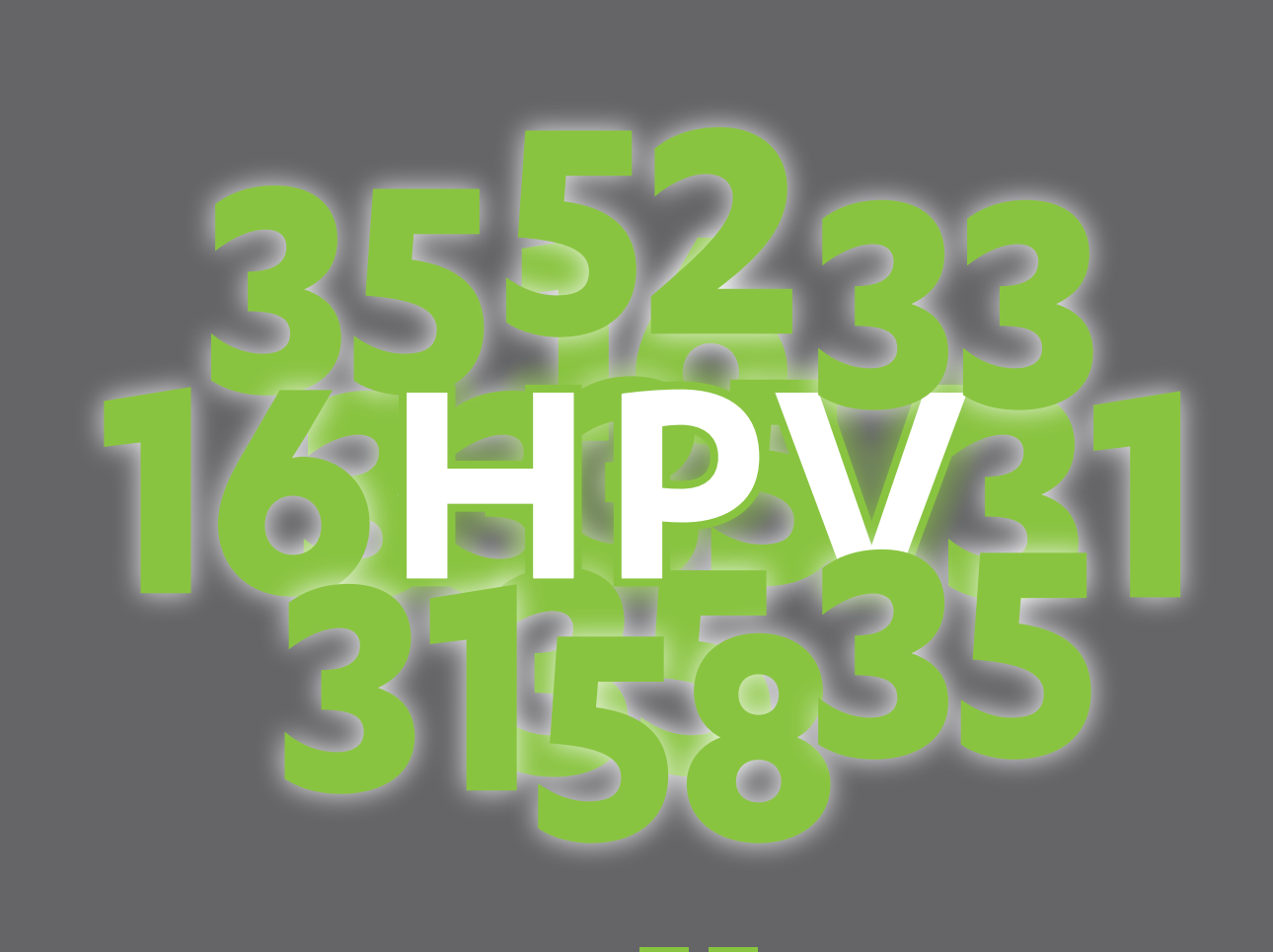

HUMAN PAPILLOMAVIRUS CLADE A9 SPECIFIC CELLULAR IMMUNITY DURING THE NATURAL COURSE OF DISEASE

Muriel van den Hende

Human papillomavirus clade A9 specific cellular immunity during the natural course of disease

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Aan mijn ouders

Ter nagedachtenis aan Henk en Clementine

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General introduction

Genital Human Papillomavirus infections

Human Papillomavirus (HPV) infection is one of the most common sexually transmitted infections worldwide. Up to 80% of sexually active individuals will be infected with HPV at some time during their life [Baseman 2005]. Data from large global meta-analyses indicate that at a given point in time 10.4% of women worldwide are positive for cervical HPV DNA. The prevalence of HPV is higher in less developed countries (15.5%) than in more developed regions (10.0%) [Clifford 2005; de Sanjose 2007; WHO/ICO information Centre 2011].

