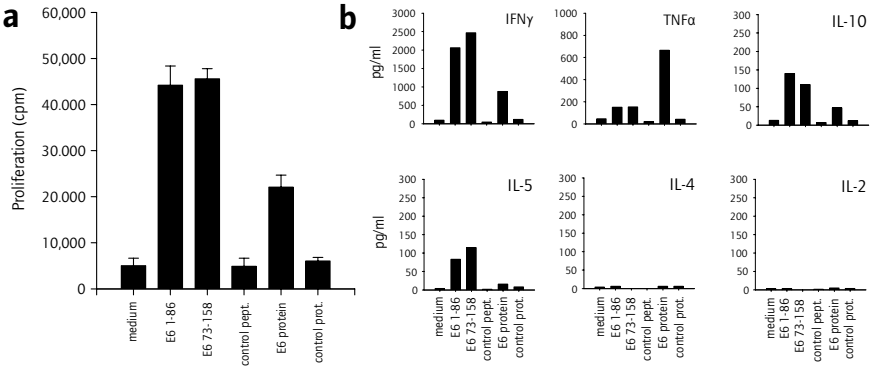

Figure 2. Strong E6-specific proliferative responses in cultured LNMC. LNMC were cultured with TCGF, IL-15 and an overlapping set of 32-mer peptides spanning the entire HPV16E6 protein (2.5 µg/ml). At day 10, the cultured LNMC were tested in a 3-day proliferation assay and showed strong recognition of both E6 peptide- and protein-pulsed autologous monocytes, as measured by [^{3}H]-thymidine incorporation (**a**). The supernatants of the proliferation assay, harvested at 48 hours, were analyzed for the presence of cytokines by cytometric bead array. The positive proliferative responses were associated with the secretion of IFNγ, TNFα, IL-10, and IL-5 (**b**).

For detailed analysis of the HPV16-specific response in LNMC of this patient, we focused on the E6 oncoprotein, because the strongest proliferation was observed against E6-derived peptides in the short-term proliferation assay. After 10 days of culture with overlapping E6 peptides, strong HPV16E6-specific responses were observed in the LNMC (Figure 2a). The LNMC proliferated in the presence of both E6 peptide- and protein-pulsed autologous monocytes, indicating the recognition of naturally processed E6 antigen. This HPV16E6-specific proliferation was associated with the secretion of a diversity of cytokines, specifically IFNγ, TNFα, IL-10 and IL-5 (Figure 2b), thus lacking a clear polarization towards either Th1 or Th2 cytokines. To gain insight in the cytokine profiles of individual E6-specific T cells generating this array of cytokines, T cell clones were derived from the LNMC culture by limiting dilution. The resultant T cell clones, which all displayed a CD4+ phenotype, were tested in a proliferation assay, and supernatants were analyzed for the presence of IL-10 and IFNγ. We primarily focused on these two cytokines, because they represent the two outer ends in the spectrum of pro- and anti-inflammatory cytokines.

An initial screening of the T cell clones was performed using irradiated autologous PBMC pulsed with saturating amounts of E6 peptide antigen, and unpulsed PBMC as a negative control. Two out of 13 E6-specific T cell clones predominantly secreted IFNγ with (almost) no IL-10, indicative of a Th1 type cytokine profile (Figure 3, cl.1 and cl.8). The remaining T cell clones all secreted considerable amounts of IL-10, accompanied by variable levels of IFNγ. Interestingly, one of the T cell clones lacked antigen-specific proliferation and IFNγ, but did secrete IL-10 in response to E6 stimulation (Figure 3, cl.3). The characteristics of the T cell clones that secreted both IL-10 and IFNγ in response to E6 stimulation, suggest that these T cells may represent a T cell subset similar to Tr1 cells, which have been found in connection to chronic infections and cancer, and display immunoregulatory functions.

Ratio of IL-10 and IFNγ secreted by HPV16E6-specific CD4+ T cells critically depends on APC type and HLA-peptide density on APC surface

Further analysis of the E6-specific T cell clones revealed that – upon optimal antigen-specific stimulation with peptide-pulsed LPS-matured dendritic cells – the majority of clones secreted low levels of IL-4 and IL-5 in addition to substantial amounts of IL-10 and IFNγ (Figure 4). CD4+ T cells with similar cytokine profiles and with regulatory properties have recently been reported in melanoma patients [14]. Cytokine analysis of supernatants derived from the peptide titration experiments

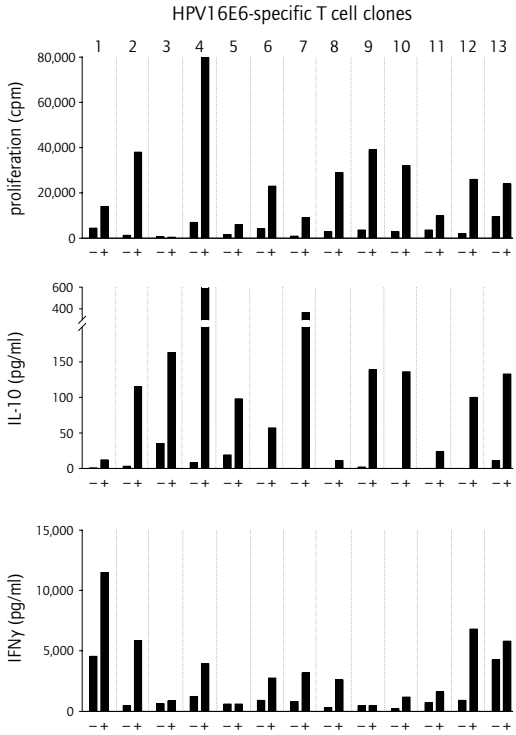

Figure 3. HPV16E6-specific T cell clones derived from tumor-draining lymph node generally secrete IL-10 and IFNγ. In an initial screening, the T cell clones derived from the HPV16E6-specific lymph node culture were tested for the recognition of E6 peptide-pulsed irradiated autologous PBMC (+). Non-pulsed PBMC were taken along as a negative control (−). Supernatants harvested at 48 hours were analyzed for the presence of IL-10 and IFNγ by ELISA, and the proliferation was measured by [^{3}H]-thymidine incorporation. The results from the T cell clones showing E6 recognition are depicted.

revealed that the peak levels of IL-10 secretion were reached at at least a 100-fold lower peptide concentration than that for IFNγ (Figure 4), and a 10-fold lower than for IL-4 and IL-5. This suggests that low antigen density on the APC (weak TCR triggering) results predominantly in IL-10 secretion, whereas a higher antigen density favors the secretion of IFNγ, IL-4 and IL-5. We decided to investigate this feature at the level of cytokine secretion by single cell analysis.

Two different types of APC were used, (1) HLA-matched LPS-matured dendritic cells (DC), which have optimal antigen presenting capacity, and (2) EBV-transformed autologous B-cells (B-LCL), which – compared to DC – are considered to be suboptimal APC [15]. These APC, pulsed with increasing concentrations of 32-mer peptides, were used as stimulator cells for the E6-specific T cell clones, after which the secretion of IL-10 and IFNγ was determined using the cytokine secretion assay. Eight out of 13 CD4+ T cell clones were available for analysis; the remaining 5 clones could not be maintained in culture. Within the 8 E6-specific CD4+ T cell clones analyzed, 3 distinct cytokine profiles were revealed. The first profile is represented by clones 3 and 13. When stimulated with peptide-pulsed B-LCL, these clones only produced IL-10 and almost no IFNγ, even at high peptide concentrations. Similarly, stimulation with peptide-pulsed DC (peptide concentration up to 1 μg/ml), triggered IL-10 secretion only. Interestingly, these T cells started to produce IFNγ in addition to IL-10 when stimulated with DC pulsed with higher concentrations (1-10 μg/ml) of peptide (Figure 5a). A second distinct cytokine profile was represented by clones 4, 7, 10, 11 and 12, in that these T cell clones displayed a shift from the secretion of only IL-10 towards secretion of IL-10 and IFNγ when incubated

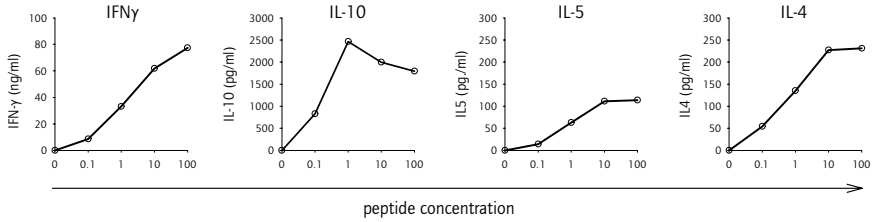

Figure 4. HPV16E6-specific T cell clones secrete low levels of IL-4 and IL-5 in addition to IL-10 and IFNγ. HLA-class II matched dendritic cells were pulsed with increasing E6 peptide concentrations and used – after LPS-induced maturation (24 hrs) – as APC in a 3-day proliferation assay. The HPV16E6-specific T cell clones were added and supernatants were harvested 6, 18, and 48 hours after stimulation. The supernatants were analyzed for the presence of IFNγ, IL-10, IL-5, and IL-4 by ELISA. The cytokine profile of clone 4 is depicted, which was representative of the profiles observed for most other E6-specific T cell clones (tested for clones 1, 2, 3, 4, 12, 13). Cytokine kinetics revealed that the highest concentration of IFNγ, IL-10 and IL-5 could be measured at 48 hours, whereas the highest concentration of IL-4 at 18 hours. The cytokine concentrations are depicted as measured at the optimal timepoint for each individual cytokine.

with B-LCL that were pulsed with increasing concentration of peptides (Figure 5b). When incubated with mature DC these T cell clones produced both IFNγ and IL-10 at low (0.01-0.1 μg/ml) peptide concentrations, with the majority of cells secreting IFNγ only. A third cytokine profile was found for clone 6; stimulation of this T cell clone with either B-LCL or DC only triggered secretion of IFNγ, almost no IL-10 (Figure 5c). Notably, high amounts of peptide (1-10 μg/ml) were required to induce the IFNγ production in this T cell clone. Even though these 3 cytokine profiles exhibit a different balance between IL-10 and IFNγ, they have one thing in common: IFNγ secretion is only observed at the most optimal stimulatory conditions, either involving B-LCL loaded with high (10 μg/ml) peptide concentration and/or mature DC (see summary in Table 1).

The highest peptide concentrations used for APC pulsing in the titration experiments will most likely result in a higher T cell epitope density on the APC surface than expected in vivo in the tumor-draining lymph node. Furthermore, the costimulatory context offered by mature DC is not likely to be found in tumor-draining LN. This suggests that IL-10 will be predominant in the overall in vivo response to tumor-derived E6 antigen in the context of the tumor-draining lymph node.

Table 1. HPV16E6-specific CD4+ T cell clones.

			B-LCL		DC		
Clone	E6	HLA	IL-10	IFNγ	IL-10	IFNγ	Profile
3	1-32[a]	DP	0.1[b]	10	0.01	1	I
13	55-86	DR	0.1	10	0.1	1	
4	73-104	DP	0.1	1	0.01	0.01	II
7	91-122	DR	n.t.	n.t.	1	1	
10	55-86	n.t.	1	10	1	1	
11	n.t.	n.t.	1	10	0.1	0.1	
12	19-50	DR	1	10	0.1	0.1	
6	55-86	n.t	10	1	10	1	III

[a] Indicated is the 32-mer E6 peptide that is recognized by the corresponding T cell clone and its HLA class II restriction.

[b] The concentrations of E6 peptide pulsed on the APC are expressed in μg/ml (32-amino acid long peptides). Indicated is the lowest peptide concentration at which the T cells clone secreted the corresponding cytokine. The cut off level for cytokine secretion was set at >2× background and ≥2%. n.t. = not tested.

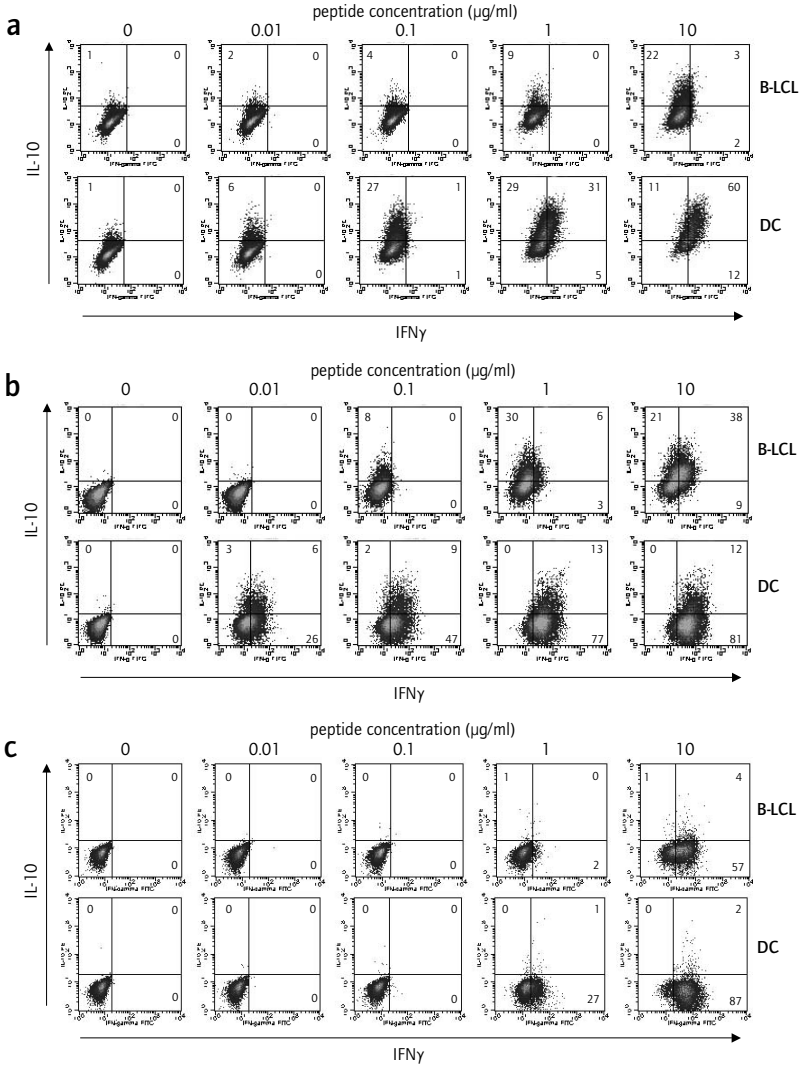

Figure 5. Single cell analysis of the HPV16E6-specific T cell clones at the level of cytokine secretion. Eight HPV16E6-specific T cell clones were analyzed for the secretion of IFNγ and IL-10 in response to autologous B-LCL and HLA-matched LPS-matured (24 h) dendritic cells pulsed with increasing peptide concentrations, using the cytokine secretion assay. This analysis revealed 3 distinct cytokine secretion patterns, depicted in **a** (clone 3), **b** (clone 4) and **c** (clone 6). The percentages of cytokine-secreting T cells are indicated in the corners of the dotplots. The cells are gated for live CD4+ T cells.

Lymph node-derived influenza M1-specific CD4+ T cell clones display predominant secretion of IFNγ

In view of our findings, we investigated whether the observed cytokine secretion profiles are a typical feature of the HPV-specific T cell response in this patient, or a more common characteristic of T cells residing in the lymph nodes, or even just a result of our in vitro culture approach. Therefore, we studied the T cell response against the influenza M1 antigen, which is commonly characterized by systemic Th1 memory in both patients and healthy individuals [16-18]. By the same in vitro culture method we obtained an influenza M1-specific polyclonal T cell culture and subsequent limiting dilu-

Table 2. Influenza M1-specific CD4+ T cell clones.

Clone	B-LCL IL-10	B-LCL IFNγ	DC IL-10	DC IFNγ	Profile
17	0.01 [a]	0.001	0.01	0.001	1
14	·	0.01	·	0.01	2
15	·	0.1	n.t.	n.t	
16	·	1	n.t.	n.t.	
18	·	1	n.t.	n.t.	

[a] The concentrations of M1 peptide pulsed on the APC are expressed in µg/ml (30-amino acid long peptides). Indicated is the lowest peptide concentration at which the T cells clone secreted the corresponding cytokine. The cut off level for cytokine secretion was set at >2× background and ≥2%. n.t. = not tested and a dot indicates non-detectable cytokine secretion.

tion of this culture resulted in a total of 5 M1-specific CD4+ T cell clones. In the initial screening by ELISA, we found that in response to stimulation with irradiated PBMC and M1 peptides, all clones secreted IFNγ (Figure 6). Furthermore, in the supernatants of 3 out of 5 M1-specific T cell clones (clones 14, 15, 17) IL-10 was detected. This IL-10 secretion in combination with IFNγ appeared similar to the cytokine profiles of the E6-specific T cell clones. However, analysis at the single cell level – using peptide-pulsed mature HLA-matched dendritic cells (DC) and autologous EBV transformed B-cells (B-LCL) as APC – revealed a different picture. Two distinct cytokine profiles were observed. The first, represented by clone 17, displayed the predominant secretion of IFNγ in response

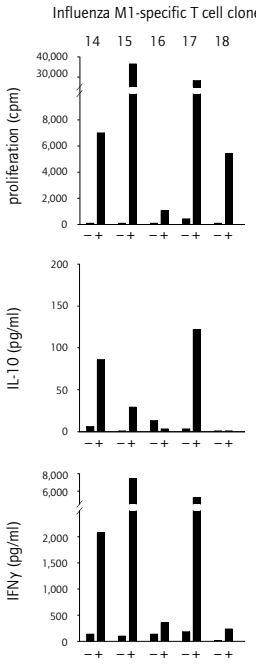

Figure 6. Influenza M1-specific T cell clones derived from tumor-draining lymph node also secrete IL-10. In an initial screening, the T cell clones derived from the influenza M1-specific lymph node culture were tested for the recognition of M1 peptide-pulsed irradiated HLA class II matched PBMC (+). Unpulsed PBMC were taken along as a negative control (-). The supernatants harvested at 48 hours were analyzed for the presence of IL-10 and IFNγ by ELISA. Only the results from the T cell clones showing M1 recognition are depicted.

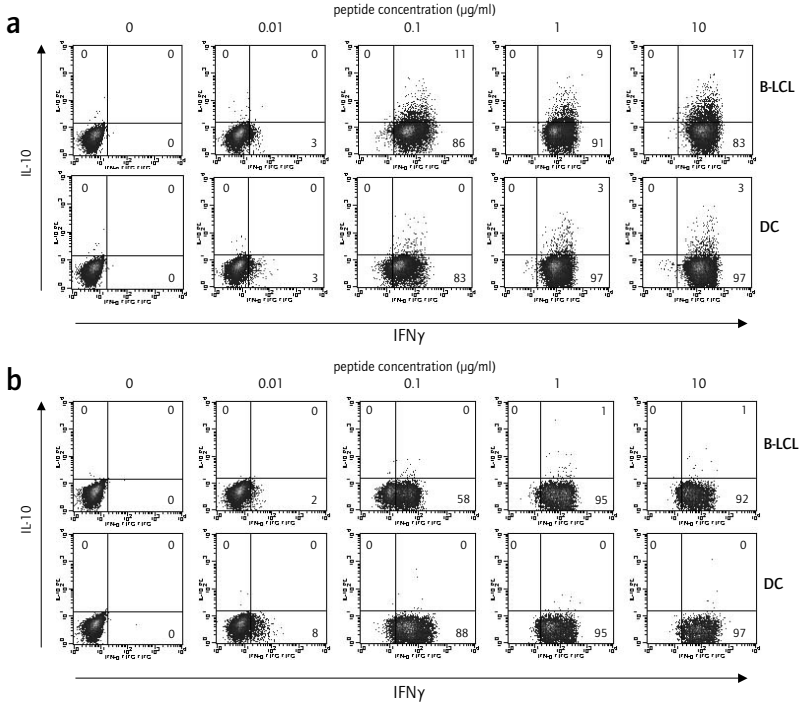

Figure 7. Single cell analysis of the Influenza M1-specific T cell clones at the level of cytokine secretion. Five influenza M1-specific T cell clones were analyzed for the secretion of IFNγ and IL-10 in response to autologous B-LCL and HLA-matched mature dendritic cells pulsed with increasing peptide concentrations, using the cytokine secretion assay. This analysis revealed 2 distinct cytokine secretion patterns, depicted in A (clone 17) and B (clone 14). The percentages of cytokine-secreting T cells are indicated in the corners of the dotplots. The cells are gated for live CD4+ T cells.

to both DC and B-LCL. IL-10 secretion was induced upon stimulation with APC pulsed with >0.01 µg/ml of peptide in a significant percentage of T cells, and was most pronounced when B-LCL were used as APC. In contrast to the IL-10-secreting E6-specific T cell clones, this M1-specific clone initiated the secretion of IFNγ at low peptide concentration (0.001 µg/ml), while IL-10 secretion was induced only at higher peptide concentrations. The second cytokine profile, represented by the remaining clones, was characterized by the secretion of IFNγ only, irrespective of the type of APC and peptide-density on the APC surface (Figure 7b); a maximum of 1% of the T cells (clones 14 and 15 upon stimulation with B-LCL) displayed the secretion of both IFNγ and IL-10. The discrepancy between this low percentage of IL-10-secreting cells and the detection of IL-10 in the culture supernatants of the stimulated cells (Figure 6), may be explained by the fact that IL-10 concentration in the culture supernatant is related to its accumulation (determined by production and consumption) in 48 hours, whereas the flowcytometric analysis measures the production of each individual cell within 3 hours, which is apparently low for IL-10 in these clones. Taken together, the cytokine profiles of the M1-specific T cell clones revealed a predominance of IFNγ and only limited IL-10 secretion at the single cell level. An overview is presented in Table 2. Three M1-specific T cell clones were tested using only B-LCL as APC. In previous experiments we consistently observed that upon stimulation with B-LCL, T cell clones are more prone to secrete IL-10 as compared to DC. Therefore, the fact that for these three clones only IFNγ was detected in response to peptide-pulsed B-LCL, makes it highly unlikely that they will secrete significant amounts of IL-10 in response to peptide-pulsed DC.

Despite the small numbers of M1 and E6-specific T cell clones analyzed, the presented data suggest a qualitative difference in the T cell response against these viral antigens in the lymph node of this patient. Whereas the E6-specific response is dominated by IL-10 secretion at suboptimal TCR triggering, the influenza M1-specific response was characterized by the secretion of IFNγ.

IL-10-secreting HPV-specific T cells present in tumor infiltrating lymphocyte (TIL) populations

The analysis of tumor-draining lymph nodes is limited to those patients with proven metastatic cervical cancer; in all other patients the lymph nodes are required for diagnostic purposes. This implies that lymph node analysis selects patients with advanced cancer, and as such, the described predominance of IL-10-secreting T cells may not apply to patients with non-metastatic disease. For this reason, we embarked on the analysis of lymphocyte populations derived from tumors of nine cervical cancer patients without tumor-positive lymph nodes.

Tumor tissue was cultured with T cell growth factor (TCGF) and IL-15, without the addition of antigen, until lymphocytes emerged from the tissue (TIL). These TIL were tested in short-term proliferation assays for HPV16 or HPV18 reactivity, as were freshly isolated PBMC derived from the same patients (Table 3). Frequently, HPV-reactivity was observed in TIL cultures, while no detectable HPV-specific responses were present in PBMC of the same patient. This implies that the absence of detectable systemic HPV immunity does not exclude the local presence of HPV-specific T cells.

TIL cultures with HPV-reactivity (6 out of 9) were subjected to cytokine analysis. The secretion of IL-10 and IFNγ by CD4+ T cells was determined at the single cell level, and in response to antigenic stimulation, these TIL cultures were all shown to harbor HPV-specific CD4+ T cells secreting IL-10, IFNγ and/or a combination of both cytokines. The balance between IL-10+ and IFNγ+ HPV-specific T cells showed a considerable variation. At chosen stimulatory conditions – involving high peptide concentrations (10 µg/ml) pulsed on GM-CSF cultured autologous monocytes – 3 out of 6 cultures showed a predominance of IL-10+ T cells in response to HPV antigens (Table 3 and Figure 8a), whereas in 2 cultures IFNγ+ T cells were more abundant (Figure 8b). In one culture similar percentages of IL-10+ and IFNγ+ HPV-specific T cells were detected.

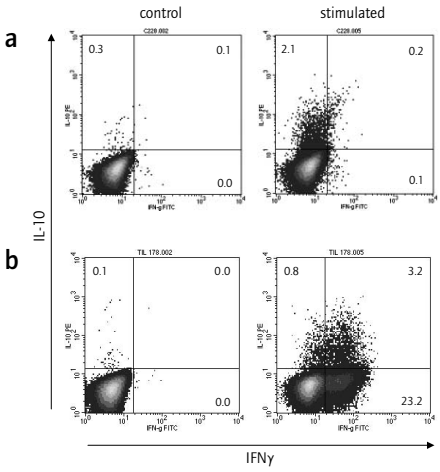

Figure 8. Single cell analysis of TIL cultures at the level of cytokine secretion. TIL cultures that showed HPV-reactivity were analyzed for IL-10 and IFNγ using the cytokine secretion assay. The TIL were incubated with GM-CSF cultured monocytes, which were pulsed with 10 µg/ml peptide or medium only (control). In response to antigenic stimulation the TIL cultures showed either a predominance of CD4+ T cells secreting IL-10 (A, patient 9) or IFNγ (B, patient 2). A representative example of each is shown. The percentages of cytokine-secreting T cells are indicated in the corners of the dotplots. The cells are gated for live CD4+ T cells.

Table 3. Tumor-derived lymphocyte cultures.

Patient	HPV type[a]	PMBC[b]	TIL[c]	IL-10[d]	IFNγ
1	HPV16	·	HPV16E6	+	+
2	HPV16	·	HPV16E7	+	++
3	HPV16/18	·	·		
4	HPV16	·	HPV16E7	+	++
5	HPV18	·	·		
6	n.t.	HPV18E7	HPV18E7	++	+
7	n.t.	·	·		
8	HPV16	·	HPV16E6	++	+
9	HPV18	HPV18E6E7	HPV18E6E7	++	+

[a] HPV type detected in tumor tissue

[b] Freshly isolated PBMC from 9 cervical cancer patients were incubated with pools of HPV16/18 E6 and E7 peptides (10 μg/ml) for 6 days after which proliferation was measured by [^{3}H]-thymidine incorporation. A proliferative response was scored positive when the proliferation of 6 out of 8 parallel microcultures exceeded the mean + 3 × SD of medium control and the stimulation index was >3.

[c] Cytokine-cultured tumor infiltrating lymphocytes (TIL) were incubated with monocytes loaded with pools of HPV16/18 E6 and E7 peptides and recombinant E6 and E7 protein. A positive response was defined as a stimulation index >3. All peptide-specific cultures showed recognition of the corresponding protein.

[d] TIL cultures displaying HPV-reactivity were analyzed at the single cell level for the antigen-specific secretion of IFNγ and IL-10 in response to monocytes pulsed with 10 μg/ml of peptide. A '+' indicates that percentage of cytokine-positive CD4+ T cells in response to HPV antigen was at least 3 times that of unstimulated control. '++' indicates the most predominant cytokine (the percentage of CD4+ T cells secreting one cytokine is at least two times that of CD4+ T cells secreting the other cytokine). A dot indicates that no HPV-specific response was detected. n.t. = not typed for HPV.

Overall, these data show that the local presence of IL-10-secreting HPV-specific CD4+ T cells is a common feature in cervical cancer patients. The implications of these findings are subject to future study, which shall address the immunoregulatory capacity of these T cells and their role in disease outcome.

Discussion

HPV16-positive cervical cancer patients frequently lack strong systemic HPV16-specific CD4+ T cell immunity associated with pro-inflammatory cytokines [3]. In this study we show that not only the HPV-specific T cells in the peripheral compartment, but also those residing in the tumor and tumor-draining lymph nodes are associated with a non-inflammatory cytokine profile. Detailed analysis of lymph node-derived HPV16E6-specific CD4+ T cell clones revealed that they predominantly secreted IL-10 and IFNγ upon antigen stimulation; the balance between both cytokines strongly depending on type of APC used for antigen presentation and the HLA class II – peptide density on the surface of the APC. At low antigen concentrations and suboptimal antigen presentation IL-10 secretion was clearly favored, whereas IFNγ was secreted only upon optimal T cell stimulation involving high peptide concentrations and fully mature DC. A similar HPV-specific T cell repertoire was found in the tumor-draining lymph node of a second patient that we recently analyzed (data not shown). Since cervical cancers do not provide the proper pro-inflammatory environment to induce potent T cell immunity, it is conceivable that T cell priming at this disease stage will affect the polarization of HPV16-specific CD4+ T cells. In contrast, influenza M1-specific T cell clones derived from the same draining lymph node showed predominant secretion of IFNγ, independent of APC type or HLA class II – peptide density. The clearly Th1 polarized cytokine profile of the influenza M1-specific T cells can be explained by the fact that these cells have been primed in a strong pro-inflammatory environment of the pulmonary lymphoid system during influenza infection and then entered the circulation.

The difference in cytokine profiles of HPV16E6 and influenza M1-specific T cell clones suggests that the observed IL-10-secreting HPV16E6-specific T cells are related to HPV-induced disease in this patient, rather than a general feature of the T cells residing in the tumor-draining lymph nodes. Furthermore, our observation that HPV16-specific T cell clones derived from the peripheral blood of healthy individuals are generally clearly Th1 (or Th2) polarized, sustains the notion that the IL-10-secreting HPV16-specific T cells reflect failing HPV-specific immunity. However, this should be confirmed by the analysis of lymph node cells from individuals who have successfully controlled HPV16 infection.

The balance between the IFNγ and IL-10 secretion of the T cell clones was dependent not only on the peptide-HLA class II density on the surface of the APC, but also on the type of APC. Stimulation with peptide-pulsed B-LCL always resulted in a higher percentage of cells secreting IL-10, than stimulation with DC. A recent study by Brady et al. showed that the cytokine profiles of melanoma-specific CD4+ T cells were skewed even more towards IL-10 secretion upon stimulation with HLA class II-positive melanoma cells than when stimulated with B-LCL [19]. This suggests that interaction between the HPV16E6-specific T cells and HLA class II-positive cervical cancer cells will most likely result in pronounced IL-10 secretion; cervical tumors have been shown to express HLA class II in approximately 80% of the cases [20]. Supporting the potential in vivo relevance of such interaction is the frequent presence in cervical cancer patients of HPV E6 and E7-specific tumor-infiltrating CD4+ T lymphocytes with similar IL-10/IFNγ secretion profiles as the lymph node-derived T cells. These T cells may thus contribute to local IL-10 secretion by interaction with HLA class II expressing tumor cells.

The isolated secretion of IL-10 in the absence of pro-inflammatory cytokines is likely to hamper effective induction of immune responses in vivo by modulating APC function, and to interfere with effector function of previously primed T cells [5, 21]. Currently, we are addressing the in vitro regulatory capacity of the HPV16E6-specific IL-10-secreting T cells. The potential immunoregulatory properties of these T cells are of major relevance for vaccination strategies. Boosting of pre-existing immunity by active vaccination may result in expansion of the IL-10-secreting T cell pool, further incapacitating the anti-tumor immune response. On the other hand, adjuvants with a strong Th1 skewing capacity may be able to redirect the immune response in favor of effective anti-tumor immunity. In a pilot study, we have attempted to skew the cytokine profiles of the IL-10-secreting HPV16E6-specific T cell clones towards a Th1 profile. However, antigen-specific stimulation of the T cell clones with mature peptide-pulsed DCs or with plate-bound anti-CD3/CD28 both combined with exogenous IL-12 did not result in a loss of IL-10 secretion in favor of IFNγ (data not shown). This suggests that the pre-existing HPV16-specific immunity present in patients is not likely to be skewed towards Th1 by vaccination. Therefore, in order to be effective, vaccination must result in de novo priming of HPV16-specific Th1 type T cells, capable of overruling the unfavorable pre-existing immunity.

References

1. Woodworth CD. HPV innate immunity. *Front Biosci,* 7: d2058-2071, 2002.
2. Ho GY, Bierman R, Beardsley L, Chang CJ, and Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med,* 338: 423-428, 1998.
3. de Jong A, van Poelgeest MIE, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJM, Kenter GG, Offringa R, and van der Burg SH. HPV16-positive cervical cancer is associated with impaired CD4+ T cell immunity against early antigens E2 and E6. *Cancer Res,* 64: 5449-5455, 2004.
4. Giannini SL, Al-Saleh W, Piron H, Jacobs N, Doyen J, Boniver J, and Delvenne P. Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression. *Clin Exp Immunol,* 113: 183-189, 1998.
5. Steinbrink K, Graulich E, Kubsch S, Knop J, and Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood,* 99: 2468-2476, 2002.
6. Muller G, Muller A, Tuting T, Steinbrink K, Saloga J, Szalma C, Knop J, and Enk AH. Interleukin-10-treated dendritic cells modulate immune responses of naive and sensitized T cells in vivo. *J Invest Dermatol,* 119: 836-841, 2002.
7. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, and Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. *Am J Pathol,* 135: 703-709, 1989.
8. Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den Hartigh J, Ottenhoff TH, and Drijfhout JW. Purification of His-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr Purif,* 18: 95-99, 2000.
9. Evans M, Borysiewicz LK, Evans AS, Rowe M, Jones M, Gileadi U, Cerundolo V, and Man S. Antigen processing defects in cervical carcinomas limit the presentation of a CTL epitope from human papillomavirus 16 E6. *J Immunol,* 167: 5420-5428, 2001.
10. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, and Offringa R. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res,* 62: 472-479, 2002.
11. van der Burg S, Kwappenberg KMC, Geluk A, van der Kruk M, Pontesilli O, Hovenkamp E, Franken KLMC, van Meijgaarden KE, Drijfhout JW, Ottenhof THM, Melief CJM, and Offringa R. 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,* 162: 152-160, 1999.
12. van der Burg SH, Menon AG, Redeker A, Franken KL, Drijfhout JW, Tollenaar RA, Hartgrink HH, van de Velde CJ, Kuppen PJ, Melief CJ, and Offringa R. Magnitude and polarization of P53-specific T-helper immunity in connection to leukocyte infiltration of colorectal tumors. *Int J Cancer,* 107: 425-433, 2003.
13. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, Franken KL, Drijfhout JW, Fleuren GJ, Kenter G, Melief CJ, Offringa R, and van der Burg SH. 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,* 63: 636-641, 2003.
14. Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, and Wang RF. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. *Immunity,* 20: 107-118, 2004.
15. Manca F, Li Pira G, Fenoglio D, Fang SP, Habeshaw A, Knight SC, and Dalgleish AG. Dendritic cells are potent antigen-presenting cells for in vitro induction of primary human CD4+ T cell lines specific for HIV gp120. *J Acquir Immune Defic Syndr,* 7: 15-23, 1994.
16. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief CJ, and Offringa, R. 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,* 91: 612-618, 2001.
17. Jameson J, Cruz J, and Ennis FA. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J Virol,* 72: 8682-8689, 1998.
18. Danke NA, and Kwok WW. HLA class II-restricted CD4+ T cell responses directed against influenza viral antigens postinfluenza vaccination. *J Immunol,* 171: 3163-3169, 2003.
19. Brady MS, Eckels DD, Lee F, Ree SY, and Lee JS. Cytokine production by CD4+ T cells responding to antigen presentation by melanoma cells. *Melanoma Res,* 9: 173-180, 1999.
20. Hilders CG, Houbiers JG, Krul EJ, and Fleuren GJ. The expression of histocompatibility-related leukocyte antigens in the pathway to cervical carcinoma. *Am J Clin Pathol,* 101: 5-12, 1994.
21. Akdis CA, and Blaser K. Mechanisms of interleukin-10-mediated immune suppression. *Immunology,* 103: 131-136, 2001.

Annemieke de Jong, T. O'Neill*, A.Y. Khan*,
Kitty Kwappenberg, S.E. Chisholm*,
N.R. Whittle*, J.A. Dobson, L.C. Jack*,
J. StClair Roberts*, Rienk Offringa,
Sjoerd van der Burg, J.K. Hickling*

* Xenova Research Ltd, Cambridge, UK.

Vaccine 20:3456-3464; 2002

6

Enhancement of HPV16 E6 and E7-specific T cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine

Abstract · TA-CIN is a vaccine that comprises the HPV16 L2, E6 and E7 as a single fusion protein. In a mouse model, TA-CIN effectively prevented outgrowth of HPV16-positive tumour cells. To assess the safety and immunogenicity of TA-CIN, a dose escalating (26 μg, 128 μg, 533 μg), double blind and placebo-controlled phase I study was conducted in 40 healthy volunteers. TA-CIN was administered without adjuvant by intramuscular injection on weeks 0, 4 and 8. No serious adverse events of the vaccination were reported during the study. Both IgG antibodies and proliferative responses against TA-CIN were elicited at all three doses. More importantly, T cell immunity against the HPV16 E6 and E7 oncoproteins was detected by IFNγ ELISPOT in 8/11 evaluable subjects vaccinated with the 533 μg dose.

Introduction

The causal link between oncogenic human papillomaviruses (HPV) and the development of cervical intraepithelial neoplasia (CIN) is now well established [1]. Current screening techniques and treatment modalities have reduced the levels of cervical cancer in the developed world significantly. However, cervical cancer remains the second most common cancer in women (after breast cancer) worldwide [2].

There are a number of observations that HPV infection is normally kept under control by the immune system and that vaccination could have a therapeutic effect in patients with HPV positive lesions. The incidence of HPV infection is often increased in immunosuppressed and immunodeficient patients [3, 4]. Furthermore, in the majority of immunocompetent individuals the HPV infection is asymptomatic, while most premalignant cervical lesions are found to regress spontaneously [5-7]. This regression is likely to be mediated by cellular immune responses, since regressing genital warts are predominantly infiltrated with CD4+ T cells and macrophages, consistent with a delayed type hypersensitivity reaction [8, 9]. The HPV E6 and E7 oncoproteins are exclusively expressed in virally infected cells, cervical intraepithelial neoplasia and cancer cells, as such these non-structural antigens form attractive targets for T-cell-based immunotherapy [10]. The use of animal models, in which vaccines inducing T cell immunity against these non-structural antigens controlled established tumours, points at the potential efficacy of therapeutic vaccination against HPV infection [11-17].

We have previously shown that a newly designed vaccine candidate, comprising the HPV16 L2, E6 and E7 as a single fusion protein (TA-CIN), elicited HPV16-specific CTL, T-helper cells and antibodies in a pre-clinical mouse model. Moreover, vaccination with TA-CIN effectively prevented outgrowth of HPV16-positive tumour cells in a prophylactic setting as well as in a minimal residual disease setting [18]. In the present study, the safety and immunogenicity of TA-CIN in healthy volunteers was analysed. Vaccination with TA-CIN was safe and well tolerated. Furthermore, TA-CIN induced HPV16-specific antibodies as well as E6 and/or E7-specific T cell responses in the majority of subjects.

Materials and Methods

TA-CIN vaccine · HPV16 DNA was isolated from the W12 cell line [19], derived from biopsy material, histologically determined to be CIN1, recovered from a 22-year old female with a history of abnormal cervical Papanicolaou smears. The HPV16 L2, E6 and E7 genes were specifically amplified from the viral DNA by PCR to generate individual products. Target genes were cloned in pET plasmids under the control of the strong bacteriophage T7 transcription and translation signals; expression was induced by providing a source of T7 RNA polymerase in the host cell. The HPV16 L2E7E6 fusion protein antigen was formed in a bacterial expression system consisting of the bacterial host Escherichia coli HMS174 (DE3) carrying the plasmid pCIN170. The construct contained a fusion gene encoding the HPV16 L2E7E6 fusion protein antigen under the global control of a Lac promoter. The construct also contained a Kanamycin resistance gene selective marker. TA-CIN protein was produced in E. coli batch culture. Inclusion bodies were disrupted using a high pressure homogeniser, and the protein product was then solubilised in guanidine hydrochloride (GuHCl) and further purified using a 5-step chromatographic process. The purified L2E7E6 protein was then filtered through a 0.2 µm membrane and stored at −80°C prior to QC testing, QA review and lot release procedures. The final product was formulated in 5 mM phosphate, 5 mM glycine buffer pH 8.0 containing 0.9 mM cysteine. The placebo consisted of vehicle alone.

Subjects · Forty healthy volunteers were recruited into the study at Pharma Bio-Research Group B.V. in the Netherlands. Thirty males and ten females (post menopausal, non-reproductive) were divided

into four groups: group 1-3 (3 × 10 males), group 4 (10 females). Volunteers were randomised to one of two arms in an 8:2 ratio: TA-CIN (n=8); placebo (n=2) using a randomisation schedule, drawn up in advance. The age of the male volunteers ranged between 19-55 years with a mean of 36.0 years. The age of the post-menopausal female volunteers ranged between 43-55. Subjects were considered to be healthy on the basis of medical history and a physical examination consisting of ECG, haematology (haemoglobin, mean cell volume, platelet count, white blood cell count and full differential), clinical chemistry (sodium, potassium, creatinin, total bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), asparatase aminotransferase (AST), urea, albumin and glucose), urine analysis and vital signs (blood pressure, pulse and oral temperature) at day 0, 28, 56 and on day 84. Female patients were also required to have a negative PAP smear at the pre-vaccination screening visit. A negative urine pregnancy test at day 0, 28 and 56 was required, and a negative PAP smear at day 84. A clinically significant value from any of these tests reported by the laboratory was entered as an adverse event. Adverse events were reported as an underlying diagnosis with the laboratory value included. Clinically significant values that were thought to be due to administration of the study drug were followed up until week 12 (i.e. the last follow-up visit), which was 4 weeks after the last vaccination.

Immunization protocol · In this phase I, double blind, randomised, placebo-controlled, dose-escalating study subjects were randomised to receive one of three dose levels, 25 µg, 125 µg and 500 µg/dose. The actual doses administered (cohort P: placebo, cohort 1: 26 µg, cohort 2: 128 µg and cohort 3 and 4: 533 µg) were based on final protein concentration measurements of filled vaccine. These varied slightly from the predicted dose levels due to the dilution required from bulk material during the filling process. Vaccine was administered without adjuvant as three consecutive intramuscular injections into the same upper arm on weeks 0, 4 and 8. The primary objective of the study was to assess the safety and tolerability of TA-CIN. Samples were taken for immunogenicity assay on week 0 (pre-vaccination), week 4 (4 weeks after the first vaccination), week 8 (4 weeks after the second vaccination) and week 12 (4 weeks after the third vaccination).

Safety monitoring · Prompted and spontaneous adverse events, injection site reactions, clinical assessments and clinical laboratory parameters were used as safety parameters. Injection site reactions were defined as induration, erythema or tenderness.

Lymphocytes · Peripheral blood mononuclear cells (PBMC) were separated from fresh citrated blood directly upon receipt (<24 hours after bleeding) using Lymphocyte Separation Medium (ICN Biomedicals) density gradients. PBMC were cryopreserved in vapour phase nitrogen until use.

T cell proliferative responses · T cell proliferation assays were set up in triplicate in 96-well round bottom plates. Cells, 2×10^5 cells/well in Dutch-modified RPMI-1640 (Gibco BRL) containing 5% (v/v) human AB serum (BioWhittaker) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (4 mM) (all Gibco BRL), were cultured with serial dilutions of TA-CIN and TA-GW (a fusion protein consisting of L2 and E7 of HPV6 [20]) (both Xenova), and optimal concentrations of the positive control antigens: tetanus toxoid (Medeva), inactivated HSV-1 (Xenova) or PHA (Murex) and incubated at 37°C in 5% CO_2 humidified atmosphere. Interassay variability was minimized by testing the samples of all subjects from one group together in the same assay. For each culture the stimulation index was calculated by dividing the mean of antigen-induced proliferation by the mean of the medium control. Wells were pulsed with 0.5 µCi of [^{3}H]-thymidine for the final 18h of a six day culture, harvested and the incorporated radioactivity was determined with a 1450 Microβ Plus counter (Wallac). Cultures were considered positive when the stimulation index (SI) of antigen-induced proliferation was ≥ 3 and of which the proliferation induced [^{3}H]-thymidine incorporation exceeded 5,000 counts per minute. Because TA-GW [20] was produced under comparable conditions as TA-CIN, this protein served as a control for HPV16 L2E7E6 specific induced T cell proliferation. Therefore, a vaccination specific response was defined as a positive proliferative response to TA-CIN of which the SI was at least 4 fold higher than that induced by TA-GW.

HPV16 E6 and E7-specific T helper responses · Analysis of T cell responses using IFNγ ELISPOT was performed as described previously [21]. Briefly, PBMC were thawed and seeded at a density of 2×10^6 cells/well of a 24 well plate (Costar, Cambridge, MA) in 1ml of Iscove's medium (Gibco) enriched with 10% FCS, in the presence or absence of indicated 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 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. The peptides used spanned the HPV16 E6 and E7 protein and consisted of fifteen E6 and nine E7 overlapping 22-mer peptides. Peptides, as indicated by their first and last amino acid in the protein, were used in pools of 4-5 peptides at a concentration of 5 µg/ml/peptide. The following pools were made E6-I: 1-22, 11-32, 21-42, and 31-52, E6-II: 41-62, 51-72, 61-82 and 71-92, E6-III: 81-102, 91-112, 101-122 and 111-132, E6-IV: 111-132, 121-142, 131-152 and 137-158, E7-I: 1-22, 11-32, 21-42, 31-52, E7-II: 41-62, 51-72, 61-82, 71-92 and 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/well of a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with a IFNγ catching antibody (Mabtech AB, Nacha, Sweden). The ELISPOT was further performed according to the instructions of the manufacturer (Mabtech). The number of spots were analysed with a fully automated computer assisted video imaging analysis system (Carl Zeiss Vision). Specific spots were calculated by subtracting the mean number of spots + $2 \times$ SD of the medium only control from the mean number of spots of experimental wells. Antigen-specific T cell frequencies were considered to be increased compared to non-responders when specific T cell frequencies were ≥ 1/10,000 PBMC [21].

Serology · L2E7E6 specific antibodies were measured by ELISA. Ninety-six well plates were coated with TA-CIN L2E7E6 fusion protein in 100 mM carbonate buffer, pH9.6, overnight at 4°C. Wells were blocked with 2% bovine serum albumin in PBS for 1 h at 37°C. Serial dilutions starting at 1:50, of test serum samples and known positive and negative samples diluted in PBS/2%BSA were added to triplicate wells and incubated for 1 h at 37°C. After washing with PBS/0.05%Tween-20, biotinylated monoclonal anti-human IgG (Sigma) was added and incubated for 1 h at 37°C. Plates were washed and incubated with horseradish peroxidase-streptavidin (Zymed) for 1 h at 37°C. Plates were washed and developed with o-phenylene diamine/H_2O_2 for 30 min at room temperature and the absorbance was measured at 490 nm. Antibody titres are recorded as the $\log_{10}$ of the reciprocal of the dilution at an absorbance of 1.0 [18].