Overall HPV is responsible for little more than 5% of the global cancer burden, the majority of which can be ascribed to cervical carcinoma and less than 0.7% of which is accounted for by cancer of the penis, vulva, vagina and anus [Parkin 2006]. Remarkably, the number of HPV (non-smoking or tobacco) related oropharyngeal cancers has more than doubled in the past 20 years [Evans 2010; Nasman 2009]. Chronic and persistent infection with HPV substantially increases the risk of development of premalignant en malignant cervical lesions and is acknowledged by the World Health Organization as a necessary cause of development of cervical cancer [Bosch 2002; Walboomers 1999; zur Hausen1996].

Cervical cancer is the third most common cancer in women worldwide with 529.800 new cases in 2008, accounting for 8% of the cancer deaths among females [Ferlay 2010; Jemal 2011]. More than 85% of these cases and deaths occur in less developed countries, mainly due to the lack of routine cervical screening programs. The highest incident rates are reported in Eastern-, Southern- and Western Africa, South-Central Asia and South America. The development of cervical cancer will take 10 to 15 years after sexual debut and HPV infection [Schiffman 2005a; Snijders 2006]. In the majority of women an HPV infection is transient and will be cleared within 2 years [Moscicki 2001; Plummer 2007]. Only a small proportion of women (~10%) will stay persistently infected for several years and are at high risk to develop (pre)malignant lesions [Kjaer 2006; Khan 2005; Schiffman 2005b]. According to the Bethesda system, precursor lesions are referred to in cytopathology as either ASC-US (Atypical squamous cells of undetermined significance), low-grade squamous intraepithelial lesions (LSIL, mild dysplasia) or high-grade squamous intraepithelial lesions (HSIL, moderate or severe dysplasia) [Solomon 2002]. Cervical intraepithelial neoplasia (CIN) grade I, II or III is the histopathologic equivalent (Figure 1) [Snijders 2006]. About 85-90% of LSIL will spontaneously regress. When persisting and progressing to HSIL and left untreated, 30% will develop in to invasive cervical carcinoma [McCredie 2008; Wheeler 2008].

Classification and prevalence of HPV types

More than 100 different HPV types have been identified and catalogued so far, of which about 30 to 40 types are known to infect the mucosa and skin of the genital tract [Bernard 2005; Chan 1995; de Villiers 2004]. The genital HPV types have,

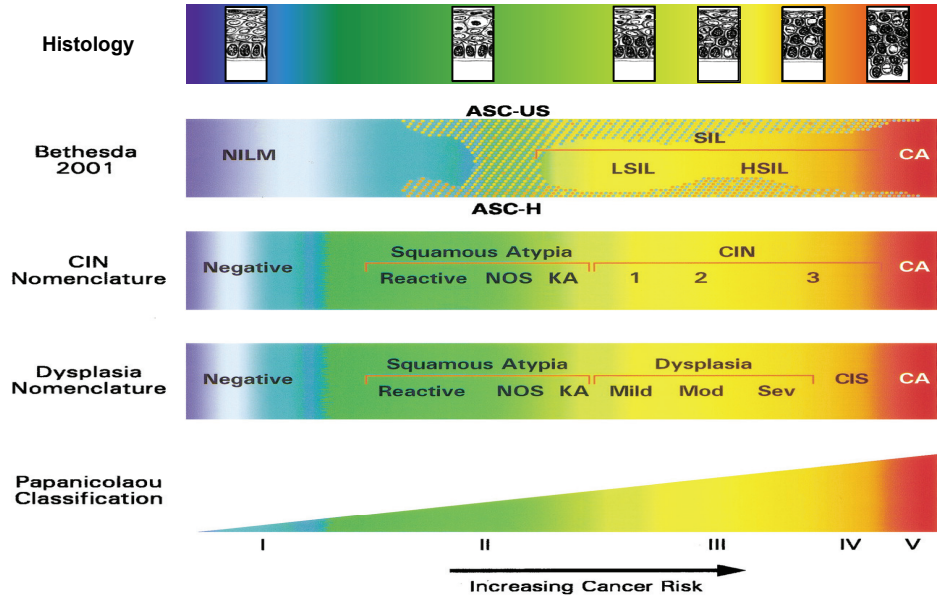

Figure 1. Schematic overview of the morphological alterations and comparative classifications of HPV-related microscopic abnormalities of normal cervical epithelial cells towards invasive cervical cancer. Modified from Sherman and Solomon et al. [Sherman 2003; Solomon 2002].