Results

Safety and tolerability of TA-CIN

To test the safety of TA-CIN in humans, 40 subjects consisting of 30 males and 10 non-reproductive females were recruited. Of these subjects 6 males and 2 females were vaccinated with placebo. The other 24 males were divided into three cohorts vaccinated with either 26 µg, 128 µg or 533 µg doses of TA-CIN. Because it was anticipated that recruitment of non-reproductive post-menopausal females would be slow, the dose-escalation study was performed in males. The 8 females were injected with 533 µg doses of TA-CIN only. Subjects were vaccinated intramuscularly, three times at 4-weeks intervals in the same upper arm.

All 40 subjects completed the study without reporting any serious or severe adverse events. The majority of events were reported within 7 days of each vaccination (Table 1). As a local injection site reaction, tenderness was the most frequently reported event. The number of subjects reporting tenderness increased when higher doses of TA-CIN were administered (Table 1). The number of subjects

reporting moderate tenderness was higher after vaccination at the two highest doses (4/8 at 128 μg; 7/16 at 533 μg) compared to placebo (0/8) or the lowest dose (0/8) of TA-CIN administered. Local reactions such as redness and swelling were rarely reported with most occurrences only lasting for one or two days. The number of females reporting injection site reactions was relatively high compared to the number of males. However, the mean age of the female volunteers was substantially higher than that of the male volunteers. The most frequently reported systemic adverse events of moderate severity, possibly related to the study medication, were headache and fatigue. The study showed no relation between the number and severity of systemic adverse events and the dose of TA-CIN administered or the number of injections given. Results obtained from vital signs, clinical chemistry, ECG parameters and physical examination revealed no clinically relevant abnormalities. In general, three administrations of TA-CIN at doses up to 533 μg were safe and well tolerated.

TA-CIN elicits specific IgG responses

It is conceivable that vaccination with whole antigen induces humoral immunity. Therefore, sera from all individuals were analysed for TA-CIN specific IgG antibodies by ELISA (Table 2). Low levels of pre-existing TA-CIN-specific IgG were seen in 2 (# 23 and 36) of the 40 individuals, although no reliable antibody titres could be determined. One of these subjects (# 36) received placebo and no further increase in specific IgG was detected. In two other subjects (# 13 and 39), a high anti-TA-CIN IgG level was detected before vaccination and this response was boosted after vaccination in subject 39 only (Table 2).

Following vaccination all but 8 of 32 subjects receiving the active vaccine produced TA-CIN specific serum IgG. Four of the non-responding subjects received the lowest dose (26 μg) of TA-CIN and as such the number of responders in this cohort was not significantly different from the placebo cohort (Fisher's Exact Test, $p=0.08$). In contrast, the subjects receiving the 128 μg or 533 μg dose vaccine responded significantly better than the placebo cohort (Fisher's Exact Test, $p<0.05$).

TA-CIN induces HPV16 specific T cell responses

T cell cultures of all subjects responded to PHA and to one or more of the control antigens tetanus toxoid and HSV-1 in the proliferation assay, indicating a generally good immune status of the tested subjects (not shown). To assess the HPV16 L2E7E6-specific T cell proliferation induced by TA-CIN, PBMC were stimulated with TA-CIN or the control protein. TA-GW, a clinical-grade HPV6 L2E7 fusion protein was used as a control because it was produced under highly comparable conditions as TA-CIN [20]. Responses were considered TA-CIN specific if the proliferation to TA-CIN exceeded the response to TA-GW at least four times. The L2 proteins of HPV6 and HPV16, in contrast to the E7 proteins of these viruses, share several regions of considerable sequence homology implicating that this manner of scoring for positive proliferation responses is primarily aimed at detecting HPV16 E6 and E7-specific T cell reactivity. TA-CIN specific proliferation was detected after the second and third vaccination in cohort 1, and after the first, second or third vaccination in cohorts 2, 3 and 4 (Table 3). The number of responders in the 128 μg and 533 μg dose treated cohorts were significantly higher than in the placebo cohort ($p< 0.01$, Fisher's Exact Test). In total 25 of the 32 vaccinated subjects responded to the vaccine in this assay.

The 26 μg and 128 μg TA-CIN dosed-cohorts were mainly used to assess safety. A more extensive analysis of TA-CIN induced T cell reactivity was performed on PBMC samples of individuals vaccinated with 533 μg TA-CIN (cohorts 3 and 4). PBMC were stimulated with pools of overlapping peptides that together covered the complete HPV16 E6 and E7 protein. We have previously demonstrated that this allows an accurate analysis of the HPV16 E6- and E7-specific T cell activity [21], which is expected to play a major role in therapeutic efficacy. Furthermore, it circumvents the possible (cross-) reactions to conserved parts of L2 or to contaminants present in TA-CIN and

Table 1. Local and systemic adverse events after vaccination with TA-CIN.

		Vaccination 1 (Day 0)					Vaccination 2 (Day 28)		
	#[a]	Injection site pain[2]	Tenderness	Redness (cm)	Swelling (cm)	Systemic	Injection site pain	Tenderness	Redness (cm)
P	14	·	·	·	·	·	·	·	·
	18	·	·	·	·	flu-like symptoms	·	·	·
	20	·	1	·	·	·	·	·	·
	25	·	·	·	·	fatigue/malaise	·	·	·
	33	·	·	·	·	·	·	·	·
	36	·	·	·	·	·	·	·	·
	43	·	·	·	·	fatigue	·	·	·
	48	·	·	·	·	·	·	·	·
I	10	·	·	·	·	·	·	·	·
	11	·	·	·	·	·	·	·	·
	12	·	·	·	·	·	·	·	·
	13	·	·	·	·	myalgia	·	·	·
	15	·	1	·	·	headache/anorexia/ drowsiness/dry mouth	1	·	·
	16	·	·	·	·	·	·	·	·
	17	·	1	·	·	headache/rhinitis	·	·	·
	19	·	·	·	·	·	1	·	·
II	21	·	2	·	·	fatigue/dizziness/ abdominal pain/syncope	·	1	·
	22	1	2	·	1.5	fatigue	·	·	·
	23	·	·	·	·	arm weakness	·	·	·
	24	·	·	·	·	nausea/dizziness/myalgia	·	1	·
	26	·	·	·	·	·	·	·	·
	27	·	2	·	·	·	·	·	·
	28	·	2	·	·	headache	·	·	·
	29	·	·	·	·	·	·	·	·
III	30	·	·	·	·	·	·	·	·
	31	·	·	·	·	·	·	·	·
	32	·	·	·	·	·	·	·	·
	34	·	1	·	·	dizziness	·	1	·
	35	·	2	·	·	·	·	1	·
	37	·	2	·	·	·	·	1	·
	38	·	·	·	·	headache	·	·	·
	39	·	·	·	·	·	·	·	·
IV	40	·	·	·	·	·	2	·	·
	41	·	2	·	·	dizziness	·	·	·
	42	·	1	·	·	·	·	1	·
	44	2	2	1.0	1.0	·	·	1	·
	45	·	·	·	·	headache	2	2	5.5
	46	·	1	·	·	·	·	·	·
	47	·	2	·	·	·	·	·	·
	49	·	2	·	·	dreaming abnormal	2	2	·

[a] Indicate the different cohorts: placebo control (14, 18, 20, 25, 33, 36, 43, 48), group I; males vaccinated with 26 µg (10-13, 15-17,19), group II; males vaccinated with 128 µg (21-24, 26-29), group III, males vaccinated with 533 µg (30-32, 34, 35, 37-39) and group IV; females vaccinated with 533 µg (40-42, 44-47, 49).

[b] Local and systemic adverse events evaluation: 1= mild, 2= moderate.

Vaccination 3 (Day 56)						
Swelling (cm)	Systemic	Injection site pain	Tenderness	Redness (cm)	Swelling (cm)	Systemic
.	.	.	.	.	.	.
.	.	.	1	.	.	.
.	.	.	.	.	.	.
.	headache/fatigue/malaise	1	1	.	.	drowsiness
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	1	1	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	rhinitis	.	.	.	.	.
.	fatigue	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	2	.	.	.	.
.	fatigue/rhinitis	.	.	.	.	headache
.	.	.	.	.	.	fatigue
.	.	.	.	.	.	.
.	nausea/abdominal pain	.	.	.	.	nausea/abdominal pain
.	.	.	.	.	.	.
.	.	.	2	0.5	0.5	headache/fatigue/inflicted injury
.	.	.	.	.	.	.
.	.	.	.	.	.	headache
.	.	.	.	.	.	.
.	headache	.	.	.	.	.
.	fatigue	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	1	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	.	.	.	.	.
.	.	1	.	.	.	.
.	.	.	2	.	.	.
1.5	.	.	.	.	.	.
.	.	.	1	.	.	pain both arms
.	.	.	2	1.0	.	menstrual disorder
.	headache×3/fatigue	.	2	.	.	headache

Table 2. Individual anti-HPV 16 L2E7E6 IgG responses.

Cohort[a]	Subject	Log titre[b] Pre-vaccination	Log titre Post-vaccination
P	14	·	·
	18	·	·
	20	·	·
	25	·	·
	33	·	·
	36	·	·
	43	·	·
	48	·	·
I	10	·	3.0
	11	·	·
	12	·	3.0
	13	2.2	2.3
	15	·	·
	16	·	·
	17	·	·
	19	·	2.4
II	21	·	2.9
	22	·	2.4
	23	·	3.0
	24	·	2.4
	26	·	·
	27	·	2.9
	28	·	1.7
	29	·	4.5
III	30	·	4.4
	31	·	2.9
	32	·	2.5
	34	·	1.4
	35	·	2.4
	37	·	2.4
	38	·	·
	39	2.1	2.9
IV	40	·	·
	41	·	3.0
	42	·	3.0
	44	·	·
	45	·	2.9
	46	·	3.0
	47	·	4.3
	49	·	4.3

[a] Indicate the different cohorts: Placebo control (P), vaccinated with 26µg (I), 128 µg (II), 533 µg (III+IV). Subjects 10-39 are males, subjects 40-49 are females.

[b] TA-CIN specific antibody titers. Titers are expressed as the log of the reciprocal serum dilution that results in an OD value of 1. Sera that displayed an OD value of which no reliable titre could be determined are marked with a centerdot (·). Post-vaccination titres are at week 12 following initial vaccination

Table 3. TA–CIN specific induced proliferative responses.

		TA-CIN				TA-GW				TA-CIN:TA-GW ratio			
Cohort[a]	Subject	0[b]	4	8	12	0	4	8	12	0	4	8	12
P	14	.[c]	·	·	·	·	·	·	·	·	·	·	·
	18	9.7	8.1	·	6.1	3.7	6.5	·	3.7	·	·	·	·
	20	6.3	9.7	12	6.3	2.7	7.5	4.8	1.1	·	·	·	5.7
	25	7.8	·	·	·	4.4	·	·	·	·	·	·	·
	33	9.3	·	·	·	2.9	·	·	·	·	·	·	·
	36	·	3.8	3.2	·	·	3.3	2.2	·	·	·	·	·
	43	·	·	5.0	8.8	·	·	3.7	6.8	·	·	·	·
	48	·	·	·	·	·	·	·	·	·	·	·	·
I	10	7.3	15	7.7	26	3.7	6.1	1.5	4.4	·	·	5.2	5.9
	11	·	16	10	3.1	·	5.7	4.3	5.5	·	·	·	·
	12	7.7	9.2	6.0	19	3.2	4.2	1.1	3.0	·	·	5.7	6.1
	13	·	5.8	·	·	·	2.3	·	·	·	·	·	·
	15	3.4	10	10	38	2.2	3.8	5.6	16				
	16	·	·	13	4.6	·	·	6.2	0.8	·	·	·	5.9
	17	4.1	25	21	31	4.1	10	5.8	12	·	·	·	·
	19	11	8.6	24	41	7.5	5.5	5.1	3.0	·	·	4.7	14
II	21	6.0	3.9	5.3	17	2.2	1.8	0.8	n.t.[d]	·	·	6.7	n.t.
	22	3.5	·	4.3	118	1.1	·	1.2	20				5.9
	23	·	3.2	8.4	58	·	0.6	0.9	3.9	·	5.1	9.7	15
	24	3.7	3.0	7.3	3.6	2.5	1.7	1.7	1.2	·	·	4.3	·
	26	·	·	10	4.9	·	·	1.3	1.7	·	4.1	7.5	·
	27	3.0	11	160	16	1.9	4.4	13	6.5	·	·	12	·
	28	·	·	5.7	33	·	·	0.9	2.7	·	·	6.1	12
	29	6.6	22	165	74	11	3.3	20	18	·	6.6	8.2	4.1
III	30	8.1	12	43	36	4.5	3.9	19	1.0	·	·	·	35
	31	3.7	3.8	93	16	2.0	1.1	2.6	0.9	·	·	35	18
	32	10	23	24	72	4.8	4.0	2.8	24	·	5.7	8.6	·
	34	·	12	13	52	·	4.5	2.1	20	·	·	6.5	·
	35	3.0	·	·	4.1	2.1	·	·	0.9	·	·	·	4.4
	37	·	11	51	22	·	2.1	3.5	2.9	·	5.1	15	7.6
	38	·	·	7.4	12	·	·	2.7	2.9	·	·	·	4.1
	39	·	5.5	5.2	36	·	1.2	1.0	2.9	·	4.7	5.2	13
IV	40	·	6.4	57	11	·	4.5	13	2.2	·	·	4.5	4.8
	41	·	·	14	7.3	·	·	7.1	4.3	·	·	·	·
	42	·	19	30	5.5	·	6.4	10	2.1	·	·	·	·
	44	·	27	4.3	9.2	·	13	1.0	2.4	·	·	4.4	4.0
	45	·	24	47	8.4	·	2.5	7.6	1.4	·	9.6	6.2	6.5
	46	·	8.6	49	44	·	1.9	5.0	3.6	·	4.5	9.9	12
	47	·	6.1	12	8.1	·	1.0	0.5	1.6	·	6.1	23	5.1
	49	19	·	68	20	14	·	33	13	·	·	·	·

[a] Indicate the different cohorts: Placebo control (P), vaccinated with 26 µg (I), 128 µg (II), 533 µg males (III) and 533 µg females (IV). Subjects 10-39 are males, subjects 40-49 are females.

[b] Different timepoints: Week 0 (pre-vaccination), week 4 (4 weeks after first vaccination), week 8 (4 weeks after second vaccination) and week 12 (4 weeks after third vaccination).

[c] Indicated are the Stimulation Indices (SI) of subject-derived PBMC after stimulation with TA-CIN (10 µg/ml) or TA-GW (a control L2E7 fusion protein of HPV type 6; 10 µg/ml) that responded with an SI ≥ 3 and of which [^{3}H]-thymidine incorporation ≥ 5000 cpm to TA-CIN. A vaccination specific response was defined as a positive proliferative response to TA-CIN of which the SI was at least 4 fold higher than that induced by TA-GW.

[d] n.t. = not tested

Table 4. Individual T-helper cell responses to pools of HPV16 E6 and E7 peptides.

Cohort[a]	Subject #	week 0[b]	week 4	week 8	week 12
III	31	·[c]	·	E6 III (26)	·
				E6 IV (55)	·
	32	·	·	·	·
	35	E7 II (10)	·	E6 II (24)	·
				E6 III (19)	·
	37	·	·	E6 IV (14)	·
	38	·	·	E6 IV (15)	E6 IV (17)
	39	·	NA	E6 IV (44)	NA
IV	41	·	·	NA	·
	42	·	·	E7 II (28)	·
	44	·	·	E6 IV (13)	E6 III (10)
					E6 IV (8)*
					E7 I (15)
	45	·	E6 IV (42)	E6 IV (49)	E6 IV (7)*
	46	·	·	·	NA

[a] Indicate the different cohorts: vaccinated with 533 µg males (III) and 533 µg females (IV). Subjects 31-39 are males, subjects 41-46 are females.

[b] Different timepoints: Week 0 (pre-vaccination), week 4 (4 weeks after first vaccination), week 8 (4 weeks after second vaccination) and week 12 (4 weeks after third vaccination).

[c] Specific spots per 100,000 PBMC are indicated. Specific spots were calculated by subtracting the mean number of spots + 2×SD of the medium control from the mean number of spots of experimental wells. Indicated are those peptide pools (and number of spots) to which specific T cell frequencies are ≥ 1/10,000 PBMC since these were considered to be increased compared to non-responders [21]. NA, not available for analysis. dot (·), no specific response to E6 or E7. * The number of specific spots is shown for comparison with previous time points.

TA-GW preparations. For extensive analysis of HPV16 E6- and/or E7-specific T cell activity sufficient amounts of high-quality PBMC samples were available of 14 subjects. Following the unblinding of treatment, 11 individuals had been given active vaccine and 3 of the subjects were vaccinated with a placebo. The three placebo-dosed subjects did not show HPV16 E6 or E7-specific reactivity. One of 14 pre-vaccination samples demonstrated T cell reactivity against HPV16 E7 pool II, suggesting T cell memory induced by a previous encounter with HPV16. After vaccination, HPV16 E6 and E7-specific T cell responses were clearly detectable in the majority of individuals given 533 µg TA-CIN (Table 4 and Figure 1). In general, detection of HPV16-specific T cells in ELISPOT coincided with detection of strong TA-CIN specific responses in the proliferation assays (compare Table 3 with Table 4, TA-CIN specific response versus ELISPOT of subjects 31, 37, 38, 39, 44 and 45). The most frequently found response in the subjects receiving active vaccine was against the E6 IV pool of peptides spanning amino acids 111 to 158. This response was present in >50% (6 out of 11) of the evaluable individuals. Sporadic responses were seen against other peptide pools post-vaccination, in particular E6 pools II and III (3 of 11 subjects) and E7 pools I and II (3 of 11 subjects). In total 8/11 (>70%) of all evaluable individuals vaccinated with the highest dose of TA-CIN showed enhancement of HPV16 E6/E7-specific INFγ-production at least at one point after vaccination.

Discussion

TA-CIN, comprising the HPV16 L2, E6 and E7 as a single fusion protein, effectively prevented outgrowth of HPV16-positive tumour cells in a prophylactic setting as well as in a minimal residual disease setting in a pre-clinical mouse model [18]. We have subsequently analysed the safety and immunogenicity of TA-CIN in a phase I double blind, randomised and placebo-controlled dose-

escalating study of three dose levels TA-CIN (26 µg, 128 µg and 533 µg) in 40 healthy volunteers. Vaccination resulted in TA-CIN specific IgG antibody and T cell proliferative responses in the majority of individuals. Moreover, in >70% of all evaluable individuals vaccinated with the highest dose, TA-CIN enhanced HPV16 E6/E7-specific immunity as demonstrated by IFNγ ELISPOT.

TA-CIN was safe and well tolerated in healthy male (cohorts 1-3) and female (cohort 4) volunteers. No serious adverse events were reported. The incidence of adverse events was low and the events that did occur were of mild or moderate severity. The total number and relative severity of the reported adverse events were lower with TA-CIN than with a related HPV 6 L2E7 vaccine (TA-GW) given by the same route of immunisation with an Alum adjuvant [20] suggesting that most of these events in the previous study may have been the result of the adjuvant used.

Pre-existing anti TA-CIN IgG was detected in 4 of the 40 subjects in this study, whereas another subject showed pre-existing HPV16 E7-specific T cell immunity. This may reflect immunological memory established by a previous infection with either the homologous virus (HPV 16) or with another HPV type. In five subjects TA-CIN specific IgG antibodies were found in the absence of a clear proliferative response. In one of these subjects a high titre of TA-CIN reactive IgG was already present before vaccination. Clearly, injection of TA-CIN neither boosted the IgG response nor the T cell response in this subject. Three other individuals showed an increase of proliferation against TA-CIN after vaccination but the TA-GW response was also boosted. Bearing in mind the homology between L2 of HPV6 and HPV16 this may well be the result of cross-reactive T cell reactivity against L2 of both types. Since we focused mainly at T cell reactivity against the oncoproteins E6 and E7, our analysis rather underestimates than overestimates TA-CIN-specific reactivity.

Animal model studies have shown that cellular immune responses are critical for the resolution of HPV induced lesions. Indeed, one vaccination with TA-CIN at the same day of challenge with TC-1 tumour cells protected mice against tumour growth [18] demonstrating the capacity of TA-CIN to induce effective anti-tumour immunity. Vaccination with TA-CIN induced T cell proliferative responses in the majority of human subjects (25 of 32) after 3 vaccinations. Six out of sixteen subjects

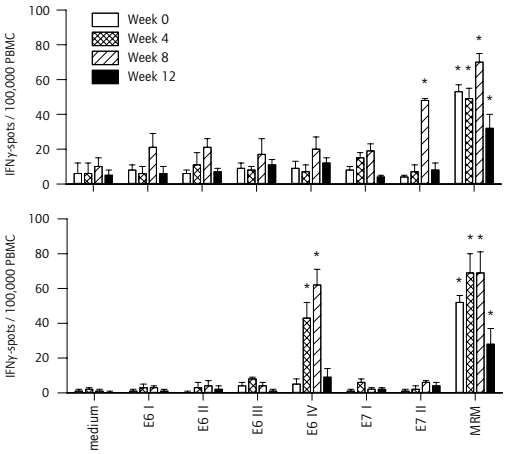

Figure 1. TA-CIN induces HPV16 E6 and E7-specific IFNγ-responses. The mean number of spots and standard error bars per 100,000 PBMC induced by the medium control or the peptides present in the E6 and E7 peptide pools are depicted. As positive control a memory recall antigen mix (see Materials and Methods) was used. Two examples, representing an HPV16 E6 and E7 response are shown. (Top) Female 42 and (Bottom) female 45, at week 0 (pre-vaccination), week 4 (4 weeks after first vaccination), week 8 (4 weeks after second vaccination) and week 12 (4 weeks after third vaccination). *, indicate antigen-specific responses with frequencies >1/10,000.

that were given a high dose of TA-CIN (533 µg) produced a proliferative response after only 1 vaccination with TA-CIN. This observation even exceeded our expectations because unadjuvanted proteins do not generally stimulate good T cell responses. The reason for the T cell stimulating capacity of TA-CIN may reside in the fact that it spontaneously forms a soluble molecular aggregate during the purification procedure.

Further analysis of the T cell response to TA-CIN using pools of overlapping 22 amino acid long E6 and E7 peptides by IFNγ ELISPOT showed that the dominant immunogenic region of the fusion protein mapped between amino acids 111 and 158 of the E6 protein. Six of eleven evaluable subjects that had received the high dose of TA-CIN responded to this C-terminal region of E6. Interestingly, E6-specific proliferative responses were found associated with clearance of HPV and regression of CIN [22].

The data presented in this paper demonstrate that TA-CIN is capable of enhancing HPV16 E6- and E7-specific immunity in humans. Continued boosting of E6/E7-immunity by TA-CIN, however, seems sub-optimal since responses tend to decrease after the last vaccination. Pre-clinical studies in mice have shown that vaccination with TA-CIN followed by vaccination with a recombinant vaccinia virus vaccine containing HPV 16 and 18 E6 and E7 (TA-HPV) stimulated greater T cell immunity than any other combination of the two vaccines [18]. Together these observations suggest that also in humans TA-CIN should preferably be applied together with TA-HPV in the context of a heterologous prime-boost regimen. In view of this notion, phase II clinical trials have been initiated looking at the TA-CIN/TA-HPV prime/boost regimen in women with high grade vulval intraepithelial neoplasia (VIN).

Acknowledgements · This work was supported by a grant from the Dutch Cancer Society (RUL 99-2024) and the Cancer Research Institute (to SvdB). The work of all the members of the TA-CIN project team at Xenova Research Ltd is gratefully acknowledged.

References

1. zur Hausen, H. Viruses in human cancers. *Science,* 254: 1167-1173, 1991.
2. Parkin DM. The global burden of cancer. *Semin Cancer Biol,* 8: 219-235, 1998.
3. Chirgwin KD, Feldman J, Augenbraun M, Landesman S, and Minkoff H. Incidence of venereal warts in human immunodeficiency virus-infected and uninfected women. *J Infect Dis,* 172: 235-238, 1995.
4. Petry KU, Scheffel D, Bode U, Gabrysiak T, Kochel H, Kupsch E, Glaubitz M, Niesert S, Kuhnle H, and Schedel I. Cellular immunodeficiency enhances the progression of human papillomavirus-associated cervical lesions. *Int J Cancer,* 57: 836-840, 1994.
5. Duggan MA, McGregor SE, Stuart GC, Morris S, Chang-Poon V, Schepansky A, and Honore L. The natural history of CIN I lesions. *Eur J Gynaecol Oncol,* 19: 338-344, 1998.
6. Evander M, Edlund K, Gustafsson A, Jonsson M, Karlsson R, Rylander E, and Wadell G. Human papillomavirus infection is transient in young women: a population-based cohort study. *J Infect Dis,* 171: 1026-1030, 1995.
7. Ho GY, Bierman R, Beardsley L, Chang CJ, and Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. *N Engl J Med,* 338: 423-428, 1998.
8. Aiba S, Rokugo M, and Tagami H. Immunohistologic analysis of the phenomenon of spontaneous regression of numerous flat warts. *Cancer,* 58: 1246-1251, 1986.
9. Coleman N, Birley HD, Renton AM, Hanna NF, Ryait BK, Byrne M, Taylor-Robinson D, and Stanley MA. Immunological events in regressing genital warts. *Am J Clin Pathol,* 102: 768-774, 1994.
10. Gilboa E. The makings of a tumor rejection antigen. *Immunity,* 11: 263-270, 1999.
11. Campo MS, Grindlay GJ, O'Neil BW, Chandrachud LM, McGarvie GM, and Jarrett WF. Prophylactic and therapeutic vaccination against a mucosal papillomavirus. *J Gen Virol,* 74 (Pt 6): 945-953, 1993.
12. Feltkamp MC, Smits HL, Vierboom MP, Minnaar RP, de Jongh BM, Drijfhout JW, ter Schegget J, Melief CJ, and Kast WM. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol,* 23: 2242-2249, 1993.
13. Feltkamp MC, Vreugdenhil GR, Vierboom MP, Ras E, van der Burg SH, ter Schegget J, Melief CJ, and Kast WM. Cytotoxic T lymphocytes raised against a subdominant epitope offered as a synthetic peptide eradicate human papillomavirus type 16-induced tumors. *Eur J Immunol,* 25: 2638-2642, 1995.
14. Lin KY, Guarnieri FG, Stavely-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, and Wu TC. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res,* 56: 21-26, 1996.
15. Selvakumar R, Borenstein LA, Lin YL, Ahmed R, and Wettstein FO. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. *J Virol,* 69: 602-605, 1995.
16. Ji H, Chang EY, Lin KY, Kurman RJ, Pardoll DM, and Wu TC. Antigen-specific immunotherapy for murine lung metastatic tumors expressing human papillomavirus type 16 E7 oncoprotein. *Int J Cancer,* 78: 41-45, 1998.
17. Rosales C, Graham VV, Rosas GA, Merchant H, and Rosales R. A recombinant vaccinia virus containing the papilloma E2 protein promotes tumor regression by stimulating macrophage antibody-dependent cytotoxicity. *Cancer Immunol Immunother,* 49: 347-360, 2000.
18. van der Burg SH, Kwappenberg KM, O'Neill T, Brandt RM, Melief CJ, Hickling JK, and Offringa R. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine, in homologous and heterologous prime-boost regimens. *Vaccine,* 19: 3652-3660, 2001.
19. Stanley MA, Browne HM, Appleby M, and Minson AC. Properties of a non-tumorigenic human cervical keratinocyte cell line. *Int J Cancer,* 43: 672-676, 1989.
20. Thompson HS, Davies ML, Holding FP, Fallon RE, Mann AE, O'Neill T, and Roberts JS. Phase I safety and antigenicity of TA-GW: a recombinant HPV6 L2E7 vaccine for the treatment of genital warts. *Vaccine,* 17: 40-49, 1999.
21. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief CJ, and Offringa R. 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,* 91: 612-618, 2001.
22. Kadish AS, Ho GY, Burk RD, Wang Y, Romney SL, Ledwidge R, and Angeletti RH. Lymphoproliferative responses to human papillomavirus (HPV) type 16 proteins E6 and E7: outcome of HPV infection and associated neoplasia. *J Natl Cancer Inst,* 89: 1285-1293, 1997.

Annemieke de Jong, Jeanette van der Hulst,
Gemma. Kenter, Jan Wouter Drijfhout,
Kees Franken, Peter Vermeij, Rienk Offringa,
Sjoerd van der Burg, Cornelis Melief

Int J Cancer. In Press

Rapid enrichment of HPV-specific polyclonal T cell populations for adoptive immunotherapy of cervical cancer

Abstract · The majority of cervical cancers are caused by human papillomavirus type 16 (HPV16). Cervical cancer is associated with an ineffective host immune response against the HPV16 oncoproteins, characterized by the lack of the strong E6-specific T-helper type 1 (Th1) immunity that is generally present in healthy individuals, the presence of improperly polarized HPV16E6- and E7-specific CD4+ T cells and increased numbers of regulatory T cells. Therefore, immunotherapeutic intervention is likely to require a modality that deletes the regulatory T cell component and enhances the HPV16-specific Type 1 T cell response. HLA-matched allogeneic stem cell transplantation may offer such a modality, because it involves the eradication of host immune cells and enables the transfer of donor-derived tumor-specific T cells to the patient. As a first step in the development of such a treatment, we evaluated the success rate of a protocol for enrichment of HPV16E6-specific CD4+ T cells from healthy donor PBMC on the basis of their IFNγ secretion. After a short in vitro stimulation with overlapping 30 amino acid long HPV16E6 peptides, we enriched the IFNγ-secreting cells by magnetic cell sorting. The obtained polyclonal CD4+ T cell populations recognized distinct epitopes within HPV16E6, as well as E6 protein, processed and presented by autologous professional antigen presenting cells. The described protocol proved successful in PBMC from more than half of the healthy adult blood donors. These HPV16E6-specific CD4+ T cells may turn out to be an essential component of future adoptive T cell therapy for advanced cervical cancer, by orchestrating CTL dependent and independent tumoricidal mechanisms.

Introduction

Cervical cancer is associated with persistent high-risk Human Papillomavirus (HPV) infection. It is the first major solid tumor that has been shown to be virally induced in essentially all cases, and HPV type 16 (HPV16) is the high-risk type most frequently found in cervical cancer [1]. The HPV16 oncoproteins E6 and E7 are constitutively expressed in tumor cells and are required to maintain the malignant phenotype [2]. Therefore, these proteins are attractive targets for immunotherapy and, as such, have inspired the development of immunotherapeutic strategies for the treatment of patients with cervical cancer [3].

Advanced cervical cancer is associated with tumor-related immunosuppression [4], the presence of increased numbers of regulatory T cells [5] and improperly polarized HPV-specific T cells [6, 7]. Under these circumstances, active therapeutic vaccination will likely fail to establish effective immunity. An alternative approach is a passive therapeutic modality involving the adoptive transfer of tumor-specific T cells. In murine tumor models, adoptive T cell therapy has proven successful for the eradication of established solid tumors [8, 9]. In patients with solid tumors, the adoptive transfer of tumor-specific T cells has mainly been applied in the treatment of metastatic melanoma. These protocols have shown a variable success rate [10], probably due to the fact that the majority of the protocols focused on the generation of large numbers of CD8+ T cells [11] and dismissed the tumor-specific CD4+ T cell population, which forms an essential component of the anti-tumor-immunity [12-14]. In addition, the autologous setting in which these adoptive T cell transfers are performed, involves two major drawbacks. Firstly, ex-vivo expanded patient-derived T cell populations (especially tumor infiltrating lymphocytes) may harbor improperly polarized and regulatory T cells that can hamper the treatment efficacy [15]. Secondly, pre-existing immunoregulatory mechanisms present in advanced cervical cancer patients may prevent the effector function of adoptively transferred tumor-specific T cells. To overcome the aforementioned barriers, HLA-matched allogeneic stem cell transplantation (allo-SCT) involving the eradication of host immune cells – including regulatory T cells – and enabling the administration of tumor-specific T cells of donor origin, may offer an alternative option. The infusion of donor lymphocytes following allo-SCT has proven relatively successful in the treatment of relapsing hematological malignancies [16, 17]. In these cases, effective therapy is often based on the in vivo induction of T cells specific for minor histocompatibility antigens expressed in host cells of hematological origin [18]. However, the incorporation of allo-SCT in the treatment of solid tumors has been less successful [19, 20]. This could be due to the lack of defined tumor-specific antigens, as well as tumor-induced tolerization of the donor-derived immune system [21, 22], which may occur during immune reconstitution. Cervical cancer has the advantage over several other solid tumors that it harbours well-defined tumor-specific antigens of viral origin. The allo-SCT can serve as a therapeutic platform to administer HPV16-specific T cells derived from the peripheral blood of the healthy stem-cell donor. The adoptive transfer of HPV16E6-specific Th1-type CD4+ T cells, which are readily detected in the peripheral blood of the majority of healthy subjects, but are often lacking in HPV16-positive cervical cancer patients [7, 23], may contribute to the anti-tumor immunity. Tumor-specific CD4+ Th1 type T cells have emerged as an essential component in anti-tumor immunity, fulfilling a multifactorial role, including the activation of antigen presenting cell (APC) 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, capable of exerting anti-tumor reactivity [12, 24]. Furthermore, the anti-angiogenic activity of CD4+ Th1 type T cell-derived IFNγ can reportedly mediate tumor rejection [25]. All of these activities orchestrated by tumor-specific CD4+ T cells are independent of MHC class II expression on the tumor.

As a first step in the development of a T cell based treatment modality, we set up a protocol for the enrichment of Th1 type HPV16E6-specific T cells from the peripheral blood of healthy blood

donors. This protocol involves brief in vitro stimulation of PBMC with long overlapping HPV16E6-derived peptides, followed by the enrichment of E6-specific memory T cells by magnetic cell sorting (MACS) on the basis of their IFNγ secretion [26]. This ensures the isolation of in vivo functionally selected T cells with the desired cytokine profile for anti-tumor immunity [27]. We here demonstrate the feasibility to obtain enriched polyclonal CD4+ T cell populations with multiple HPV16E6 specificities within a time span of 2-3 weeks.

Materials and Methods

Blood bank donors · Buffycoats and plasma from healthy blood donors were obtained after informed consent. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-amidotrizoate density gradient centrifugation. Citrated plasma was recalcified by adding 0.011 mM Ca^{2}+ (=3.3 mg calciumlaevulate) per ml of plasma.

Antigens and antibodies · A set of 8 overlapping peptides spanning the HPV16E6 antigen was used for the in vitro stimulation of memory T cell responses. The length of the peptides was 32 amino acids with an overlap of 14 residues. For the experiments with influenza matrix protein as a model antigen, a set of 16 peptides with a length of 30 amino acids, and an overlap of 15 amino acids (C-terminal peptide with an overlap of 18 aa), were derived from influenza A/PR/8/34 (M1). The peptides were synthesized and dissolved as described previously [28]. Recombinant HPV16 E6 protein and HPV16 E7 protein (the latter protein served as negative control protein in proliferation assays) were produced in recombinant *E. coli* transformed with pET-19b-HPV16 E6 and pET-19b-HPV16 E2 [29]. The antibodies for flowcytometric analysis were obtained from BD Biosciences and were used in the indicated dilutions: anti-CD4 FITC (cat.no. 345768, 1:12); anti-CD8 APC (cat.no. 345775, 1:50); anti-CD8 PerCP (cat.no.345774, 1:12); anti-CD3 FITC (catno. 345763, 1:20); anti-CD16 PE (cat.no. 347617, 1:20); anti-IFNγ PE (554552, 1:50). The antibodies used for T cell expansion were anti-CD3 (Orthoclone, OKT3, Janssen-Cilag) and anti-CD28 (BD Biosciences, cat.no. 348040).

Isolation of antigen-specific T cells directly ex-vivo by magnetic cell sorting (MACS) based on IFNγ secretion · 9×10^{7} freshly isolated PBMC derived from healthy blood donors were suspended in 18 ml IMDM (Cambrex, Verviers, Belgium) supplemented with 10% recalcified autologous plasma and seeded at a density of 15×10^{6} per well in a 6 well plate (Costar, Corning, USA). The total set of overlapping HPV16 E6- or influenza M1-derived peptides were added at a concentration of 2.5 µg/ml/peptide. An unstimulated sample was taken along as a negative control for FACS analysis. After 16 hours, the cells were harvested and the IFNγ-secreting c cells were isolated by MACS (IFNγ secretion assay, Miltenyi Biotech, Bergisch Gladbach, Germany), according to manufacturers' instruction. The samples for FACS analysis the cells were counterstained with anti-CD4 FITC, and propidium iodide (0.4 µg/ml) was added prior to analysis. The isolated cells were cultured with irradiated autologous PBMC at a ratio of 1:10 (isolated cell culture:irradiated PBMC) and 5% T cell growth factor (TCGF, Zeptometrix, NY, USA) without any further addition of antigen. TCGF was used as a source of IL-2 in the cell cultures. TCGF is not a well-defined source of cytokine, and therefore not directly suitable for clinical ex vivo applications. For several cell cultures we compared TCGF with the combination of IL-2 (10 IU/ml, Proleukin, Chiron) and IL-15 (5 ng/ml, PeproTech, USA). IL-15 has been described to prevent T cell apoptosis [30], and we previously observed that the combination of IL-2 and IL-15 resulted in an increased expansion of memory T cells compared to IL-2 alone (unpublished observations). Both TCGF and IL-2/IL-15 resulted in similar percentages of antigen-specific cells, but TCGF was superior to IL-2/IL-15 in terms of cell expansion.

Isolation of antigen-specific T cells after extended period of in vitro stimulation · 9×10^{7} freshly isolated PBMC were incubated with HPV16E6-derived peptides as described above. One day after

stimulation, TCGF (Zeptometrix, NY, USA) was added at a final concentration of 5%. The enrichment procedure was performed 8-11 days after stimulation. Two days before enrichment autologous monocytes were derived from PBMC by adherence to a 24-well plate during 2 hours at 37°C in x-vivo15 medium (Cambrex). Adherent monocytes were cultured with GM-CSF (Leucomax, Novartis) 500 IU/ml to increase their accessory cell function [31], and then used as antigen presenting cells for the second stimulation of the T cell cultures. The autologous monocytes were incubated with E6 peptides (1 µg/ml), after which the T cell cultures were added at a ratio of approximately 10:1 (T cell:monocyte). An unstimulated sample (incubated with monocytes without peptides) was taken along as a negative control for FACS analysis. Sixteen hours after stimulation, the T cells producing IFNγ in response to the E6 peptides were enriched by MACS (IFNγ secretion assay, Miltenyi Biotech, Bergisch Gladbach, Germany), according to manufacturers' instruction. The isolated cells were cultured with irradiated autologous PBMC at 1:10 ratio and 5% TCGF without any further addition of antigen.

Intracellular cytokine staining (ICS) · Autologous monocytes were isolated from PBMC by adherence to a flat-bottom 96-wells plate during 2 hours in x-vivo15 medium (Cambrex) at 37°C. Adherent monocytes were cultured for 2 days with GM-CSF (Leucomax, Novartis) 500 IU/ml, before they were used as APC in the ICS assay. 50 µl of peptide solution (10 µg/ml) or 50 µl of medium (negative control) was added to the monocytes, prior to the addition of 50 µl of cell suspension (4×10^6 cells/ml). After 1 hour of incubation at 37°C, the Golgi-mediated secretion of cytokine was inhibited by the addition of 100 µl of Brefeldin A (Sigma; final concentration of 10 µg/ml), after which the cells were incubated for another 5 hours. The cells were then harvested, transferred into a V-bottom 96-wells plate, washed twice with ice-cold PBS and fixed with 50 µl paraformaldehyde 4% for 4 minutes on ice. Following fixation, the cells were washed once with cold PBS and once with PBS/NaAz 0.02%/BSA 0.5%/Saponin 0.1% for permeabilization. This was followed by an incubation in 50 µl PBS/NaAz 0.02%/BSA 0.5%/Saponin 0.1%/FCS 10% for 10 minutes on ice. Cells were washed twice with PBS/NaAz 0.02%/BSA 0.5%/Saponin 0.1% and supernatant was removed before 25 µl of PBS/NaAz 0.02%/BSA 0.5%/Saponin 0.1% containing 2 µl of anti-CD4 FITC, 0.5 µl IFNγ PE and 2 µl anti-CD8 PerCP was added. Following 30 minutes of incubation on ice, the cells were washed, suspended in 100 µl 1% paraformaldehyde and analysed by flow cytometry.

Proliferation assay · The polyclonal T cell cultures were tested for recognition of E6 peptides (5 µg/ml/peptide) and protein (10 µg/ml) in a 3-day proliferation assay. Autologous adherent monocytes in a flat-bottom 96-well plate were used as antigen presenting cells. Per well 25,000 responder cells were seeded, and proliferation was measured in triplicate by [^{3}H]-thymidine incorporation.

Expansion of polyclonal T cell cultures · Round-bottom 96-well culture plates were coated overnight with anti-CD3 and anti-CD28 1 µg/ml/Ab in PBS at 4°C. 10^6 E6-specific cells were mixed with 10^7 irradiated autologous PBMC and TCGF 5% in 10 ml IMDM supplemented with 10% autologous plasma. The cells were seeded at 10^5 cells/well in a round-bottom 96-well culture plate. After 4 days the stimulated cells were transferred to an uncoated culture plate. The cell cultures were split whenever required.

Results

Enrichment of influenza M1-specific T cells after stimulation with 30-mer peptides

In order to set up a protocol for the enrichment of antigen-specific T cells, we first made use of the memory T cell population specific for the influenza M1 protein, which is readily detectable in the peripheral blood of most healthy donors. Freshly isolated PBMC from healthy blood donors were stimulated for 16 hours with a set of synthetic 30-mer peptides spanning the M1 protein, after which the IFNγ producing cells were isolated by MACS. The frequencies of the IFNγ-secreting cells in the CD4+ fraction in response to M1 peptides varied from <0.01% (detection limit of our FACS analysis)

Table 1. Enrichment of influenza M1-specific T cells after 16 hr stimulation with overlapping M1 peptides

donor	CD4-positive PBMC control	+M1 peptides	after MACS	CD4-negative PBMC control	+M1 peptides	after MACS
1	<0.01[a]	0.08	7.4	0.05	0.42	14.9
2	0.01	0.07	7.2	0.05	0.20	12.4
3	0.02	0.01	13.7	0.06	0.09	22.3
4	0.02	<0.01	2.1	0.05	0.06	4.4
5	0.02	0.01	1.0	0.03	0.05	7.7
6	0.01	0.02	2.2	0.03	0.09	7.9
7	0.01	0.06	7.9	0.05	0.15	8.4
8	0.01	0.07	9.1	0.04	0.05	6.5
9	0.01	0.02	8.7	0.01	0.06	13.5
10	0.01	0.05	11.3	0.05	0.07	5.3

[a] Indicated are the percentages of IFNγ+ cells in the CD4 positive and negative fraction of PBMC, before and after enrichment by magnetic cell sorting (MACS). The controls represent the percentages of IFNγ-secreting cells in the absence of the M1-derived peptides.

to 0.08 % (Table 1 and Figure 1). These T cell frequencies were increased up to 13.7 % after magnetic sorting of the IFNγ-secreting cells (Table 1). No clear correlation was observed between the pre-enrichment percentages of influenza-specific IFNγ-secreting cells and the magnitude of enrichment.

The enriched IFNγ-secreting cells were expanded without further antigenic stimulation for approximately 12 days, after which the success of the enrichment procedure was determined by the enumeration of M1-specific T cells using intracellular cytokine staining. The use of a complete set of overlapping peptides covering the M1 protein, allowed the establishment of an immunological 'fingerprint', as illustrated by our data concerning charting of the CD4+ T cell immunity against various antigens [23, 32, 33]. For a given antigen, these 'fingerprints' differ considerably between PBMC isolates from different donors, indicating that the peptides within a certain antigen serve as each other's controls. T cell cultures derived from all 10 donors displayed CD4+ responses against multiple M1-derived peptides (Table 2 and Figure 2). The total percentage of M1-specific CD4+ T cells was in all cases higher after culture than directly after isolation, indicating the selective outgrowth of the stimulated M1-specific memory CD4+ T cells. Seven out of 10 donors also showed CD8+ responses against one or more M1-derived peptides, indicating that the protocol resulted in the enrichment of M1-specific T cells of both CD4+ and CD8+ lineage (Table 2 and Figure 2). However, the percent-

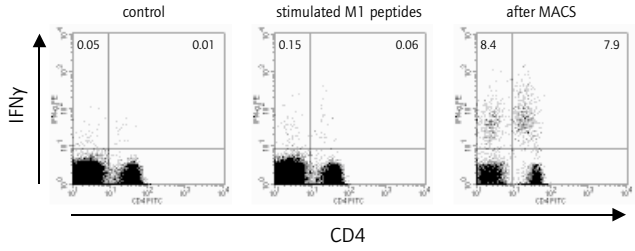

Figure 1. Enrichment of IFNγ+ influenza M1-specific cells after overnight peptide stimulation. Freshly isolated PBMC were stimulated for 16 hours with a set of overlapping peptides spanning the influenza M1 protein. One representative example is shown (Donor 7, Table 1). Both in the CD4 positive and negative fraction IFNγ was secreted, which was increased upon peptide stimulation. The IFNγ+ cells were strongly enriched after magnetic cell sorting (MACS). The percentages of IFNγ+ cells in the CD4 positive and the CD4 negative PBMC fraction are depicted in the corresponding corners of the figures.