based on large epidemical studies, been subdivided into low-risk and high-risk or carcinogenic HPV types. Infections with low-risk HPV types (6, 11, 40, 42, 44, 54, 61, 70, 72, 81 and CP6108) have been associated with benign lesions of the anogenital region (e.g. condylomata accuminata, LSIL) and high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) are frequently associated with (pre) malignant cervical lesions [Munoz 2003]. Infection with one of the high-risk HPV types is prevalent in more than 96% of cervical carcinomas [Clifford 2003; Clifford 2006; de Sanjose 2010; Munoz 2004; Smith 2007b]. HPV16 and HPV 18 are estimated to account for 70% of cervical cancer cases worldwide (54.4% and 16.5% respectively), with only marginal differences between developed and less developed regions [WHO/ ICO information Centre 2011]. The other most common high-risk HPV types 31, 33, 35, 45, 52 and 58, account for another 20% of cases. The eight high-risk HPV types are not only detected most frequently in cervical cancer, but to a lesser extend also in pre-cancerous lesions and even in women with normal cytological findings (Figure 2). Respectively, 44.1% of high-grade lesions (HSIL), 18.5% of low-grade lesions (LSIL) and 2.7% of women with normal cytology are estimated to be HPV16 positive. The relative importance of HPV18, 31, 33, 35, 45, 52 and 58 vary a little by region and cytopathological result.

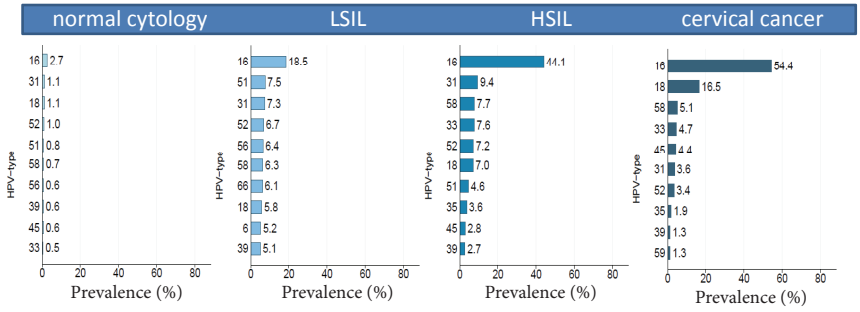

Figure 2. Ten most frequent HPV types among women with and without cervical lesions in the world. Modified from figure 22 of the summary report of the WHO/ICO information Centre on HPV and Cervical cancer [WHO/ICO information Centre 2011].

HPV biology and infection

HPV is a small non-enveloped circular double stranded DNA virus of almost 8000 base pairs belonging to the family of Papillomaviridae. The genome encodes for late structural genes (L1 and L2) and six early non-structural or regulatory genes (E1, E2, E4-E7). Briefly, L1 and L2 encode for viral capsid proteins, E1 and E2 enable viral transcription and the oncogenes E5, E6 and E7 modulate the transformation process. The L1 gene is the most conserved part of the genome between different HPV types and has been used to stratify the 118 HPV types identified so far in a phylogenetic tree (Figure 3) [de Villiers 2004; Doorbar 2006]. The genital HPV types belong to the