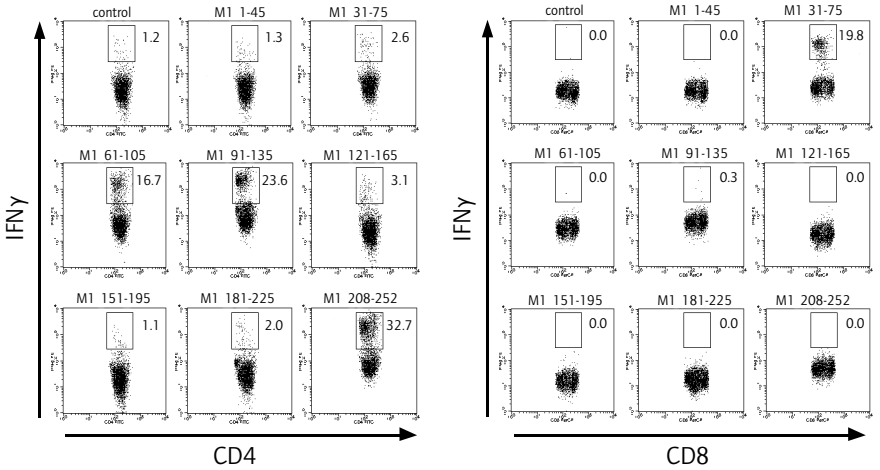

Figure 2. Both CD4+ and CD8+ T cells specific for influenza M1 are present in isolated cell cultures. The specificities of the polyclonal M1-specific cultures were tested in an intracellular IFNγ staining assay against 8 pools of influenza M1 peptides. The peptide pools consisted of 2 peptides per pool and are indicated by the first and last amino acid of the region of the protein covered by the peptides. One representative example is shown (Donor 4, Table 2), gated for CD4+ and for CD8+ T cells. Multiple peptide specificities were observed in the CD4+ T cell fraction, whereas in the CD8+ T cell fraction cells specific for one pool $M1_{31\text{-}75}$ was observed.

Table 2. Influenza M1-specific T cells in isolated cell cultures.

donor	control	M1 1–45	31–75	61–105	91–135	121–165	151–195	181–225	211–255	peptide pool
	CD4+									
1	0.7[a]	**9.2**	**11.7**	**11.4**	**7.6**	**14**	**18.5**	**27.7**	**18.5**	·
2	1.1	·	·	·	·	·	·	·	·	**37.5**[b]
3	0.4	0.8	0.9	1.0	**39.4**	**1.8**	**5.7**	**3.0**	**4.3**	·
4	1.2	1.3	2.6	**16.7**	**23.6**	3.1	1.1	2.0	**32.7**	·
5	12.7	·	·	·	·	·	·	·	·	**73.6**
6	0.3	0.4	5.2	**4.4**	**4.5**	0.1	**5.0**	**2.3**	**3.0**	·
7	0.1	0.2	0.1	**2.1**	**1.8**	0.2	0.1	**26.1**	**23.8**	·
8	0.6	·	·	·	·	·	·	·	·	**15.8**
9	0.2	**3.9**	**21.0**	**4.6**	**14.7**	**2.1**	**11.2**	**2.7**	**4.1**	·
10	0.1	**17.9**	**22.7**	**9.4**	**5.9**	0.2	**7.1**	**0.8**	**12.2**	·
	CD8+									
1	0.2	0.2	**13.0**	0.4	0.3	0.3	0.4	0.2	0.4	·
2	1.0	·	·	·	·	·	·	·	·	**15.8**
3	0.7	0.6	1.9	2.0	**4.5**	**9.6**	0.8	0.6	0.9	·
4	<0.1	<0.1	**19.8**	<0.1	**0.3**	<0.1	<0.1	<0.1	<0.1	·
5	–[c]	·	·	·	·	·	·	·	·	·
6	0.2	0.2	0.4	0.1	0.1	0.2	0.1	0.1	0.1	·
7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	·
8	0.3	·	·	·	·	·	·	·	·	**2.0**
9	0.5	0.3	**9.9**	0.5	**3.8**	**1.9**	**1.7**	0.5	0.7	·
10	<0.1	<0.1	0.1	<0.1	**0.3**	**0.3**	<0.1	<0.1	<0.1	·

[a] Influenza M1-specific T cells were analysed by intracellular IFNγ staining 12 days after enrichment, and prior to further in vitro restimulation. Indicated are the percentages of cells producing IFNγ in the CD4+ respectively CD8+ T cell fraction. Positive responses, defined as a percentage of IFNγ+ T cells >3× unstimulated control, are depicted in bold. The overlapping M1-derived peptides were pooled by 2 peptides, each pool indicated by the first and last amino acid of the region covered by the 2 peptides.

[b] Donors 2, 5 and 8 were analysed against a pool consisting of all M1 peptides because of limited cell numbers.

[c] Insufficient cell number for analysis

ages of M1-specific T cells in the CD8+ fraction were in all cases lower than in the CD4+ fraction, in contrast to their frequencies in the peripheral blood (unpublished observations). This suggests that the described protocol involving ex vivo stimulation with long peptides favors the enrichment and outgrowth of antigen-specific CD4+ T cells.

Peptide stimulation enables the enrichment of HPV16E6-specific CD4+ T cells.

Having shown that M1-specific T cells can be isolated directly ex-vivo using long overlapping peptides, we applied the same protocol for the isolation of HPV16 E6-specific T cells. Previously, we have demonstrated the presence of Th1 type HPV16E6-specific T cells in the CD4+CD45RO+ PBMC fraction of the majority of healthy individuals ([23] and unpublished data). This suggests that a similar approach – involving a short in vitro stimulation and enrichment IFNγ-secreting memory T cells – is feasible in a panel of randomly selected blood donors.

Both upon peptide stimulation and after enrichment by MACS, the percentages of IFNγ+ T cells in the CD4+ fraction were significantly lower in the E6 than in the M1-stimulated cell populations (Table 3). This corresponds with our previous observation that the frequency of M1-specific T cells is higher than E6-specific T cells in PBMC of healthy subjects [23]. Following in vitro culture after enrichment, reactivity against one or more E6-derived peptides was observed in the CD4+ fraction of 5 out of 8 enriched T cell cultures (Table 4). In general, the percentages of E6-specific CD4+ T cells were approximately 1% of the CD4+ T cells, with one exception showing as much as 9.8% of the CD4+ T cells specific for $E6_{73\text{-}104}$ (Figure 3).

In contrast to the M1-stimulated cultures, no E6-specific CD8+ T cells were detected after culture (Table 4 and Figure 3), despite the occasional detection of an increase in IFNγ+ cells in the CD4 negative fraction upon stimulation of PBMC with E6 peptides (Table 3). Direct ex-vivo flow cytometric analysis of PBMC revealed that IFNγ was generally secreted by a CD4–CD8– double negative population (data not shown). These CD4–CD8– cells were detected in both the M1 and E6-specific MACS isolated cell populations and they co-expanded upon culture. Further analysis revealed that the CD4–CD8– cell population was CD3–CD16+, consistent with an NK cell like phenotype. The percentage of these cells after culture was variable and did not differ significantly between the E6 and M1-stimulated cultures (Figure 4).

In conclusion, the ex-vivo stimulation of healthy donor PBMC with HPV16 E6-derived peptides allows the isolation of E6-specific memory CD4+ T cells but not CD8+ T cells. The relatively low percentages of E6-specific CD4+ T cells in the resulting cell cultures prompted the further optimisation of the enrichment protocol.

Table 3. Enrichment of HPV16E6-specific T cells after 16 hr stimulation with overlapping E6 peptides.

donor	CD4-positive PBMC control	+E6 peptides	after MACS	CD4-negative PBMC control	+E6 peptides	after MACS
3	0.02[a]	<0.01	0.1	0.06	0.05	3.8
4	0.02	<0.01	0.3	0.05	0.03	3.3
6	0.01	0.02	4.2	0.03	0.10	16.6
7	0.01	0.01	0.3	0.05	0.05	6.4
9	0.01	<0.01	1.9	0.01	0.05	6.8
10	<0.01	0.02	0.8	0.05	0.05	3.9

[a] Indicated are the percentages of IFNγ+ cells in the CD4 positive and negative fraction of PBMC, before and after enrichment by magnetic cell sorting (MACS). The controls represent the percentages of IFNγ-secreting cells in the absence of the E6-derived peptides.

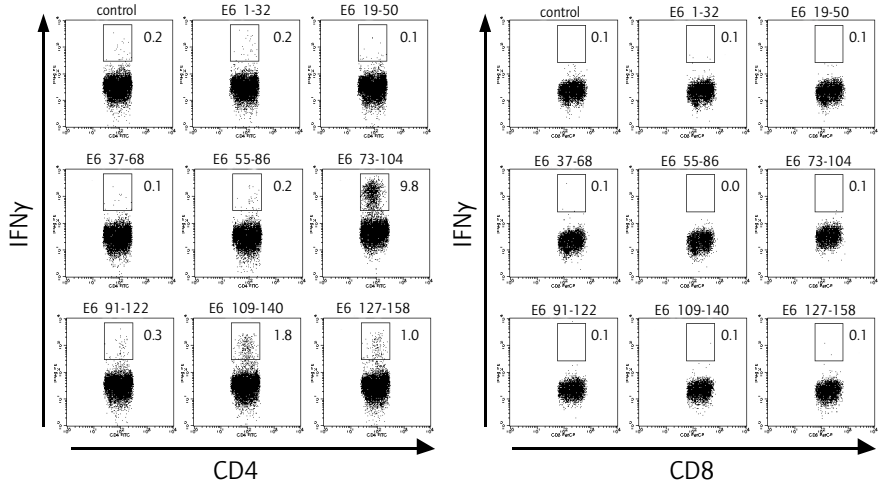

Figure 3. HPV16E6-specific CD4+ T cells present in isolated cell cultures. The specificities of the polyclonal E6-specific cultures, isolated after 16-hour stimulation with E6 peptides, were tested in an intracellular IFNγ staining assay against 8 peptides spanning the HPV16 E6 antigen. One example (Donor 3, Table 4), with the highest percentage of E6-specific T cells, is shown, gated for CD4+ and for CD8+ T cells. The CD4+ fraction harbours T cells specific for multiple E6 epitopes.

Table 4. HPV16E6-specific T cells in isolated cell cultures.

donor	control	E6 1–32	19–50	37–68	55–86	73–104	91–122	109–140	127–158	peptide pool
	CD4+									
3	0.2[a]	0.2	0.1	0.1	0.2	**9.8**	0.3	**1.8**	**1.0**	·
4	6.6	4.3	4.5	5.1	5.1	6.6	4.7	6.4	4.8	·
6	0.1	0.1	0.2	0.2	0.1	**1.8**	0.2	0.2	0.1	·
7	<0.1	0.1	**1.6**	0.1	0.1	0.1	0.1	**1.3**	0.1	·
9	–[b]	·	·	·	·	·	·	·	·	·
10	1.6	·	·	·	·	·	·	·	·	1.9[c]
11	0.2	**0.6**	·	**0.6**	·	0.2	·	0.2	·	·
12	0.1	<0.1	·	0.1	·	**0.9**	·	**0.7**	·	·
	CD8+									
3	0.1	0.1	0.1	0.1	<0.1	0.1	0.1	0.1	0.1	·
4	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	·
6	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1	·
7	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.2	·
9	–[b]	·	·	·	·	·	·	·	·	·
10	0.7	·	·	·	·	·	·	·	·	0.9[c]
11	0.2	0.2	·	0.2	·	0.2	·	0.3	·	·
12	0.2	0.3	·	0.3	·	0.3	·	0.3	·	·

[a] HPV16E6-specific T cells were analysed by intracellular IFNγ staining 12 days after enrichment, and prior to further in vitro restimulation. Indicated are the percentages of cells producing IFNγ in the CD4+ respectively CD8+ T cell fraction. Positive responses, defined as a percentage of IFNγ+ T cells >3× unstimulated control, are depicted in bold. The overlapping E6–derived peptides were tested individually and are indicated by the first and last amino acid.

[b] Insufficient cell amount for analysis

[c] Cell culture from donor 10 was analysed against a pool consisting of all E6 peptides, and cell cultures from donors 11 and 12 were analysed against pools of 2 peptides (1–32 + 19–50 etc), because of a limited amount of cells. Donors 11 and 12 are not depicted in Table 3, because no FACS analysis was performed of the enrichment of these cell cultures.

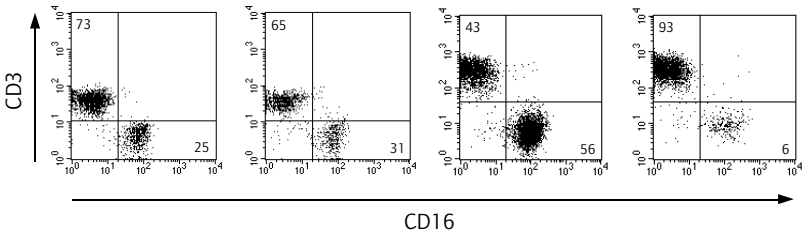

Figure 4. Isolation and outgrowth of NK cells in isolated cell cultures. Both in the E6- and M1-stimulated PBMC, enrichment of IFNγ+ cells after 16 hours of stimulation resulted in the isolation of NK-like cells in addition to antigen-specific cells. These cells grew out to a variable extent upon culture, and the percentages of NK-like cells after culture did not differ significantly between M1 and E6-stimulated cultures. Four representative examples are shown of cell cultures approximately 10 days after enrichment. The cell cultures were stained for CD3 and CD16, and the percentages of CD3+ respectively CD16+ cells are depicted in the corners of the figures.

Optimised protocol for the enrichment of HPV16E6-specific T cells

In order to increase the frequency of HPV16E6-specific CD4+ T cells, the stimulation period before enrichment was extended. PBMC from 17 healthy blood donors were stimulated for approximately 10 days with E6 peptides and −16 hours after an additional peptide stimulation − the IFNγ-secreting cells were isolated by MACS (time scheme Figure 5). The extended stimulation period before isolation generally resulted in a higher frequency of E6-specific CD4+ T cells before isolation (mean 2.4% of CD4+ T cells, range 0–17.4%, n=17), compared to the cultures that were stimulated for 16 hours only (mean 0.01%, Table 3). Magnetic cell sorting of these cell populations resulted in a strongly enriched IFNγ+ CD4+ T cell fraction (Figure 6). In contrast to direct ex-vivo isolation of IFNγ-secreting cells, which often showed high percentages of NK-like cells, a stimulation period before isolation generally resulted in the isolation and outgrowth of >95% T cells (data not shown).

No E6-specific T cells could be isolated from PBMC of 4 out of 17 donors and the isolated cell populations of another 3 donors showed no E6-specific proliferative reactivity after the culture period. The cultures of the remaining 10 donors demonstrated strong E6-specific responses with multiple specificities (Table 5). Furthermore, all T cell cultures displaying E6 peptide reactivity also recognized recombinant E6 protein after exogenous uptake and presentation by APC (Table 5). This capacity to recognize naturally processed E6 epitopes is a prerequisite for the E6-specific CD4+ T cells in order to activate professional APC that have taken up and processed E6 antigen in vivo. In general, the proliferation in response to protein-pulsed monocytes was lower than in response to peptide-pulsed monocytes, possibly due to the fact that on a molar basis the amount of peptide used was higher than that of protein.

We then questioned whether it was possible to expand the isolated E6-specific T cell cultures without loss of antigen-specificity using plate-bound anti-CD3/CD28 (Figure 5). Following 4 days of incubation, the stimulated cells were transferred to non-coated plates and cultured for another 10 days. By this method we obtained up to 10^8 cells, corresponding with an expansion of 20-75 fold. In 9 out of 10 cultures the E6-specificity was retained after expansion, with percentages of antigen-specific T cells in the CD4+ fraction ranging from 13.6–45% (Figure 7a) as determined by intracellular cytokine staining. Five out of 8 of the evaluable E6-specific T cell cultures retained a broad reactivity against multiple peptides, whereas 3 cultures showed loss of several specificities (Table 5, Figure 7b and data not shown). The cell culture of donor D25 was only tested against peptide pool in the proliferation assay, therefore not allowing conclusions regarding loss of specificity. Taken together, aspecific expansion by plate-bound anti-CD3/CD28 generally retains a high percentage of HPV16E6-specific T cells. Occasionally, the breadth of the T cell response may be reduced, which suggests that limited in vitro manipulation is preferred after the enrichment of HPV16E6-specific T cells.

Table 5. Proliferation assay of HPV16E6-specific T cell cultures.

donor	E6 1-32	19-50	37-68	55-86	73-104	91-122	109-140	127-158	E2 1-30	E6 protein	E2 protein
14	**7.6**	**6.0**	**10.3**	**13.1**	**13.8**	**8.7**	**4.0**	**10.6**	2.2	**5.7**[a]	1.9
15	1.2	**4.0**	**6.0**	**14.3**	**13.9**	**9.9**	**10.4**	**13.8**	1.0	**10.0**	2.7
16	1.8	2.2	**13.6**	**10.5**	**13.6**	**14.8**	1.7	1.5	1.1	**4.3**	0.6
21	1.6[b]	·	**14.6**	·	1.7	·	**7.6**	·	1.5	**5.0**	**3.1**
22	**3.4**	·	**5.8**	·	**4.2**	·	**6.6**	·	1.3	**5.1**	1.2
24	0.9	0.8	1.0	**6.7**	**19.7**	0.9	**5.4**	**10.3**	1.1	**7.4**	1.9
25	**4.0**	·	·	·	·	·	·	·	1.0	**6.9**	2.2
26	1.1	1.2	1.1	2.4	**3.2**	0.9	**3.8**	**4.7**	0.9	**3.0**	0.7
27	1.4	1.5	1.1	1.4	**5.2**	0.9	1.8	**3.2**	1.2	**4.5**	1.2
28	0.9	0.9	1.3	2.3	2.9	2.1	1.0	**4.1**	1.1	**7.5**	1.0

[a] HPV16E6-specific T cell cultures were tested in short-term proliferation assays for the recognition of E6 peptides and naturally processed E6 protein. HPV16E2-derived peptide and recombinant E2 protein were used as negative controls. Indicated are the stimulation indices (SI), defined as proliferation in the test wells divided by the proliferation in the medium only control wells. In bold are positive proliferative responses with a SI > 3.

[b] Cell cultures from donors 21 and 22 were tested against pools of 2 peptides, and cell culture from donor 25 was tested against the pool of all E6 peptides, because of limited amount of cells.

The percentages of CD8+ T cell were low in the isolated cell populations and these cells showed variable outgrowth upon expansion. Intracellular cytokine staining revealed that none of the cell cultures displayed HPV16E6-specific IFNγ secretion in the CD8+ fraction (data not shown). In conclusion, the stimulation of PBMC with HPV16E6-derived peptides for approximately 10 days before isolation of the antigen specific cells results in E6-specific CD4+ T cell cultures with a better enrichment than upon direct ex-vivo isolation. The HPV16E6-specific cell cultures generally display a broad specificity, and recognize naturally processed E6 protein.

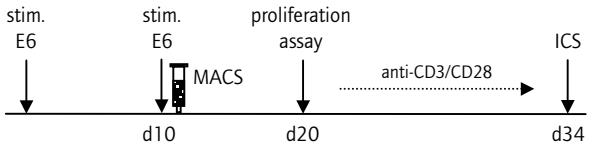

Figure 5. Time scheme of culture and analysis of HPV16E6-specific T cells.

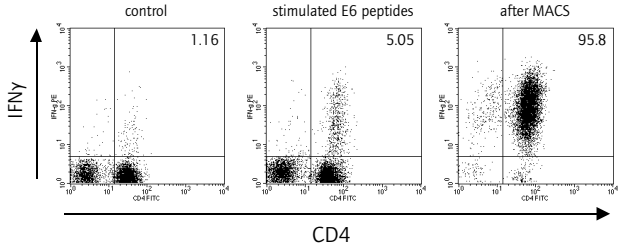

Figure 6. Increased purity of HPV16E6-specific CD4+ T cells by extended culture period before isolation of antigen-specific cells. After a 10 day culture period of PBMC with HPV16 E6 peptides, the T cells were restimulated for 16 hours after which the IFNγ+ fraction was isolated. One representative example is shown. IFNγ+ cells were observed mainly in the CD4+ fraction and these cells were strongly enriched upon magnetic cell sorting. In the right-hand corner of each figure the percentage of IFNγ+ cells in the CD4+ fraction is depicted.

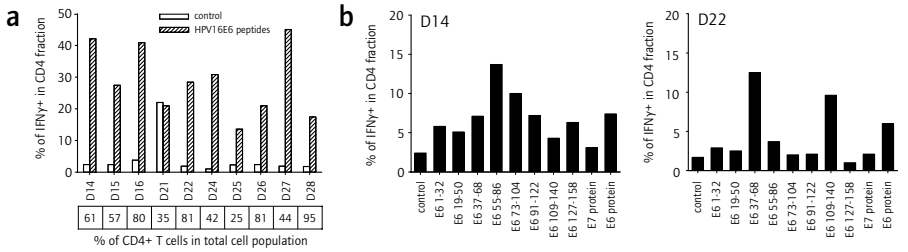

Figure 7. HPV16E6-specific T cell cultures retain antigen-specificity after expansion by plate-bound anti-CD3/CD28 stimulation. Polyclonal HPV16E6-specific T cell cultures derived from 10 different donors were expanded by anti-CD3/CD28 stimulation for approximately 14 days after which the percentages of HPV16E6-specific CD4+ T cells were determined by intracellular IFNγ staining. The cell cultures were stimulated with autologous monocytes pulsed with a pool of E6 peptides, and a pool of E2-derived peptides was taken along as a negative control (a). The percentages of E6-specific T cells in the CD4+ fraction are depicted in the graph. The percentages of CD4+ T cells in the total cell culture were variable and are indicated in the graph below each individual donor. The T cell cultures were also tested against the individual peptides. Compared with the specificity before expansion (Table 5), several cell cultures retained a broad antigen-specificity, whereas others showed a partial loss of specificity after expansion. Representative examples are shown (b). Indicated are the percentages of IFNγ+ cells in the CD4+ fraction.

Discussion

In view of the limited treatment options and poor prognosis of advanced HPV16+ cervical cancer, it is of interest to investigate whether allo-SCT followed by the transfer of donor-derived HPV16-specific T cells can make a significant clinical impact on this disease. We have studied the feasibility to enrich T cells that recognize the viral tumor antigen HPV16E6 from the peripheral blood of healthy donors.

Enrichment of HPV16-specific T cells from the peripheral blood of in vivo primed healthy blood donors based on IFNγ secretion has several advantages. First of all, Th1 type responses – associated with the pro-inflammatory cytokine IFNγ – are of major importance in anti-tumor immunity [13, 34]. Therefore, enrichment of antigen-specific T cells secreting this cytokine ensures selection of T cells with the desired cytokine profile [27]. Secondly, in contrast to protocols involving the in vitro induction of HPV-specific T cells by multiple in vitro stimulations [35, 36], direct ex-vivo stimulation results in the reactivation of memory T cells that have been selected for function in vivo. Finally, enrichment of antigen-specific T cells is desirable in the light of minimizing the alloreactivity that may occur following the adoptive transfer of donor T cells. Although this aspect has not been addressed in the present study, the inclusion of an HPV16-specific enrichment step will most likely minimize the risk of severe graft-versus-host-disease (GVHD). Donor-derived polyclonal T cell lines specific for cytomegalovirus (CMV) were shown to establish effective suppression of CMV disease without causing severe GVHD [37]. Although the required number of HPV16-specific T cells for adoptive transfer remains to be established, we obtained similar numbers of specific T cells as described in several successful adoptive cellular therapy protocols [37, 38]. These studies suggest that large numbers of T cells are not a prerequisite for effective adoptive T cell therapy, as long as the in vivo expansion capacity of the cells is retained.

The stimulation of healthy donor-derived PBMC with influenza M1 or HPV16E6-derived peptides for a short period in vitro results in the reactivation of specific memory T cells. With the influenza M1 antigen we demonstrated that stimulation of PBMC with long overlapping peptides allows the isolation of both CD4+ and CD8+ memory T cells specific for M1. With the same approach for the isolation of HPV16E6-specific T cells, we were unable to isolate CD8+ T cells specific for the E6 antigen from these healthy donors. A major reason for the absence of E6-specific CD8+ T cells in the isolated cell cultures could be that HPV16E6-specific memory CD8+ T cells are present at very low frequency

in the peripheral blood of healthy individuals, as has been shown for T cells against the E7 antigen [23, 39], and that the applied in vitro method relying on the use of long peptides – requiring uptake and cross-presentation – is only suitable for the stimulation of strong, high frequency memory CD8+ responses. In order to increase the frequencies of the HPV16-specific T cells in the peripheral blood, vaccination of the stem cell donors could be considered. Several clinical grade HPV16 vaccines are currently available or being developed [40-42]. These vaccines have the capacity to boost both E6 and E7-specific immunity, thereby facilitating the isolation of T cells against both HPV16 oncoproteins. Furthermore, in some cases we failed to isolate or expand HPV-specific CD4+ T cells from the healthy subjects. On one hand, this may be due to the fact that these individuals simply have not encountered HPV16, hence no memory T cell immunity was induced. On the other hand, the number of isolated cells was in some cases too small to allow proper expansion within 2 weeks. Vaccination of stem cell donors may thus also facilitate an increase in the number of individuals from whom HPV-specific T cells can successfully be enriched and expanded.

Tumor-specific CD4+ T cells have been shown to play a pivotal role in anti-tumor immunity [12-14, 43, 44]. In the case of HPV16-positive cervical cancer, future studies are required to address the efficacy of donor-derived HPV16E6-specific CD4+ T cells in the allo-SCT setting. Ideally, these Th1 type T cells contribute to the establishment of an effective anti-tumor immune response by proper polarization and maturation of dendritic cells, thereby preventing tumor-induced tolerization of newly generated T cells [21, 22], and supporting the activation of CD8+ CTL responses against HPV16 or other unique tumor antigens. Furthermore, the HPV-specific CD4+ T cells at the site of the tumor may attract and activate innate immune effectors, capable of exerting anti-tumor activity [24].

In conclusion, by providing a method for the rapid enrichment HPV16E6-specific CD4+ T cells from healthy donor PBMC, this study is a first step in the development of a T cell-based therapy for advanced cervical cancer in an allo-SCT setting.

Acknowledgements · We thank all healthy blood donors participating in this study. We also thank W. Benckhuijsen for peptide synthesis, and Prof. F. Falkenburg for critical reading of the manuscript. This work was supported by a grant from the Dutch Cancer Society (NKB:RUL 99-2024 to R.O. and C.J.M.M).

References

1. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, Schiffman MH, Moreno V, Kurman R, and Shah KV. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J Natl Cancer Inst*, 87: 796-802, 1995.
2. zur Hausen H. Papillomavirus infections-a major cause of human cancers. *Biochim Biophys Acta*, 1288: F55-78, 1996.
3. Galloway DA. Papillomavirus vaccines in clinical trials. *Lancet Infect Dis*, 3: 469-475, 2003.
4. Ressing ME, van Driel WJ, Brandt RM, Kenter GG, de Jong JH, Bauknecht T, Fleuren GJ, Hoogerhout P, Offringa R, Sette A, Celis E, Grey H, Trimbos BJ, Kast WM, and Melief CJ. 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*, 23: 255-266, 2000.
5. Fattorossi A, Battaglia A, Ferrandina G, Buzzonetti A, Legge F, Salutari V, and Scambia G. Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients. *Gynecol Oncol*, 92: 106-115, 2004.
6. Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, and Huang SC. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol*, 167: 2972-2978, 2001.
7. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, Kenter G, Offringa R, and van der Burg SH. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T cell immunity against early antigens E2 and E6. *Cancer Res*, 64: 5449-5455, 2004.
8. Vierboom MP, Nijman HW, Offringa R, van der Voort EI, van Hall T, van den Broek L, Fleuren GJ, Kenemans P, Kast WM, and Melief CJ. Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. *J Exp Med*, 186: 695-704, 1997.

9. Melief CJ, and Kast WM. Efficacy of cytotoxic T lymphocytes against virus-induced tumors. *Cancer Cells,* 2: 116-120, 1990.
10. Dudley ME, and Rosenberg SA. Adoptive-cell-transfer therapy for the treatment of patients with cancer. *Nat Rev Cancer,* 3: 666-675, 2003.
11. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, and Greenberg PD. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc Natl Acad Sci U S A,* 99: 16168-16173, 2002.
12. Toes REM, Ossendorp F, Offringa R, and Melief CJM. CD4 T cells and their role in antitumor immune responses. *J Exp Med,* 189: 753-756, 1999.
13. Ossendorp F, Toes RE, Offringa R, van der Burg SH, and Melief CJ. Importance of CD4(+) T helper cell responses in tumor immunity. *Immunol Lett,* 74: 75-79, 2000.
14. Pardoll DM, and Topalian SL. The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol,* 10: 588-594, 1998.
15. Wang HY, Lee DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, and Wang RF. Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy. *Immunity,* 20: 107-118, 2004.
16. Marks DI, Lush R, Cavenagh J, Milligan DW, Schey S, Parker A, Clark FJ, Hunt L, Yin J, Fuller S, Vandenberghe E, Marsh J, Littlewood T, Smith GM, Culligan D, Hunter A, Chopra R, Davies A, Towlson K, and Williams CD. The toxicity and efficacy of donor lymphocyte infusions given after reduced-intensity conditioning allogeneic stem cell transplantation. *Blood,* 100: 3108-3114, 2002.
17. Badros A, Barlogie B, Morris C, Desikan R, Martin SR, Munshi N, Zangari M, Mehta J, Toor A, Cottler-Fox M, Fassas A, Anaissie E, Schichman S, Tricot G, and Aniassie E. High response rate in refractory and poor-risk multiple myeloma after allotransplantation using a nonmyeloablative conditioning regimen and donor lymphocyte infusions. *Blood,* 97: 2574-2579, 2001.
18. Marijt WA, Heemskerk MH, Kloosterboer FM, Goulmy E, Kester MG, van der Hoorn MA, van Luxemburg-Heys SA, Hoogeboom M, Mutis T, Drijfhout JW, van Rood JJ, Willemze R, and Falkenburg JH. Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci U S A,* 100: 2742-2747, 2003.
19. Bishop MR. Nonmyeloablative allogeneic hematopoietic stem cell transplantation for metastatic breast cancer. *Clin Breast Cancer,* 4: 39-45, 2003.
20. Barge RM, Osanto S, Marijt WA, Starrenburg CW, Fibbe WE, Nortier JW, Falkenburg JH, and Willemze R. Minimal GVHD following in-vitro T cell-depleted allogeneic stem cell transplantation with reduced-intensity conditioning allowing subsequent infusions of donor lymphocytes in patients with hematological malignancies and solid tumors. *Exp Hematol,* 31: 865-872, 2003.
21. Stavely-O'Carroll KF, Sotomayor E, Montgomery J, Borrello I, Hwang L, Fein S, Pardoll DM, and Levitsky HI. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Immunology,* 95: 1178-1183, 1998.
22. Cuenca A, Cheng F, Wang H, Brayer J, Horna P, Gu L, Bien H, Borrello IM, Levitsky HI, and Sotomayor EM. Extra-lymphatic solid tumor growth is not immunologically ignored and results in early induction of antigen-specific T cell anergy: dominant role of cross-tolerance to tumor antigens. *Cancer Res,* 63: 9007-9015, 2003.
23. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, Franken KL, Drijfhout JW, Fleuren GJ, Kenter G, Melief CJ, Offringa R, and van der Burg SH. 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,* 63: 636-641, 2003.
24. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, and Levitsky H. The central role of CD4+ cells in the antitumor immune response. *J Exp Med,* 188: 2357-2368, 1998.
25. Casares N, Arribillaga L, Sarobe P, Dotor J, Lopez-Diaz de Cerio A, Melero I, Prieto J, Borras-Cuesta F, and Lasarte JJ. CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-γ-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J Immunol,* 171: 5931-5939, 2003.
26. Brosterhus H, Brings S, Leyendeckers H, Manz RA, Miltenyi S, Radbruch A, Assenmacher M, and Schmitz J. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. *Eur J Immunol,* 29: 4053-4059, 1999.
27. Becker C, Pohla H, Frankenberger B, Schuler T, Assenmacher M, Schendel DJ, and Blankenstein T. Adoptive tumor therapy with T lymphocytes enriched through an IFN-γ capture assay. *Nat Med,* 7: 1159-1162, 2001.
28. van der Burg S, Kwappenberg KMC, Geluk A, van der Kruk M, Pontesilli O, Hovenkamp E, Franken KLMC, van Meijgaarden KE, Drijfhout JW, Ottenhof THM, Melief CJM, and Offringa R. 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,* 162: 152-160, 1999.
29. Franken KL, Hiemstra HS, van Meijgaarden KE, Subronto Y, den Hartigh J, Ottenhoff TH, and Drijfhout JW. Purification of His-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. *Protein Expr Purif,* 18: 95-99, 2000.
30. Mueller YM, Makar V, Bojczuk PM, Witek J, and Katsikis PD. IL-15 enhances the function and inhibits CD95/Fas-induced apoptosis of human CD4+ and CD8+ effector-memory T cells. *Int Immunol,* 15: 49-58, 2003.
31. Smith PD, Lamerson CL, Wong HL, Wahl LM, and Wahl SM. Granulocyte-macrophage colony-stimulating factor stimulates human monocyte accessory cell function. *J Immunol,* 144: 3829-3834, 1990.
32. van Der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief CJ, and Offringa R. 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,* 91: 612-618, 2001.
33. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, and Offringa R. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res,* 62: 472-479, 2002.
34. Ikeda H, Old LJ, and Schreiber RD. The roles of IFN-γ in protection against tumor development and cancer immunoediting. *Cytokine Growth Factor Rev,* 13: 95-109, 2002.
35. Nonn M, Schinz M, Zumbach K, Pawlita M, Schneider A, Durst M, and Kaufmann AM. Dendritic cell-based tumor vaccine for cervical cancer I: in vitro stimulation with recombinant protein-pulsed dendritic cells induces specific T cells to HPV16 E7 or HPV18 E7. *J Cancer Res Clin Oncol,* 129: 511-520, 2003.
36. Santin AD, Hermonat PL, Ravaggi A, Chiriva-internati M, Zhan D, Pecorelli S, Parham GP, and Cannon MJ. Induction of Human Papillomavirus-specific CD4+ and CD8+ lymphocytes by E7-pulsed autologous dendritic cells in patients with Human Papillomavirus type 16- and 18-positive cervical cancer. *J Virol,* 73: 5402-5410, 1999.
37. Peggs KS, Verfuerth S, Pizzey A, Khan N, Guiver M, Moss PA, and Mackinnon S. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T cell lines. *Lancet,* 362: 1375-1377, 2003.
38. Einsele H, Roosnek E, Rufer N, Sinzger C, Riegler S, Loffler J, Grigoleit U, Moris A, Rammensee HG, Kanz L, Kleihauer A, Frank F, Jahn G, and Hebart H. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood,* 99: 3916-3922, 2002.
39. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, and Man S. 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,* 60: 365-371, 2000.
40. van der Burg SH, Kwappenberg KM, O'Neill T, Brandt RM, Melief CJ, Hickling JK, and Offringa R. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine, in homologous and heterologous prime-boost regimens. *Vaccine,* 19: 3652-3660, 2001.
41. de Jong A, O'Neill T, Khan AY, Kwappenberg KM, Chisholm SE, Whittle NR, Dobson JA, Jack LC, St Clair Roberts JA, Offringa R, van der Burg SH, and Hickling JK. Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. *Vaccine,* 20: 3456-3464, 2002.
42. Zwaveling S, Ferreira Mota SC, Nouta J, Johnson M, Lipford GB, Offringa R, van der Burg SH, and Melief CJ. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. *J Immunol,* 169: 350-358, 2002.
43. Qin Z, and Blankenstein T. CD4+ T cell-mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN-γ receptor expression by nonhematopoietic cells. *Immunity,* 12: 677-686, 2000.
44. Surman DR, Dudley ME, Overwijk WW, and Restifo NP. Cutting edge: CD4+ T cell control of CD8+ T cell reactivity to a model tumor antigen. *J Immunol,* 164: 562-565, 2000.

8

General discussion

Summary · Our studies addressing the HPV16-specific cellular immune response in healthy individuals revealed that CD4+ memory T cells specific for the viral proteins E2 and E6 were present in a large fraction of the healthy population (Chapters 2-4). These reports are the first to describe T cell memory against the HPV early proteins in non-diseased subjects. In contrast to HPV16E2 and E6-specific T cell responses, E7-specific responses were rarely observed, and this was reflected in the predominant detection of E6-specific and not E7-specific responses upon vaccination of healthy volunteers with an HPV16L2E7E6 fusion protein vaccine (Chapter 6). HPV16-specific T cell responses in patients with HPV16-induced cervical cancer lacked the strong pro-inflammatory cytokine profiles generally associated with HPV16-specific responses in healthy individuals, suggesting an impairment of HPV16-specific immunity in patients (Chapter 4). This was supported by detailed analysis of the local immune response in these patients, which pointed at an abundance of HPV-specific CD4+ T cells with unfavorable anti-inflammatory cytokine profiles, secreting IL-10 in response to the viral antigens (Chapter 5). Finally, as a first step in the development of immunotherapeutic strategies for the treatment of advanced cervical cancer, we set up a protocol for the enrichment of Th1 type HPV16E6 CD4+ T cells from the peripheral blood of healthy individuals, based on their IFNγ secretion. Future treatment protocols may involve the adoptive transfer of these donor T cells following HLA-matched allogeneic stem cell transplantation (Chapter 7).

HPV16-specific immune success versus failure

The infection rate with oncogenic HPV types is extremely high and even in the pre-screening era the rate of cervical cancer has been only a fraction of that of HPV exposure [1, 2]. An important factor in the natural history of HPV infection is adaptive immunity, which is thought to play a major role in the control of HPV infections, preventing viral persistence and subsequent development of lesions. The results described in this thesis suggest that systemic Th1/Th2 CD4+ T cells specific for the HPV16 early antigens E2 and E6 are a hallmark of successful control of HPV16 infection. In patients with HPV16-positive cervical carcinoma, the impaired systemic HPV16-specific immunity and the local predominance of IL-10 secretion by HPV16-specific CD4+ T cells, likely represent HPV16-specific immune failure that allows outgrowth of HPV16-induced malignancies. Based on results described in this thesis and on previously published data, this chapter presents a hypothesis of the role of HPV-specific CD4+ T cells in HPV infection and disease. This hypothesis is depicted in Figure 1, and the background of several aspects visualized in this figure is provided throughout this chapter. In addition to HPV-specific CD4+ T cell immunity, which is the main focus of this thesis, the possible roles in HPV-induced disease of HPV-specific CD8+ T cells and capsid-specific antibodies are briefly discussed. Finally, a personal view is given on the implications of the findings for rational immunotherapeutic intervention in HPV-induced disease.

Successful HPV16-specific immunity

Estimates of cervical HPV16 infection prevalence by repeated sampling have revealed a 1 year cumulative prevalence as high as 67%, whereas single-point measurements showed markedly lower percentages of infected individuals [3]. This suggests that active HPV infection (detectable HPV DNA) flares up periodically and is present in a sub-clinical or latent state the remaining time, a notion that is sustained by the fluctuating HPV DNA presence observed during the menstrual cycle [4]. Adaptive immunity is likely of major importance in the continuous control of HPV infection, given the fact that a reduced cellular immunocompetence rapidly results in the appearance of active HPV infections and lesions [5-8].

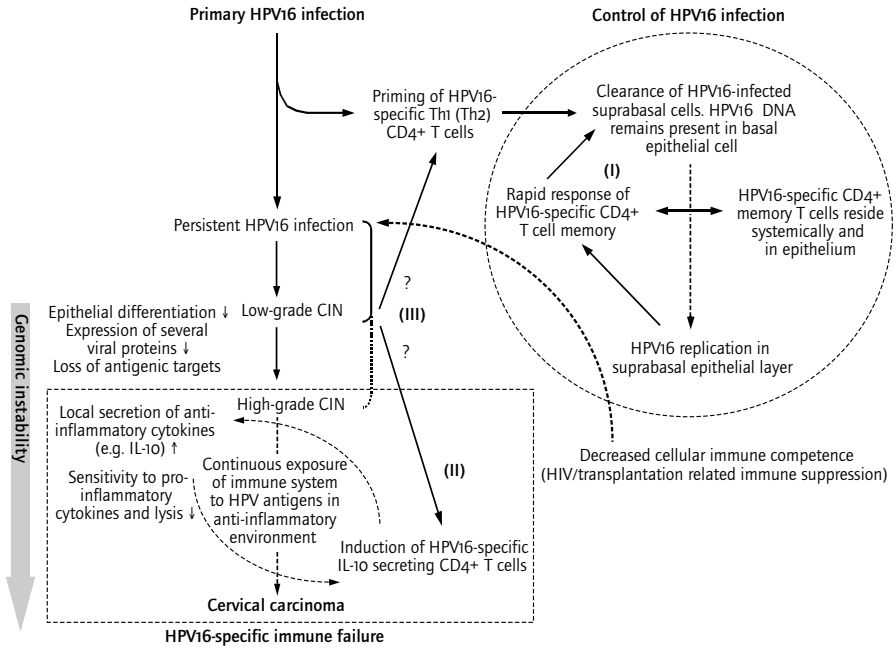

Figure 1. Hypothesis: The CD4+ T cell response in HPV infection and disease

The presence of HPV16-specific CD4+ memory T cells with a strong pro-inflammatory cytokine profile in the peripheral blood of a large fraction of healthy individuals (Chapters 2,3,4 and 7) suggests that these cells participate in the control of HPV16 infection (Figure 1). Longitudinal analysis of this T cell immunity along the course of infection, persistence or clearance is required in order to establish its exact role in the prevention of HPV16 disease progression. Memory CD4+ T cells specific for HPV16 early antigens are proposed to contribute to clearance of HPV16 infected keratinocytes by direct and/or indirect effector mechanisms (Figure 1, I). Indirectly, once activated through the recognition of HPV antigens presented by e.g. Langerhans cells, the CD4+ memory T cells attract and activate macrophages and other immune effectors by local cytokine secretion [9]. Alternatively, HPV-positive HLA class II expressing keratinocytes are directly recognized and killed (e.g. via Fas-FasL) by resident CD4+ effector T cells [10, 11]. Various inflammatory mediators, especially interferons, have been shown to upregulate HLA class II on cervical epithelial cells, which would enable direct recognition by CD4+ T cells [12]. The release of type I interferons by keratinocytes themselves can be triggered by viral infection [13]. The direct mechanism of viral control by CD4+ T cells has been suggested for epithelial Herpes simplex virus (HSV) infection, by demonstrating that HSV-specific cytotoxic CD4+ T cells targeted infected keratinocytes in an HLA class II restricted fashion [14, 15].

HPV16-specific memory CD4+ T cells generally target the early antigens E2 and E6 (Chapters 2, 3 and 4). Interestingly, both in the COPV and the CRPV model the systemic proliferative responses against early antigens, in particular E2, were strongly induced around the time of papilloma regression ([16] and M. Stanley, personal communication). This indicates that in these animal models the regression of a local epithelial infection is reflected in the peripheral blood by a PV-specific T cell response. Simultaneously, local epithelial infiltrates of T cells – predominantly CD4+ – are observed [17]. The therapeutic efficacy of vaccination against the viral early antigens in both models suggest that T cells specific for these antigens play an important role in the control of infection, and do not merely reflect responses that have been primed as a result of viral antigen release along with papilloma regression [18, 19].

Role of HPV-specific CD8+ T cells and antibodies in control of HPV infection. In general, effective long-term immunity against viral infections involves both CD4+ and CD8+ T cells specific for the viral antigens [20-22]. Although limited data on HPV16-specific CD8+ T cells are presented in this thesis (Chapter 7), we have investigated the presence of these T cells both in healthy individuals and cervical cancer patients. In healthy individuals we were unable to detect CD8+ T cells specific for HPV16 E2 or E6. Previously, others have failed to detect CD8+ T cells specific for HPV16E6 and E7 epitopes in asymptomatic individuals [23-27]. By contrast, Nakagawa et al. described the presence of HPV16-specific CTL in the peripheral blood of healthy subjects. In this study, lysis of autologous vaccinia E6E7 infected EBV-transformed B cells was demonstrated after culture of donor PBMC with E6 and E7 protein, but the analyses lacked the proper controls to conclude that the observed target cell lysis was attributable to CD8+ T cells [28]. Several other studies describe the detection of HPV-specific CD8+ T cells in healthy donor PBMC, however only after extensive in vitro culture [29-31]. Based on cumulative data we cannot exclude that HPV16-specific memory CD8+ T cells are present in the peripheral blood of asymptomatic individuals, but – if present – their frequency is expected to be low. In view of the epithelial restriction of HPV infections, it is possible that HPV16-specific CD8+ T cells reside primarily in the epithelium. However, the relative abundance of HPV16-specific memory CD4+ T cells in the peripheral blood indicates that the local superficial epithelial infection does result in systemic CD4+ T cell immunity (Chapters 2-4), and the same would be expected for CD8+ T cells. For instance, epithelial herpes simplex and influenza virus infection both result in the induction of a systemic CD4+ and CD8+ T cell response against the viral antigens [32-35]. Although analysis of the local HPV-specific CD8+ T cell immunity or of systemic CD8+ T cell responses against HPV16 antigens other than those studied (E2, E6 and E7) may reveal a different picture, the results so far point towards a limited role for CD8+ T cells in the control of transient HPV16 infection.

Besides the cellular immune response, in transient HPV infection the humoral immune response may also contribute to the control of infection. HPV capsid-specific IgA is frequently detected in the cervical mucosa during HPV infection, the levels of which rapidly decline after resolution of infection [36]. This suggests that local HPV-specific antibodies induced in response to natural infection are not likely to protect against novel HPV infection. They may, however, assist the resolution of established HPV infection by limiting viral spread and reinoculation via neutralisation of released viral particles. This notion is supported by the observation that vaccine induced HPV16L1-specific neutralising antibodies prevented viral persistence, despite the fact that they did not in all cases prevent HPV16 infection [37]. Although the latter refers to vaccine induced antibodies and can therefore not readily be extrapolated to infection-induced humoral immunity, it does suggest that HPV-specific antibodies are capable of intervening in present HPV infection and thus may play a role in viral control.

HPV16-specific immune failure

In the peripheral blood of approximately half of the patients with HPV16-positive cervical cancer proliferative responses against E2 and E6 were observed, whereas the other half lacked HPV16-specific proliferation altogether (Chapter 4). Compared to previous studies, relatively few cervical cancer patients showed responses against the E7 oncoprotein [38, 39]. This may be due to the readout (proliferation) and the relatively high cut off level in our study. Indeed, when a stimulation index of 2 was taken as a cut off, a significantly larger fraction of patients responded to the E7 antigen as compared to healthy females. Cumulative data suggest that, in contrast to E2 and E6-specific T cells, T cells specific for the E7 antigen are not frequently induced in transient infection, but rather during disease progression [40]. The underlying reason for this could be that the availability of E7 protein for antigen processing and presentation is low in infection – due to limited expression and/or rapid degradation – whereas this availability is increased after viral integration [41, 42].