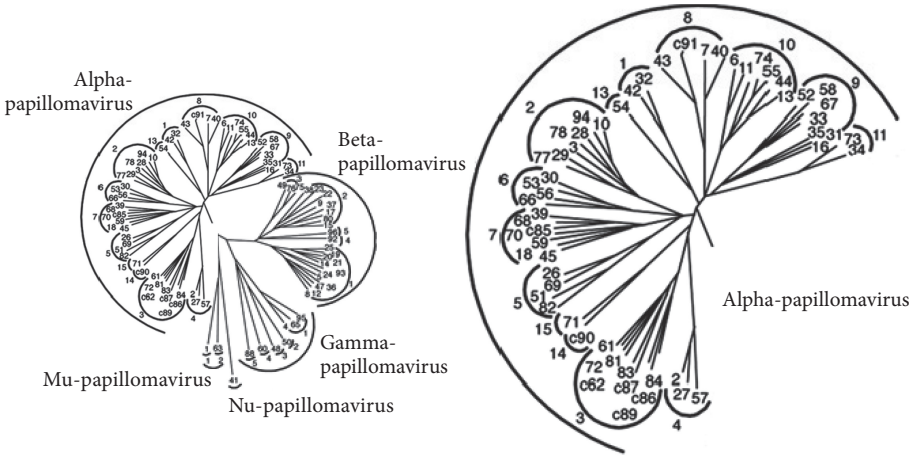

Figure 3. Philogenetic tree of HPV types. Alpha-papillomaviruses primary infect the genital mucosa and are subdivided in 15 different species or clades. HPV16, 31, 33, 35, 52 and 58 belong to the α9 species [de Villiers 2004; Doorbar 2006].

genus of alpha-papillomavirus, which is subdivided in 15 different species (subgroups). HPV16, 31, 33, 35, 52 and 58 belong to the α9 species or clade A9; HPV18 and HPV45 are a member of clade A7. Viruses within a species share between 71%-89% nucleotide homology within the L1 gene [de Villiers 2004].

Papilloma viruses probably infect the basal cells of the epithelium via micro-abrasions of the cervical transformation zone (i.e. squamous epithelium of the ectocervix to glandular epithelium of the endocervix). This leads to the expression of the early genes E1, E2, E5, E6 and E7 and replication of episomal viral DNA in the basal keratinocytes. Structural proteins L1 and L2 are assembled and mature virions are shredded and released from the superficial epithelium and may re-infect the host or patient (LSIL/ CIN I). Persistent infection with a high-risk HPV type might result in integration of the viral DNA into the host cellular DNA, and subsequently to overexpression of the E6 and E7 oncoproteins thereby promoting genetic abnormalities. These cells become dysplastic and if left untreated will progress to HSIL or (micro) invasive carcinoma [Doorbar 2005; Woodman 2007].

Immune responses to HPV

As described, infection with HPV is very common (lifetime incidence ~80%) and the majority of infected individuals will spontaneously clear the virus without even developing premalignant lesions of the cervix. Only a small proportion woman will become persistently infected and are at risk to develop HPV related malignancies. The immune system plays an important role in the balance between viral clearance (immunity) and viral persistence (immune tolerance).

Immunity is a result of the interaction between the innate or non-specific immune system and antigen-specific or adaptive immune system. The innate immune system provides immediate defense against infection by epithelial barriers, local inflammation and cytokines, complement system and phagocytes. Innate immunity has no memory, but is crucial for activating the adaptive immune response by antigen presentation. Adaptive immunity generates antigen-specific effector and memory cells (B and T lymphocytes). B cells play a role in humoral, or antibody related immune responses and T cells are involved in cell-mediated immunity.

The cellular immune system comprises many different subsets of T cells, including CD4+ T helper (Th) cells, CD4+ regulatory T cells (Treg) and CD8+ cytotoxic T lymphocytes (CTL). Th cells are key players in regulating immune responses and represent a much more heterogeneous population than previously suggested: Th1, Th2, Th9, Th17 and follicular T helper cells have been identified so far. Th1 cells regulate cell-mediated immune responses (i.e. activation of CD8+ cytotoxic T cells and effector cells of the innate immune system like macrophages and natural killer cells) and a.o. secrete IFNγ. Th2 cells produce interleukin (IL)-4, -5 and help the humoral effector immune response (i.e. help or stimulate B-lymphocytes to produce antibodies or

Immunoglobulin (Ig) to neutralize foreign antigen and prevent infection). Th17 cells play a key role in autoimmune diseases, mediate antitumor immunity indirectly by changing the tumor microenvironment (secretion of cytokines), and facilitate the recruitment of tumor-infiltrating CD8+ T cells and natural killer cells. Regulatory T cells play a role in immune tolerance by suppressing immune responses including CD8+ cytotoxic T lymphocytes and Th1 mediated responses. Both B- and T lymphocytes are capable of inducing immunological memory.