In the group of cervical carcinoma patients, the supernatants of HPV16-specific microcultures often lacked detectable levels of pro-inflammatory cytokines, and occasionally the sole secretion of IL-10 was observed (Chapter 4). Detailed analysis of patients' tumor-draining lymph nodes confirmed the notion that the HPV16-specific CD4+ T cell response was characterized by prominent production of the immunoregulatory cytokine IL-10 (Figure 1, II). This phenomenon was likely HPV16-specific in this patient, given the strong Th1 cytokine profile of the influenza M1-specific CD4+ T cell response in the same lymph node (Chapter 5). Note that the HPV-specific T cells did have the capacity to secrete pro-inflammatory cytokines in addition to IL-10, however, only at optimal stimulatory conditions. Therefore, we chose to refer to these T cells as IL-10-secreting HPV-specific T cells. The fact that strongly polarized HPV16-specific Th1 cells can readily be isolated from the peripheral blood of healthy subjects (Chapter 7), whereas they appear to be lacking in cervical carcinoma patients, suggests a qualitative difference in the HPV-specific CD4+ T cell response and points at the predominance of IL-10 secreting HPV-specific T cells as a hallmark of HPV-induced disease. Further analysis, of cervix-draining lymph nodes of females undergoing surgery for reasons other than HPV-induced disease, is required to confirm this notion.

Not only in the tumor-draining lymph node, but also in the tumor itself, we have detected CD4+ T cells specifically secreting IL-10 in response to the viral oncoproteins (Chapter 5). These tumor infiltrating lymphocytes (TIL) were isolated from tissue samples by culturing with cytokine only (IL-15 and TCGF) and were detected in TIL both from patients with and without detectable HPV-specific T cells in the peripheral blood. Therefore, the absence of detectable systemic HPV-specific T cell responses does not exclude their overall presence. The direct ex-vivo proliferative capacity of the type of T cells with predominant IL-10 secretion is likely to be low, which precludes their clear detection in short-term proliferation assays. Culture of IL-10 secreting T cells (e.g. Tr1 cells) with IL-15 has been shown to increase the in vitro proliferative capacity of these T cells [43, 44], which may explain their outgrowth in our lymph node and tumor tissue cultures (Chapter 5). Recently, we have obtained evidence of the

in vitro immunosuppressive capacity of the IL-10 secreting HPV-specific T cells. Co-culture of these T cells with polyclonal CD4+ T cell populations and anti-CD3 showed a cell-dose dependent suppression of proliferation (data not shown). Importantly, this implies that the HPV16-specific CD4+ T cell response in cervical cancer patients does not only lack a pro-inflammatory component, but is essentially actively suppressed. The mechanism (IL-10 and/or cell-cell contact dependent) of this immunosuppressive potential is subject to further study.

It remains to be explored how and at which stage of disease these IL-10 secreting HPV16-specific CD4+ T cells have been primed (Figure 1, III). Conceivably the anti-inflammatory (IL-10/TGF-β) cytokine environment in cervical (pre)malignancy contributed to their induction by the modulation of the resident antigen presenting cells, as IL-10 treated dendritic cells have been shown to induce IL-10 secreting T cells with immunoregulatory properties [45]. Alternatively, these T cells may be the result of direct priming of naive T cells by HLA class II positive keratinocytes harbouring HPV antigens. The frequent presence of lymphoid follicles in high-grade lesions suggests that at this stage of disease these lesions are accessible to naïve T cells [46, 47], and this mode of priming in the absence of proper co-stimulation can result in T cells with regulatory activity [48, 49].

HPV-specific CD8+ T cells in HPV-induced disease. In high-grade CIN and cervical cancer, CD4+ T cells are clearly not the only players in the HPV-specific immune response. Several studies have demonstrated the presence of CD8+ T cells specific for the HPV16 oncoproteins in the peripheral blood of patients with HPV16-positive lesions [23-27, 50]. Their presence was generally associated with increasing disease severity, suggesting they are primed in later stages of disease. Underlying reason for this could be an increased availability of oncoprotein for cross-priming of CD8+ T cells in high-grade CIN and cervical carcinoma as compared to HPV infection [51, 52]. Furthermore, the extension of viral antigen expression to basal epithelial layer and the presence of micro-invasions in cervical carcinoma are likely to increase the accessibility of viral antigens to submucosal dendritic cells, which can then participate in the induction (cross-priming) of HPV-specific T cell responses. Interestingly, in our recent TIL analyses we have isolated HPV16/18 E6E7 specific CD8+ T cells from tumor biopsies of several patients (data not shown). In contrast to the HPV-specific CD4+ TIL, the CD8+ T cells generally secreted IFNγ only in response to antigenic stimulation, although IL-10 secretion by CD8+ T cells was also occasionally observed. We and others have demonstrated the capacity of HPV-specific CD8+ T cells to lyse HLA-matched cervical tumor cell lines *in vitro* [26, 50]. Indirect evidence for the *in vivo* functionality of these CD8+ T cells is given by the allelic loss of HLA class I in a large fraction of primary cervical tumors and metastases, which points at a selective immune pressure of the HPV-specific CD8+ T cells on the tumor [53, 54]. In contrast, the HPV-specific CD4+ T cells are not expected to pose direct selective immune pressure on the tumor, given the fact that many cervical tumors and high-grade CIN lesions show de novo expression of HLA class II on their surface [55-57].

Implications for immune intervention

In Figure 1 (Hypothesis) the HPV response is presented as a dichotomy; IL-10 secreting CD4+ T cells representing immune failure and polarized Th1 (and Th2) T cells representing effective immunity. As with most hypotheses, the true situation is likely to be more subtle, and will in this case rather suggest that 'success' versus 'failure' depends on a balance between HPV-specific CD4+ T cells with a pro-inflammatory cytokine profile (generally present in healthy donors) and those predominantly secreting IL-10 (present in carcinoma patients). Supporting the presence of such a balance is the fact that several HPV-specific T cell clones derived from the patients' lymph nodes had cytokine profiles that were more or less polarized towards Th1 or Th2. Interesting in this respect is a recent study describing the immune response against common allergens in non-allergic and allergic individuals. This study demonstrated that in both groups three subsets of CD4+ T cells specific for the allergen could be detected; Th1, Th2

and Tr1 (selection based in the secretion of IFNγ, IL-4 and IL-10 respectively in response to allergen). The net outcome of exposure to allergen (allergic reaction or tolerance) was dependent on the balance between the allergen-specific Th2 and Tr1 subsets [58, 59]. It is tempting to draw parallels to immune control of epithelial HPV infection. The epithelial exposure to HPV antigens may induce similar subsets of Th1, Th2 and IL-10 secreting T cells against the viral antigens. Similarly, the relative frequencies of these T cell subsets and the timing of their induction may play a crucial role in the outcome of HPV infection. In individuals failing to sufficiently prime polarized Th1 (Th2) HPV-specific CD4+ T cells during active viral infection, the balance may tip towards IL-10 secreting HPV-specific CD4+ T cells, which are induced instead and subsequently expanded during persistent HPV infection.

Persistent infection and low-grade CIN. If a balance between the pro- and anti-inflammatory cytokine profiles of HPV-specific CD4+ T cells indeed determines the efficacy of HPV control, it is important to 'tip the scale' towards predominance of HPV-specific CD4+ T cells with a pro-inflammatory cytokine profile at an early (premalignant) stage of disease. For inducing regression of low-grade lesions, it may prove sufficient to induce a local pro-inflammatory cytokine milieu, without the need for antigen-specific vaccination. In persistent low-risk HPV infections (e.g. genital warts), the local topical application of imiquimod, an immunomodulator, has shown to trigger the regression of the warts as compared to placebo [60]. Imiquimod acts as a ligand for toll-like receptor 7, activating macrophages and dendritic cells to release IFNα and other pro-inflammatory cytokines, and the establishment of local inflammation results in the attraction of immune effectors [61]. The modulation of the cytokine environment in favour of Th1-inducing milieu may trigger an effective adaptive immune response against the viral antigens. However, it is not clear if imiquimod treatment indirectly triggers the induction of an HPV-specific T cell response or that the mechanism of regression relies primarily on the action of innate effectors. The fact that recurrence rates with imiquimod treatment are relatively low, suggests that the establishment of effective HPV-specific immune memory may indeed occur [62].

Taken together, the induction of a local pro-inflammatory environment in HPV-induced lesions, resulting in the attraction and activation of immune effectors may prove sufficient for inducing regression of lesions. However, to ensure the prevention of recurrence, an optimal therapeutic modality should additionally contain an HPV specific component that induces long-term HPV-specific T cell memory. Therapeutic vaccination in low-grade CIN can potentially target a number of viral antigens, because at this stage productive viral infection still takes place and the whole array of viral proteins is expressed. In the COPV model, representing low-grade / low-risk PV disease, targeting of E1 or E2 with a codon-optimized DNA vaccine has proven therapeutically successful [18]. However, in lesions caused by high-risk HPV types, targeting of these antigens should always be combined with targeting of E6 and E7 oncoproteins. Keratinocytes containing integrated HPV DNA may else selectively escape immune attack, because the expression of other viral early proteins is often reduced or absent in these cells [63]. Even in low-grade CIN the integration of high-risk viral DNA is occasionally observed [64]. Pre-clinical data suggest that targeting of the oncoproteins only may also prove effective. In the CRPV model, a vaccine comprising overlapping peptides spanning the E6 and E7 CRPV oncoproteins, effectively prevented the outgrowth of established CRPV induced papillomas [65]. Due to increased genomic instability, high-grade CIN will evidently pose a greater challenge than low-grade lesions in terms of effective immunotherapeutic intervention. Furthermore, the variable expression of viral genes other than E6 and E7 in high-grade lesions renders the oncoproteins the only consistent viral targets for immune intervention at this stage of disease [66].

Cervical cancer. After malignant transformation has taken place, therapeutic vaccination will be an adjuvant therapy at best, because it has to overcome several important barriers that can hamper its efficacy. These barriers involving both the tumor and the immune system – which mutually affect one another – include: 1. The increasingly unfavourable local cytokine environment for the function

of effector cells [57, 67, 68]; 2. The genomic instability of the HPV-positive keratinocytes, readily allowing immune escape mechanisms (e.g. reduced sensitivity to pro-inflammatory cytokines and lysis, downregulation of HLA class I molecules, reviewed in [63]); and 3. The dominance of IL-10 secreting T cells in the HPV-specific CD4+ T cell repertoire (Chapter 5).

These barriers should be taken into account when strategically designing immunotherapeutic protocols. Ideally, the intervention involves: 1. the establishment of a local pro-inflammatory cytokine/chemokine milieu or at least neutralization of the local anti-inflammatory cytokines, for instance the combination of a toll-like receptor ligand in combination with IL-10R blocking [69], 2. the provision of large numbers of E6 and E7-specific CD4+ and CD8+ T cells targeting multiple epitopes, thereby minimizing the chance of immune escape. HPV-specific Th1 type CD4+ T cells will be essential for CTL dependent and independent effector mechanisms, the latter becoming increasingly important in case of allelic loss of HLA class I in cervical tumors, and 3. overruling of the unfavourable IL-10 dominated immune response (by skewing the overall immune response towards Th1). Immunotherapeutic approaches can involve either active vaccination or the passive transfer of ex-vivo cultured HPV16-specific T cells. Active vaccination should result in abundant de novo priming and expansion of a type 1 HPV-specific immune response and/or skewing of the resident IL-10 secreting HPV-specific CD4+ T cells towards a Th1 phenotype. Despite the fact that the latter may not prove feasible (Chapter 5), the general prospects for overruling an overall antigen-specific regulatory response are promising, as the counterpart has proven successful; allergen-specific desensitisation shifted the balance from either Th1-based contact hypersensitivity or Th2-based allergy towards IL-10-mediated immunoregulation [70, 71]. An important issue to address is whether vaccination also results in the in vivo expansion of the IL-10 secreting HPV-specific T cell subset, as this would severely counteract effective vaccination. Overall, vaccination is likely to be adversely affected by both HPV-specific and non-specific regulatory T cells. In light of this, treatment with anti-CD25 before vaccination may improve vaccine efficacy, given the fact that naturally occurring and most induced regulatory T cells express CD25 [72]. Alternatively, for advanced cervical cancer, the adoptive transfer of donor-derived HPV-specific T cells in the allogeneic setting may be taken into consideration. In this setting several of the aforementioned barriers can be overcome at least partially, because conditioning of the patient before stem cell transplantation eradicates the host immune cells, including the regulatory component, and allows the administration of a broad repertoire of HPV16 oncoprotein-specific IFNγ-secreting T cells, which can generally be derived from peripheral blood of healthy donors (Chapter7).

Final remark

Although the presence of IL-10 secreting HPV-specific CD4+ T cells provides an explanation for the apparent impaired HPV-specific immunity in cancer patients, it does not reveal as to why the patients failed to mount an effective immune response in the first place. The subsequent establishment of persistent HPV infection may have been influenced by a combination of genetic and environmental factors. In the case of genetic factors related to the innate or adaptive immune system, the 'defects' are expected to be subtle, because subjects with persistent PV infections generally do not show increased susceptibility to other opportunist pathogens. Several studies have addressed the contribution of polymorphisms in certain cytokine genes (e.g. IL-10, TNFα) to the risk of developing cervical cancer, but so far this has not revealed a clear correlation [73-75]. This is not surprising, given that fact that in most studies one single polymorphism was investigated, and it is probable that genetic involvement will consist of large numbers of alleles conferring modest levels of risks [76]. Future efforts should determine the polymorphisms of numerous genes simultaneously, enabling the comprehensive assessment of immune gene pathways rather than a limited number of candidate genes. Finally, microarray technology for gene-and protein-expression may prove a powerful tool for the characterization of the cervical microenvironment in response to HPV infection, allowing the assessment of susceptibility factors for HPV persistence.

References

1. Solomon D. Chapter 14: Role of triage testing in cervical cancer screening. *J Natl Cancer Inst* Monogr 97-101, 2003.
2. Schiffman M, and Castle PE. Human papillomavirus: epidemiology and public health. *Arch Pathol Lab Med,* 127: 930-934, 2003.
3. Schneider A, Kirchhoff T, Meinhardt G, and Gissmann L. Repeated evaluation of human papillomavirus 16 status in cervical swabs of young women with a history of normal Papanicolaou smears. *Obstet Gynecol,* 79: 683-688, 1992.
4. van Ham MA, Melchers WJ, Hanselaar AG, Bekkers RL, Boonstra H, and Massuger LF. Fluctuations in prevalence of cervical human papillomavirus in women frequently sampled during a single menstrual cycle. *Br J Cancer,* 87: 373-376, 2002.
5. Bouwes Bavinck JN, and Berkhout RJ. HPV infections and immunosuppression. *Clin Dermatol,* 15: 427-437, 1997.
6. Ho GY, Burk RD, Fleming I, and Klein RS. Risk of genital human papillomavirus infection in women with human immunodeficiency virus-induced immunosuppression. *Int J Cancer,* 56: 788-792, 1994.
7. Palefsky JM, and Holly EA. Chapter 6: Immunosuppression and coinfection with HIV. *J Natl Cancer Inst* Monogr 41-46, 2003.
8. Penn I. Cancers of the anogenital region in renal transplant recipients. Analysis of 65 cases. *Cancer,* 58: 611-616, 1986.
9. Pardoll DM, and Topalian SL. The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol,* 10: 588-594, 1998.
10. Arnold R, Seifert M, Asadullah K, and Volk HD. Crosstalk between keratinocytes and T lymphocytes via Fas/Fas ligand interaction: modulation by cytokines. *J Immunol,* 162: 7140-7147, 1999.
11. Traidl C, Sebastiani S, Albanesi C, Merk HF, Puddu P, Girolomoni G, and Cavani A. Disparate cytotoxic activity of nickel-specific CD8+ and CD4+ T cell subsets against keratinocytes. *J Immunol,* 165: 3058-3064, 2000.
12. Ljunggren G, and Anderson DJ. Cytokine induced modulation of MHC class I and class II molecules on human cervical epithelial cells. *J Reprod Immunol,* 38: 123-138, 1998.
13. Biron CA. Initial and innate responses to viral infections-pattern setting in immunity or disease. *Curr Opin Microbiol,* 2: 374-381, 1999.
14. Mikloska Z, and Cunningham AL. Herpes simplex virus type 1 glycoproteins gB, gC and gD are major targets for CD4 T-lymphocyte cytotoxicity in HLA-DR expressing human epidermal keratinocytes. *J Gen Virol,* 79 (Pt 2): 353-361, 1998.
15. Cunningham AL, and Noble JR. Role of keratinocytes in human recurrent herpetic lesions. Ability to present herpes simplex virus antigen and act as targets for T lymphocyte cytotoxicity in vitro. *J Clin Invest,* 83: 490-496, 1989.
16. Selvakumar R, Ahmed R, and Wettstein FO. Tumor regression is associated with a specific immune response to the E2 protein of cottontail rabbit papillomavirus. *Virology,* 208: 298-302, 1995.
17. Nicholls PK, Moore PF, Anderson DM, Moore RA, Parry NR, Gough GW, and Stanley MA. Regression of canine oral papillomas is associated with infiltration of CD4+ and CD8+ lymphocytes. *Virology,* 283: 31-39, 2001.
18. Moore RA, Walcott S, White KL, Anderson DM, Jain S, Lloyd A, Topley P, Thomsen L, Gough GW, and Stanley MA. Therapeutic immunisation with COPV early genes by epithelial DNA delivery. *Virology,* 314: 630-635, 2003.
19. Selvakumar R, Borenstein LA, Lin YL, Ahmed R, and Wettstein FO. Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. *J Virol,* 69: 602-605, 1995.
20. Dong C, and Flavell RA. Th1 and Th2 cells. *Curr Opin Hematol,* 8: 47-51, 2001.
21. Woodland DL. Cell-mediated immunity to respiratory virus infections. *Curr Opin Immunol,* 15: 430-435, 2003.
22. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, and Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature,* 421: 852-856, 2003.
23. Evans EM, Man S, Evans AS, and Borysiewicz LK. Infiltration of cervical cancer tissue with Human Papillomavirus-specific cytotoxic T-lymphocytes. *Cancer Res,* 57: 2943-2950, 1997.
24. Nimako M, Fiander AN, Wilkinson GWG, Borysiewicz LK, and Man S. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. *Cancer Res,* 57: 4855-4861, 1997.
25. Evans M, Borysiewicz LK, Evans AS, Rowe M, Jones M, Gileadi U, Cerundolo V, and Man S. Antigen processing defects in cervical carcinomas limit the presentation of a CTL epitope from human papillomavirus 16 E6. *J Immunol,* 167: 5420-5428, 2001.
26. Ressing ME, van Driel WJ, Celis E, Sette A, Brandt MP, Hartman M, Anholts JD, Schreuder GM, ter Harmsel WB, Fleuren GJ, Trimbos BJ, Kast WM, and Melief CJ. 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,* 56: 582-588, 1996.
27. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, Verheijen RH, Stukart MJ, Meijer CJ, Scheper RJ, Stacey SN, Duggan-Keen MF, Stern PL, Man S, Borysiewicz LK, and Walboomers JM. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. *Int J Cancer,* 88: 92-98, 2000.
28. Nakagawa M, Stites DP, Patel S, Farhat S, Scott M, Hills NK, Palefsky JM, and Moscicki AB. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. *J Infect Dis,* 182: 595-598, 2000.
29. Schoell WM, Mirhashemi R, Liu B, Janicek MF, Podack ER, Penalver MA, and Averette HE. Generation of tumor-specific cytotoxic T lymphocytes by stimulation with HPV type 16 E7 peptide-pulsed dendritic cells: an approach to immunotherapy of cervical cancer. *Gynecol Oncol,* 74: 448-455, 1999.
30. Ressing ME, Sette A, Brandt RM, Ruppert J, Wentworth PA, Hartman M, Oseroff C, Grey HM, Melief CJ, and Kast WM. Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. *J Immunol,* 154: 5934-5943, 1995.
31. Thornburg C, Boczkowski D, Gilboa E, and Nair SK. Induction of cytotoxic T lymphocytes with dendritic cells transfected with human papillomavirus E6 and E7 RNA: implications for cervical cancer immunotherapy. *J Immunother,* 23: 412-418, 2000.
32. Danke NA, and Kwok WW. HLA class II-restricted CD4+ T cell responses directed against influenza viral antigens postinfluenza vaccination. *J Immunol,* 171: 3163-3169, 2003.
33. Niemialtowski MG, and Rouse B T. Cytotoxic T lymphocyte response to herpes simplex virus type 1 is composed of both CD8+ and CD4+ T cell phenotypes in acute and memory states. *Arch Immunol Ther Exp (Warsz),* 42: 319-324, 1994.
34. Posavad CM, Wald A, Hosken N, Huang ML, Koelle DM, Ashley RL, and Corey L. T cell immunity to herpes simplex viruses in seronegative subjects: silent infection or acquired immunity? *J Immunol,* 170: 4380-4388, 2003.
35. Jameson J, Cruz J, and Ennis FA. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. *J Virol,* 72: 8682-8689, 1998.
36. Sasagawa T, Rose RC, Azar KK, Sakai A, and Inoue M. Mucosal immunoglobulin-A and -G responses to oncogenic human papilloma virus capsids. *Int J Cancer,* 104: 328-335, 2003.
37. Koutsky LA, Ault KA, Wheeler CM, Brown DR, Barr E, Alvarez FB, Chiacchierini LM, and Jansen KU. A controlled trial of a human papillomavirus type 16 vaccine. *N Engl J Med,* 347: 1645-1651, 2002.
38. Luxton JC, Rowe AJ, Cridland JC, Coletart T, Wilson P, and Shepherd PS. 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,* 77: 1585-1593, 1996.
39. de Gruijl TD, Bontkes HJ, Walboomers JM, Stukart MJ, Doekhie FS, Remmink AJ, Helmerhorst TJ, Verheijen RH, Duggan-Keen MF, Stern PL, Meijer CJ, and Scheper RJ. 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,* 58: 1700-1706, 1998.
40. de Gruijl TD, Bontkes HJ, Stukart MJ, Walboomers JMM, Remmink AJ, Verheijen RHM, Helmerhorst TJM, Meijer CJLM, and Scheper RJ. 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,* 77: 2183-2191, 1996.
41. Wagatsuma M, Hashimoto K, and Matsukura T. Analysis of integrated human papillomavirus type 16 DNA in cervical cancers: amplification of viral sequences together with cellular flanking sequences. *J Virol,* 64: 813-821, 1990.
42. Jeon S, and Lambert PF. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proc Natl Acad Sci* U S A, 92: 1654-1658, 1995.
43. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, and Roncarolo MG. A CD4+ T cell subset inhibits antigen-specific T cell responses and prevents colitis. *Nature,* 389: 737-742, 1997.
44. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, and Levings MK. Type 1 T regulatory cells. *Immunol Rev,* 182: 68-79, 2001.
45. Steinbrink K, Graulich E, Kubsch S, Knop J, and Enk AH. CD4(+) and CD8(+) anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood,* 99: 2468-2476, 2002.
46. Kobayashi A, Darragh T, Herndier B, Anastos K, Minkoff H, Cohen M, Young M, Levine A, Grant LA, Hyun W, Weinberg V, Greenblatt R, and Smith-McCune K. Lymphoid follicles are generated in high-grade cervical dysplasia and have differing characteristics depending on HIV status. *Am J Pathol,* 160: 151-164, 2002.