Innate immunity: immune evasion strategy of HPV

More and more aspects of immune mediated clearance of the HPV infection have become known, however the exact role of interactions of the (host) immune system and HPV remain undetermined. Time taken to spontaneous clearance of a high-risk HPV infection is a relatively long: generally 8 to 16 months [Trottier 2006]. HPVs are very successful in inducing chronic infections. The virus remains undetected by the host immune system for long periods of time because it is able to replicate in the stratified epithelia without inducing overt cell death, danger signals (cytokines) and local inflammation. Therefore triggering and activation of dendritic cells or Langerhans' cells (APC) and initiation of the adaptive immune response does not occur. There is no blood-borne phase or systemic viraemia since virus particles are shredded by the squamous epithelium through normal wear and tear, with no vascular or lymphatic channels nearby where immune responses are initiated. In addition, it has been reported that Langerhans' cells are reduced in women with CIN and do not get activated due to an altered phenotype [Fausch 2005; Hubert 2005]. Increased expression of Toll like receptors (TLRs) in cervical epithelium is associated with HPV clearance, while persistence was associated with reduced levels [Daud 2011]. High-risk HPV types down regulate the type 1 interferon (IFNα and β) secretion and thereby inhibit the activation of the adaptive immune response [Kanodia 2007]. A recent genome-wide expression profiling of HPV infected keratinocytes (KC) versus non-infected keratinocytes revealed that HPV is able to dampen the downstream signaling of the viral recognition receptors in infected cells. Many of the genes downregulated in HPV-positive KCs involve components of the antigen-presenting pathway, the inflammasome, the production of antivirals, pro-inflammatory and chemotactic cytokines, and components downstream of activated pathogen receptors. Notably, gene and/or protein interaction analysis revealed the down regulation of a network of genes that was strongly interconnected by IL-1β, a crucial cytokine to activate adaptive immunity [Karim 2011]. Together this shows that HPV can efficiently evade the innate immune system but many details and underlying mechanisms on how HPV mediates this remain unclear and require in depth study.

Adaptive immunity: role of humoral immune response in HPV infections

HPV type -specific antibodies produced by memory B cells may neutralize and opsonize the virus and prevent re- infection of the cervical epithelium. Serum-neutralizing antibody levels are low in natural infections and sero-conversion is detected within 8 to 18 months after infection [Carter 1996; Carter 2000; Dillner 1999; Ho 2004]. Not all

infected women sero-convert, HPV specific antibodies can be detected in 50 to 60% of women and are mainly directed against epitopes of the major capsid protein L1. The low or weak humoral immune response after natural infection is not surprising since the infection is intraepithelial and there is hardly systemic viraemia.

Adaptive immunity: local and systemic cell mediated immune responses to HPV
80% to 90% of the genital HPV infections resolve with time, suggesting that the adaptive immune response eventually is activated and the infection is controlled by cellular mediated immune responses. Increased prevalence of persistent HPV infections and progression of disease in immunocompromised subjects illustrate the important role of cell mediated immune responses [Palefsky 2006]. A protective immune response to chronic viral infections comprises CD4+ helper T cells that activate and sustain the function of CD8+ cytotoxic T cells as well as innate effector cells [Cardin 1996; Harari 2006; Matloubian 1994; Walter 1995; Zajac 1998]. Immune responses in relation to progression and regression of HPV related diseases have intensively been studied and substantiate this notion. HPV-specific CD4+ and CD8+ T-cell responses have been detected in the peripheral blood of healthy, HPV-negative individuals (i.e. cleared infection in the past) and women with regression of their cervical lesions [Bontkes 2000; de Jong 2002a; de Jong 2004; Farhat 2009; Nakagawa 1997; Nakagawa 2000; Seresini 2007; Welters 2003; Welters 2006]. Immunohistological analysis of spontaneously regressing HPV-related genital warts demonstrated infiltration of large numbers of both CD4+ and CD8+ T cells and macrophages [Coleman 1994]. Woo et al. showed that infiltration of CD8+ cytotoxic T cells into LSIL lesions is related with subsequent regression of the lesion (i.e. protects) whereas the number of CTLs is substantially lower in persisting or progressing lesions [Woo 2008]. In *chapter3*, we will show that both HPV-specific CD4+ Th1/Th2 and CD8+ T cells are able to migrate from the circulation into the skin after intradermal peptide challenge in healthy individuals [van den Hende 2008]. In contrast, partial or complete failure of these types of HPV-specific immune responses is associated with viral persistence and progression of disease. Patients with cervical cancer and (recurrent) high-grade lesions (HSIL) either lacked or showed a dysfunctional and sometimes even suppressive systemic CD4+ T-cell response while CD8+ CTLs were rarely detected in the blood [Bontkes 2000; de Jong 2004; de Gruijl 1998; de Vos van Steenwijk PJ 2008; Nakagawa 2000; Steele 2005; Trimble 2010a; Youde 2000; Welters 2006]. The number of infiltrating CD4 and CD8 T cells is reduced in lesions progressing to HSIL or cervical cancer [Monnier-Benoit 2006; Woo 2008]. Trimble et al. recently showed that the lack of local or intra-lesional vascular expression of mucosal addressin cell adhesion molecule (MAdCAM) is correlated with the influx of CD8+ T-cells and regression of CIN2/3 lesions, whereas progressing lesions showed a dysregulated expression of MAdCAM [Trimble 2010b]. HPV-specific CD4 and CD8 T cells have been detected in tumor and cervical lesions as well as in tumor draining lymph nodes [de Vos van Steenwijk 2008; de Vos van Steenwijk 2010; Piersma 2008; van der Burg 2007]. Tumor infiltrating intraepithelial CD8+ T-cells is associated with a lack of lymph node metastases and a low CD8+/Treg ratio is an independent unfavorable prognostic factor in cervical cancer survival [Jordanova 2008; Piersma 2007].

HPV-specific regulatory CD4 T cells (Treg) are suggested to play a role preventing or suppressing immunological clearance of the HPV infection and associated malignancies [Adurthi 2008; Jordanova 2008; Molling 2007; Piersma 2007; van der Burg 2007].

Immunological approaches to combat HPV infections

Since infection with HPV is a necessary cause of development of cervical cancer, vaccination is assumed an effective mechanism to prevent infection and control HPV related disease. Two different treatment modalities have been developed: prophylactic vaccines that aim at prevention of HPV infection by antibodies or humoral immune responses and therapeutic vaccines that aim at regression of HPV induced (pre) malignant lesions by cell-mediated immune responses.

Prophylactic vaccination

Nowadays, two prophylactic HPV vaccines are licensed: Gardasil® and Cervarix®. Both use L1 VLPs as immunogens. Gardasil® is a quadrivalent vaccine consisting of HPV6, 11, 16 and 18 L1 VLPs and Cervarix® is a bivalent vaccine of HPV16 and HPV18 L1 VLPs. The vaccines make use of a different adjuvant. Both vaccines are administered by intramuscular injection on three occasions in six months and are well tolerated. Both vaccines are highly immunogenic and induce more than 98% sero-conversion and antibody titers 80-100 fold higher than detected after natural infection [Einstein 2009a; Villa 2006]. In fully vaccinated women, both vaccines are highly effective and provide protection of CIN lesions (associated with the HPV types in the vaccine) up to 6.4 and 8.5 years [Frazer 2011; Kjaer 2009; Paavonen 2009; Stanley 2010]. Vaccination will theoretically reduce 70% of the cervical cancer burden (e.g. HPV16 and HPV18 cervical carcinomas). These highly effective vaccines have been shown to reduce HPV-related disease. Nonetheless, long-term duration of protection after vaccination needs to be confirmed (ongoing long-term follow up studies). Mathematical models of the bivalent vaccine however suggest that antibodies can be detected up to 50 years after vaccination [Rowhani-Rahbar 2009].

In natural infections, the antibodies are type-specific and do not appear to be cross-protective [Palmroth 2010]. However, both VLP vaccines do not only induce type-specific antibodies but also cross-protection against related HPV types is observed (i.e. decrease of infections with related HPV types in vaccinated women compared to controls, but also cross-reactive and cross-neutralizing antibodies) [Bonanni 2009; Brown 2009; Jenkins 2008; Kemp 2011; Smith 2007a; Wheeler 2009]. Cross-reactivity has been observed between HPV16, 31 and 58 and HPV18 and 45. This is probably based on the high antibody concentrations as generated by the vaccines and the L1 epitope homology between the different HPV genotypes from the same species or clade.

Although prophylactic vaccination is currently licensed in most countries worldwide [WHO/ICO information Centre 2011], it will take many years before the prevalence of HPV infections among the population and HPV related (pre)malignant lesions will

decrease. Notably, current practice reveals that vaccination coverage among 13-17 year old females is lower as expected: little more than half of the young women (56.4%) in The Netherlands have been fully vaccinated and in the USA, only 26.7 to 32.0% of the young women have completed HPV vaccination [Centers for Disease and Prevention 2010 and 2011; de Hoogh 2011]. This suggests that it may take even longer before an effect of the vaccine at the population level will be noticeable. As mentioned, VLP vaccines were designed to prevent HPV infection. In women already infected with HPV, vaccination with the bivalent vaccine did not increase viral clearance, and therefore the vaccine has no therapeutic effect [Hildesheim 2007]. This underlines that there is still a need to develop therapeutic vaccines that aim at eradicating or reducing these lesions by cell mediated immunity.