47. Demetter P, Van Huysse JA, De Keyser F, Van Damme N, Verbruggen G, Mielants H, De Vos M, Veys EM, and Cuvelier CA. Increase in lymphoid follicles and leukocyte adhesion molecules emphasizes a role for the gut in spondyloarthropathy pathogenesis. *J Pathol*, 198: 517-522, 2002.
48. Vermeiren J, Ceuppens JL, Van Ghelue M, Witters P, Bullens D, Mages HW, Kroczek RA, and Van Gool SW. Human T cell activation by costimulatory signal-deficient allogeneic cells induces inducible costimulator-expressing anergic T cells with regulatory cell activity. *J Immunol*, 172: 5371-5378, 2004.
49. Cao Y, Zhou H, Tao J, Zheng Z, Li N, Shen B, Shih TS, Hong J, Zhang J, and Chou KY. Keratinocytes induce local tolerance to skin graft by activating interleukin-10-secreting T cells in the context of costimulation molecule B7-H1. *Transplantation*, 75: 1390-1396, 2003.
50. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, and Man S. 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*, 60: 365-371, 2000.
51. von Knebel Doeberitz M. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *Eur J Cancer*, 38: 2229-2242, 2002.
52. Sotlar K, Selinka HC, Menton M, Kandolf R, and Bultmann B. Detection of human papillomavirus type 16 E6/E7 oncogene transcripts in dysplastic and nondysplastic cervical scrapes by nested RT-PCR. *Gynecol Oncol*, 69: 114-121, 1998.
53. Koopman LA, Corver WE, van der Slik AR, Giphart MJ, and Fleuren GJ. Multiple genetic alterations cause frequent and heterogeneous human histocompatibility leukocyte antigen class I loss in cervical cancer. *J Exp Med*, 191: 961-976, 2000.
54. Hilders CG, Munoz IM, Nooyen Y, and Fleuren GJ. Altered HLA expression by metastatic cervical carcinoma cells as a factor in impaired immune surveillance. *Gynecol Oncol*, 57: 366-375, 1995.
55. Hilders CG, Houbiers JG, Krul EJ, and Fleuren GJ. The expression of histocompatibility-related leukocyte antigens in the pathway to cervical carcinoma. *Am J Clin Pathol*, 101: 5-12, 1994.
56. Chil A, Sikorski M, Bobek M, Jakiel G, and Marcinkiewicz J. Alterations in the expression of selected MHC antigens in premalignant lesions and squamous carcinomas of the uterine cervix. *Acta Obstet Gynecol Scand*, 82: 1146-1152, 2003.
57. Mota F, Rayment N, Chong S, Singer A, and Chain B. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. *Clin Exp Immunol*, 116: 33-40, 1999.
58. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, Kegel C, Disch R, Schmidt-Weber CB, Blaser K, and Akdis CA. Immune Responses in Healthy and Allergic Individuals Are Characterized by a Fine Balance between Allergen-specific T Regulatory 1 and T Helper 2 Cells. *J Exp Med*, 199: 1567-1575, 2004.
59. Cavani A, Mei D, Guerra E, Corinti S, Giani M, Pirrotta L, Puddu P, and Girolomoni G. Patients with allergic contact dermatitis to nickel and nonallergic individuals display different nickel-specific T cell responses. Evidence for the presence of effector CD8+ and regulatory CD4+ T cells. *J Invest Dermatol*, 111: 621-628, 1998.
60. Syed TA, Hadi SM, Qureshi ZA, Ali SM, and Kwah MS. Treatment of external genital warts in men with imiquimod 2% in cream. A placebo-controlled, double-blind study. *J Infect*, 41: 148-151, 2000.
61. Stanley MA. Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential. *Clin Exp Dermatol*, 27: 571-577, 2002.
62. Moore RA, Edwards JE, Hopwood J, and Hicks D. Imiquimod for the treatment of genital warts: a quantitative systematic review. *BMC Infect Dis*, 1: 3, 2001.
63. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer*, 2: 342-350, 2002.
64. Gallo G, Bibbo M, Bagella L, Zamparelli A, Sanseverino F, Giovagnoli MR, Vecchione A, and Giordano A. Study of viral integration of HPV-16 in young patients with LSIL. *J Clin Pathol*, 56: 532-536, 2003.
65. Vambutas A, De Voti J, Nouri M, Drijfhout JW, Lipford GB, Bonagura VR, van der Burg SH, and Melief CJM. 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 preclinical Cottontail Rabbit Papillomavirus model. *submitted*, 2004.
66. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. *J Natl Cancer Inst*, 92: 690-698, 2000.
67. Sheu BC, Lin RH, Lien HC, Ho HN, Hsu SM, and Huang SC. Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer. *J Immunol*, 167: 2972-2978, 2001.
68. El-Sherif AM, Seth R, Tighe PJ, and Jenkins D. Quantitative analysis of IL-10 and IFN-γ mRNA levels in normal cervix and human papillomavirus type 16 associated cervical precancer. *J Pathol*, 195: 179-185, 2001.
69. Vicari AP, Chiodoni C, Vaure C, Ait-Yahia S, Dercamp C, Matsos F, Reynard O, Taverne C, Merle P, Colombo MP, O'Garra A, Trinchieri G, and Caux C. Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. *J Exp Med*, 196: 541-549, 2002.
70. Francis JN, Till SJ, and Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol*, 111: 1255-1261, 2003.
71. Girolomoni G, Gisondi P, Ottaviani C, and Cavani A. Immunoregulation of allergic contact dermatitis. *J Dermatol*, 31: 264-270, 2004.
72. Sutmuller RP, Offringa R, and Melief CJ. Revival of the regulatory T cell: new targets for drug development. *Drug Discov Today*, 9: 310-316, 2004.
73. Roh JW, Kim MH, Seo SS, Kim SH, Kim JW, Park NH, Song YS, Park SY, Kang SB, and Lee HP. Interleukin-10 promoter polymorphisms and cervical cancer risk in Korean women. *Cancer Lett*, 184: 57-63, 2002.
74. Stanczuk GA, Sibanda EN, Perrey C, Chirara M, Pravica V, Hutchinson IV, and Tswana SA. Cancer of the uterine cervix may be significantly associated with a gene polymorphism coding for increased IL-10 production. *Int J Cancer*, 94: 792-794, 2001.
75. Stanczuk GA, Sibanda EN, Tswana SA, and Bergstrom S. Polymorphism at the -308-promoter position of the tumor necrosis factor-α (TNF-α) gene and cervical cancer. *Int J Gynecol Cancer*, 13: 148-153, 2003.
76. Wang SS, and Hildesheim A. Chapter 5: Viral and host factors in human papillomavirus persistence and progression. *J Natl Cancer Inst* Monogr 35-40, 2003.

9

Samenvatting

Humaan papillomavirus (HPV) en baarmoederhalskanker

Genitale HPV infecties zijn een van de meest voorkomende sexueel overdraagbare aandoeningen; naar schatting komt ruim 80% van de sexueel actieve vrouwen in contact met dit virus gedurende hun leven. HPV infecties van de baarmoedermond zijn over het algemeen vrij onschuldig, aangezien het merendeel van deze infecties na enige tijd door de afweer wordt opgeruimd. Als een HPV infectie echter voortduurt, bestaat de kans op ontwikkeling van baarmoederhalskanker. Er zijn vele verschillende HPV typen, waarvan enkele in staat zijn deze kwaadaardige ontwikkeling van cellen te bevorderen. De grootste boosdoener is type 16 (HPV16), dat in meer dan de helft van de tumoren van de baarmoederhals wordt teruggevonden. HPV16 kan op allerlei plekken de slijmvliezen infecteren, maar doet dit bij voorkeur ter plekke van de baarmoedermond. Hier heeft het virus toegang tot de zogenaamde basaalcellen van het slijmvlies, die mogelijk ook dienen als reservoir voor het virus. Humaan papillomavirussen hebben zich wonderlijk goed aangepast aan hun gastheer. In tegenstelling tot vele andere virusinfecties leidt HPV infectie niet tot massale inflammatie en verstoort het virus tijdens zijn vermenigvuldiging nauwelijks het normale proces van celdeling en differentiatie van het slijmvlies. Gevolg hiervan is dat een HPV infectie lange tijd onopgemerkt kan blijven voor het immuunsysteem (de afweer). Daarnaast zijn enkele HPV eiwitten in staat om te interfereren met signaaleiwitten die gewoonlijk het immuunsysteem activeren op het moment van virale infectie.

Ondanks het feit dat papillomavirussen verschillende strategieën gebruiken om activering van het immuunsysteem te voorkomen, worden uiteindelijk de meeste HPV infecties effectief door de afweer bestreden. Wanneer dit niet gebeurt en de HPV infectie blijft voortduren, bestaat de kans dat het virale DNA ingebouwd wordt in het DNA van de gastheercel. Een belangrijk gevolg van deze integratie is dat twee HPV eiwitten (E6 E7) in verhoogde mate worden geproduceerd. E6 en E7

kunnen binden aan cellulaire eiwitten die betrokken zijn bij het controleren van de celcyclus. Door deze binding raakt de celcyclus ontregeld, met als gevolg ongecontroleerde celdeling en uiteindelijk de vorming van kwaadaardig weefsel.

De rol van het immuunsysteem

Bij het bestrijden van virusinfecties speelt de zogenaamde 'adaptieve' of 'specifieke' afweer een belangrijke rol. Zoals de naam al suggereert, reageert deze component van het immuunsysteem zeer specifiek op afwijkende of lichaamvreemde structuren en vormt het een soort 'geheugen'. Bij een volgend contact met dezelfde ziekteverwekker reageert de specifieke afweer sneller en krachtiger waardoor infectie wordt voorkomen of sneller wordt bestreden. Binnen de specifieke afweer zijn twee celtypen van belang: B en T cellen. Iedere B en T cel bezit een unieke receptor waarmee zeer specifiek een bepaald lichaamsvreemd eiwitfragment kan worden herkend. De extreme diversiteit van deze receptoren zorgt ervoor dat vrijwel elke lichaamsvreemde structuur potentieel herkend kan worden. Het principe van het geheugen van het immuunsysteem is onder andere gebaseerd op het feit dat bij een bepaalde infectie juist die B en T cellen die specifiek zijn voor de ziekteverwekker zich vermenigvuldigen, waardoor de dichtheid van deze specifieke cellen in het bloed sterk toeneemt.

B cellen maken na activatie antilichamen. Bij een virusinfectie zijn antilichamen – gericht tegen het eiwitomhulsel van het virus – in staat het binnendringen van het virus in de cel te voorkómen. Deze antilichamen hebben echter een geringe functie wanneer een virus eenmaal de cel is binnengedrongen. Dit is waar de T cel een rol speelt. T cellen 'herkennen' met behulp van hun receptor stukjes eiwit (peptiden) die gebonden zijn aan zogenaamde HLA moleculen. Deze HLA moleculen bevinden zich op het oppervlak van vrijwel alle cellen en de peptiden die aan deze HLA moleculen gebonden zijn vormen een afspiegeling van de eiwitten binnenin de betreffende cel. Bijvoorbeeld, wanneer een cel geïnfecteerd is met een virus, dan zal het HLA ook peptiden afkomstig van virale eiwitten op het oppervlak presenteren, die vervolgens herkend kunnen worden door T cellen. Binnen de T cellen kunnen globaal de volgende soorten worden onderscheiden: CD8+ T cellen, die in staat zijn om andere cellen te doden en CD4+ T cellen die ook incidenteel cellen kunnen doden, maar hoofdzakelijk cytokinen produceren. Cytokinen zijn celsignaalstoffen die onmisbaar zijn voor het op gang brengen en houden van afweerprocessen. Binnen CD4+ T cellen is er weer onderscheid te maken tussen 'helper' T cellen, die ontstekingsbevorderende cytokinen produceren en 'regulatoire' T cellen, die juist ontstekingsonderdrukkende cytokinen produceren.

De exacte rol van de specifieke afweer bij de bescherming tegen en het onder controle houden van van HPV infecties is niet geheel bekend. Wel is vanuit epidemiologische studies duidelijk geworden dat T cellen en met name CD4+ T cellen een belangrijke rol hierin spelen. Patiënten met algemeen verminderde cellulaire afweer (HIV geïnfecteerden / transplantatiepatiënten) en specifiek verminderde CD4+ populaties (idiopathische CD4 lymfocytopenie) vertonen een extreme toename in voorkomen en ernst van HPV infecties.

Dit proefschrift

De 'link' tussen HPV infectie en baarmoederhalskanker biedt een belangrijk aangrijpingspunt voor mogelijke behandeling van deze vorm van kanker met behulp van immunotherapie. Belangrijk hierbij is het feit dat virale eiwitten tot expressie komen in de tumorcellen. Het activeren van T cellen gericht tegen de virale eiwitten, zal mogelijk een effectieve 'aanval' op tumorcellen in gang zetten.

Effectieve HPV-specifieke afweer. Voor het ontwikkelen van effectieve immunotherapeutische strategieën is het van belang vast te stellen hoe de HPV-specifieke T cel immuniteit er uit ziet bij individuen

die in staat zijn geweest een HPV16 infectie op te ruimen en hoe deze T cel immuniteit zich kwantitatief en kwalitatief verhoudt ten opzichte van die in vrouwen met HPV16-geïnduceerde baarmoederhalskanker of voorstadia daarvan. Bij het beantwoorden van deze vraag zijn we eerst uitgegaan van de gezonde populatie waarin zich – gezien de hoge incidentie van HPV16 infecties – een groot aantal individuen bevindt die in het verleden een HPV16 infectie hebben doorgemaakt. In eerste instantie hebben we gekeken naar de afweer tegen het E2 eiwit, dat vroeg in infectie hoog tot expressie komt. Al gauw werd duidelijk dat bij ruim de helft van de gezonde mensen zogenaamde 'memory' CD4+ T cellen tegen E2 in het bloed aanwezig waren. Hiermee werd voor het eerst aangetoond dat T cellen tegen eiwitten van HPV16 zeer frequent aanwezig zijn in individuen zonder HPV16-geïnduceerde ziekte. Ook tegen het E6 eiwit van HPV16 werden memory CD4+ T cellen gedetecteerd in het bloed van gezonde bloeddonoren. Dit gold echter niet voor T cel responsen tegen het E7 eiwit, mogelijk omdat E7 minder accumuleert in de geïnfecteerde cellen en minder beschikbaar is voor presentatie aan het immuunsysteem. Het gegeven dat memory CD4+ T cellen tegen HPV16 E2 en E6 zeer frequent voorkomen in gezonde individuen suggereert dat deze cellen een rol spelen bij het onder controle houden van HPV16 infectie. Dit zal echter nog bevestigd moeten worden in een zogenaamde longitudinale studie, waarin de ontwikkeling van HPV16-specifieke T cel responsen gedurende een periode wordt bekeken in relatie tot het al dan niet opruimen van de HPV16 infectie.

Falende HPV-specifieke afweer. Bij patiënten met baarmoederhalskanker (of voorstadia) veroorzaakt door HPV16 verschilde het beeld van de virus-specifieke T cel immuniteit opmerkelijk van die bij gezonde individuen. Hoewel T cel responsen tegen HPV16 E2 en E6 (en ook E7) wel gezien werden bij een deel van de patiënten, waren deze responsen zwakker en vaak geassocieerd met een sterk verminderde productie van cytokinen. Dit terwijl deze patiënten meestal niet een significant verminderde afweer hadden tegen andere ziekteverwekkers. Ook het soort cytokinen dat geproduceerd werd verschilde; terwijl bij gezonde vrouwen overwegend ontstekingsbevorderende cytokinen werden gemeten (interferon-gamma (IFNγ) en interleukine-5 (IL-5)), werd bij patiënten vaak het ontstekingsonderdrukkende cytokine, interleukine-10 (IL-10), gemeten.

Bij een aantal patiënten met baarmoederhalskanker hebben we in detail HPV-specifieke T cellen kunnen bestuderen die zich in de tumor of de drainerende lymfeklieren bevonden. Het merendeel van deze HPV16-specifieke T cellen produceerde zowel het ontstekingsonderdrukkende IL-10 als het ontstekingsbevorderende IFNγ. Belangrijke observatie hierbij was dat de balans tussen deze 2 cytokinen afhing van de hoeveelheid HPV16 peptide waarmee de T cellen in contact kwamen. Bij lage concentraties maakten de T cellen alleen IL-10, terwijl pas bij hogere concentraties IL-10 én IFNγ werden geproduceerd. Hoewel de betekenis van deze bevindingen nog niet helemaal duidelijk is; lijkt het er wel op dat bij patiënten de balans van de HPV16-specifieke afweer bij patiënten in het voordeel is van IL-10, hetgeen suggereert dat de afweer tegen HPV16, en dus tegen de tumor, specifiek wordt onderdrukt. Recentelijk hebben we de eerste aanwijzingen gevonden dat de HPV-specifieke T cellen afkomstig uit de baarmoederhalstumoren en lymfeklieren inderdaad in staat zijn om activatie en deling van andere CD4+ T cellen te onderdrukken.

Tot slot • De bevindingen beschreven in dit proefschrift geven inzicht in de afweer tegen HPV in gezonde individuen en in patiënten met baarmoederhalskanker. De observatie dat bij patiënten de afweer tegen HPV niet alleen verminderd is, maar mogelijk zelfs specifiek onderdrukt, heeft consequenties voor de aanpak van baarmoederhalskanker met behulp van immunotherapie. Dit betekent dat de gekozen behandelingsstrategie niet alleen gericht moet zijn op het activeren van 'nieuwe' T cellen tegen HPV. Ook het inactiveren of verwijderen van reeds aanwezige IL-10 producerende HPV-specifieke T cellen is van belang, aangezien deze mogelijk de effectiviteit van de immunotherapie kunnen verminderen.

10

List of Publications

1. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, Melief CJ, and Offringa R. 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,* 91: 612-618, 2001.

2. de Jong A, van der Burg SH, Kwappenberg KM, van der Hulst JM, Franken KL, Geluk A, van Meijgaarden KE, Drijfhout JW, Kenter G, Vermeij P, Melief CJ, and Offringa R. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. *Cancer Res,* 62: 472-479, 2002.

3. de Jong A, O'Neill T, Khan AY, Kwappenberg KM, Chisholm SE, Whittle NR, Dobson JA, Jack LC, St Clair Roberts JA, Offringa R, van der Burg SH, and Hickling JK. Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. *Vaccine,* 20: 3456-3464, 2002.

4. van der Burg SH, de Jong A, Welters MJ, Offringa R, and Melief CJ. The status of HPV16-specific T cell reactivity in health and disease as a guide to HPV vaccine development. *Virus Res,* 89: 275-284, 2002.

5. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, Franken KL, Drijfhout JW, Fleuren GJ, Kenter G, Melief CJ, Offringa R, and van der Burg SH. 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,* 63: 636-641, 2003.

6. Offringa R, de Jong A, Toes RE, van der Burg SH, and Melief CJ. Interplay between human papillomaviruses and dendritic cells. *Curr Top Microbiol Immunol,* 276: 215-240, 2003.

7. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, Kenter G, Offringa R, and van der Burg SH. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T cell immunity against early antigens E2 and E6. *Cancer Res,* 64: 5449-5455, 2004.

8. de Jong A, van der Hulst JM, Kenter GG, Drijfhout JW, Franken KL, Vermeij P, Offringa R, van der Burg SH, and Melief CJ. Rapid enrichment of human papillomavirus (HPV)-specific polyclonal T cell populations for adoptive immunotherapy of cervical cancer. *Int J Cancer,* 2004.

Curriculum vitæ

De schrijfster van dit proefschrift werd op 4 oktober 1971 geboren te Amsterdam. Na het behalen van haar VWO diploma (Rijnlands Lyceum te Oegstgeest), begon zij in 1990 met de studie Farmacie aan de Universiteit Utrecht. Tijdens de doctoraalfase, welke in 1995 werd afgerond, heeft zij onderzoek gedaan naar bepalingsmethoden voor bot-turnover markers bij metabole botziekten aan The Institute of Medical and Veterinary Science, afdeling Biochemie, Adelaide, Australië. Het postdoctoraal apothekersdiploma behaalde zij in 1997, waarna zij in dienst trad als projectapotheker bij de afdeling Klinische Farmacie en Toxicologie van het Leids Universitair Medisch Centrum. In het kader van het zogenaamde Zapico curriculum (promotieonderzoek gecombineerd met de opleiding tot ziekenhuisapotheker) begon zij in april 1999 aan een promotieonderzoek op de afdeling Immunohematologie & Bloedtransfusie. Bij de sectie Tumorimmunologie van deze afdeling heeft zij tot eind 2003 onderzoek gedaan naar de cellulaire afweer tegen Humaan Papillomavirussen, waarvan de resultaten beschreven staan in dit proefschrift. Dit onderzoek werd begeleid door prof. dr. C.J.M. Melief, dr. R. Offringa en dr. S.H. van der Burg. Gedurende deze 'onderzoeksjaren' heeft zij haar toekomstplannen gewijzigd. In plaats van de opleiding tot ziekenhuisapotheker heeft zij gekozen voor een verdere carrière in wetenschappelijk onderzoek. In 2004 heeft zij nog wel een bescheiden 'come-back' gemaakt in de Farmacie. Gedurende dit jaar was zij werkzaam op de Interdivisionele GMP faciliteit (IGFL) van de afdeling Klinische Farmacie & Toxicologie en was daar betrokken bij de ontwikkeling en kwaliteitsborging van cellulaire therapeutica en peptidevaccins. Per maart 2005 begint zij in Boston als postdoctoral fellow in de groep van Branch Moody, Division of Rheumatology, Immunology and Allergy van Brigham & Women's Hospital en Harvard Medical School. Zij zal daar werken aan CD1-gemediëerde T cel immuniteit.

11

Nawoord

In de continue stroom die wetenschap heet, is het voltooien van een proefschrift niet meer dan een gestold moment. *Het boek is klaar, maar het onderzoek is nooit af.* Desondanks is het voor mij een bijzonder moment en wil ik even stilstaan bij de mensen die bij de totstandkoming van dit proefschrift een belangrijke rol hebben gespeeld.

De 'HPV club' van de sectie Tumorimmunologie is in de periode van mijn promotieonderzoek qua omvang gegroeid van een onderzoeksduo tot een volwaardige club enthousiaste onderzoekers. Mijn dank gaat in het bijzonder uit naar Kitty die mij vanuit 'niets' alle praktische beginselen van T cel immunologie heeft bijgebracht. Ook wilde ik Marij, Mariëtte en Susan danken voor de bijdragen aan enkele hoofdstukken in dit proefschrift. Jeanette voor de grote hoeveelheid werk, enthousiasme en gezelligheid en natuurlijk voor de bereidheid om paranimf te zijn.

Verder was dit onderzoek niet mogelijk geweest zonder de goede samenwerking met de afdelingen Gynaecologie en Pathologie en de bereidheid van patiënten en gezonde vrijwilligers om te participeren in de studies. Buiten de HPV club heb ik de sectie Tumorimmunologie en ook de verdere IHB afdeling ervaren als een zeer stimulerende werkomgeving. Hier is bij mij een groot enthousiasme voor de immunologie ontstaan en de afdeling mag het denk ik als een compliment beschouwen dat ik door dit enthousiasme een toekomst in de ziekenhuisfarmacie heb verruild voor een onderzoekspad in de immunologie. Dank ook aan alle medewerkers van de afdeling Klinische Farmacie en Toxicologie. Al was ik het afgelopen jaar slechts tijdelijk terug bij de afdeling, heb ik mij nog steeds welkom gevoeld.

Ik wilde Sam bedanken voor de mooie vormgeving van dit proefschrift en de organisatie rondom het drukwerk.

Tot slot, veel dank aan lieve vrienden en familie voor alle belangstelling en bovenal voor de afleiding en het relativeren!