Therapeutic vaccination

HPV-infected epithelial cells of HPV-associated (pre)malignant lesions overexpress E6 and E7 oncoproteins and therefore these proteins are attractive targets for the development of immunotherapeutic vaccines. Animal studies using transplantable tumors did show promising results (reviewed by [Frazer 2011]), however despite of all clinical trials (> than 40 publications) so far only Kenter et al. show a compelling therapeutic effect (e.g. 47% complete clinical response) after vaccination [Kenter 2009]. All literature reports presenting results of immunotherapeutic vaccines (i.e. adjuvant protein or peptide vaccines, recombinant viral vectors, and polynucleotide vaccines) in HPV-related clinical trials have been thoroughly reviewed by Frazer et al [Frazer 2011].

In the aforementioned phase II clinical trial, we have recently reported that patients with HPV-16 induced grade 3 vulvar intraepithelial neoplasia (VIN), treated with a highly immunogenic vaccine comprise HPV16E6 and E7 synthetic long overlapping peptides (HPV16 SLP) show clinical response [Kenter 2009]. At 24 months of follow up, complete regression of the HPV-induced lesions was observed in 47% of the patients. Success was paralleled by the induction of a strong and broad HPV-specific CD4+ and CD8+ T-cell response that peaked after the first vaccination [Welters 2010]. The size of the lesion at study entry was associated with clinical response, as non-responders displayed larger lesions and mount a weaker effector T-cell response, which coincided with a stronger HPV-specific regulatory T-cell response [Welters 2010]. Interestingly, this data sustained the notion that protection against chronic viral infections is mediated by both CD4+ and CD8+ T-cells as formulated in earlier reports based on studies of the spontaneous HPV-specific immune response in healthy individuals and patients with HPV-induced lesions [de Jong 2002a; de Jong 2004; Welters 2003; Welters 2006]. The induction of a local innate immune response might enhance the effect of the peptide vaccine in these larger lesions. Local pro-inflammatory stimuli (like Imiquimod) enhance the influx of CD8+ effector cells and suppress regulatory T cells, which might favor clinical outcome [Wagstaff 2007]. Recently, Daayana et al. reported that the effect of vaccine-induced responses was boosted by local treatment with Imiquimod of the VIN lesion [Daayana 2010].

Outline of the thesis

Infections with HPV are very common (~ 80% lifetime incidence) and the immune system plays an important role in the balance between viral clearance and viral persistence. The aim of this thesis was to gain further insight into HPV-specific cellular immunity in relation to the natural course of HPV related disease. HPV clade A9-specific T-cell responses were studied in relation to virological status (i.e. clearance or persistence) and clinical outcome (i.e. regression or progression of premalignant lesions). Furthermore, cross-protective immunity to related HPV types was so far only described for humoral immune responses. No data was available on cross-reactivity in HPV-specific cellular immune responses. Insight in this matter is important, not only for correct interpretation of analysis of HPV specific T-cell responses in relation to health and disease, but also to inform us whether the impact of therapeutic HPV-specific vaccines would be limited to the HPV types comprised (type specific vaccination) or could simultaneously target multiple high-risk HPV types.

To address these aims we conducted a detailed study of cellular immune responses against the highly immunogenic E6 antigen of the closely related HPV types of clade A9 (HPV16, 31, 33, 35, 52 and 58) and found that HPV-specific cross reactive CD4+ T cells are rare and are not likely to affect the interpretation of immune assays. The response pattern in HPV16 vaccinated VIN patients resembles that of healthy controls suggesting that vaccine induced CD4+ T-cell responses are unlikely to mediate cross protection and indicate that therapeutic efficacy of vaccines is type specific (*chapter 2*). *Chapter 3* reports on a pilot study of the use of a delayed type hypersensitivity (DTH) skin test to detect HPV-specific immune responses *in vivo*. This test might be useful to screen spontaneous and vaccine-induced responses to HPV in prospective cohort studies in low resource areas: where the prevalence of HPV infections is high and the access to specialized laboratories to perform *in vitro* assays is limited. Intradermal injection of HPV16 synthetic long peptides is safe and results in migration of HPV specific T cells into the skin as well as an increase of circulating HPV16 specific T cells. In both, *chapter 4 and 5* we report on HPV-specific T-cell responses in relation to virological and clinical outcome in three prospective cohort studies. *Chapter 4* describes the outcome in two developing countries (Haiti and South Africa) where the burden of HPV related disease is high. A prospective study in the United Kingdom on the natural course of HPV16 related low-grade cervical lesions is described in *chapter 5*. The outcome of these studies revealed that the failure of a HPV-specific T-cell response is associated with persistent infection of that specific HPV type and development of progressive disease. Interestingly, while no correlation could be detected between HPV type-specific immune responses and viral clearance, both studies revealed a trend between the regression of a low-grade lesion and the presence of type-specific immunity. Finally, the results of this thesis and directions for further research are discussed in *chapter 6*, and a summary in Dutch is provided in *chapter 7*.

Evaluation of immunological cross-reactivity between clade A9 hrHPV-types on basis of E6-specific CD4+ memory T-cell responses

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Adapted from J Infect Dis 2010; 202:1200-1211

Abstract

CD4+ T-cell responses against the E6 oncoprotein of HPV16 and 5 closely related members of clade A9 (HPV31, 33, 35, 52, 58) were charted in PBMC cultures from healthy subjects and patients that underwent HPV16E6/E7 specific vaccination. Initial analyses with overlapping peptide arrays showed that approximately half of the responding subjects displayed reactivity against corresponding E6 peptides from two or more HPV types. This suggested immunological cross-reactivity and complicated retrospective evaluation of the infection history of the healthy subjects concerned. Importantly, further dissection of the response by means of enriched and clonal T-cell cultures, using protein antigen instead of peptides, revealed that CD4+ T cells capable of efficiently reacting against E6 antigen from multiple HPV types are rare, and only occur when epitope sequences are highly conserved. Our data indicate that natural and vaccine-induced HPV16E6-specific CD4+ T-cell responses are unlikely to mediate efficient cross-protection against other clade A9 members.

Introduction

Comparison of HPV-specific T-cell immunity in patients and healthy controls revealed that HPV16-positive cervical neoplasia is associated with failure of the CD4+ T-cell response against HPV16 early antigens E6, E7 and E2. In contrast, healthy subjects commonly display strong CD4 Th1/Th2 memory responses against HPV16, bearing witness to encounter and successful immune control of this virus [de Jong 2002a; de Jong 2004; van Poelgeest 2006; Welters 2003]. The frequency by which the latter responses are detected (approximately 50%) is in line with the estimated lifetime incidence of HPV16 infections [Jenkins 1996]. Our recent finding of a strong correlation between the strength of vaccine-induced HPV16E6/E7-specific CD4+ T-cell responses and full regression of HPV16-positive lesions in patients [Kenter 2009] lends further support to the functional importance of CD4 T-cell responses against HPV early antigens.

The existence of multiple closely related high-risk (hr) HPV types [Munoz 2003], the antigens of which display up to 70% homology, suggests that a considerable degree of immunological cross-reactivity towards these viruses may exist. Increased insight in this matter is important for two reasons. First, this will facilitate correct interpretation of analyses of the HPV-specific T-cell response in relation to health and disease, such as for assessing the role of T-cell immunity in clearance of HPV infections and regression of premalignant lesions. Second, detailed information on the specificity of hrHPV-specific T-cell responses can inform us on whether the impact of HPV-specific vaccines would be limited to the HPV types comprised, or could target a broader range of hrHPV types. We therefore charted the incidence of CD4+ memory responses against HPV16 and highly similar hrHPV types [Bernard 2005; Chan 1995; de Villiers 2004] in healthy donors as well as in patients that received HPV16 E6/E7 specific vaccination. We focused on clade A9, encompassing HPV types 16, 31, 33, 35, 52, 58, because HPV16 is the most prevalent type in cervical carcinoma and anogenital neoplasia [De Vuyst 2009; Smith 2007]. Furthermore, the degree of sequence conservation within this clade is higher than that between clades A9 and A7, the latter of which comprises HPV types 18 and 45. We choose to study responses against the E6 antigen, because its expression is associated with all stages of neoplasia, and T-cell responses against E6 are more commonly found than against E7 in both healthy subjects and patients [de Jong 2004; Welters 2003].

Materials and Methods

Subjects

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy blood donors (n = 44), as well as from a group (n = 10) of HPV16 positive patients with high-grade vulvar intraepithelial neoplasia (VIN) who participated in our immunotherapeutic vaccination trial [Kenter 2009], after informed consent.

Antigens

To examine HPV clade A9 specific T-cell responses, sets of overlapping 32-mer peptides (14-amino acid overlap) spanning the entire E6 proteins of HPV16, 31, 33, 35, 52 and 58 were used (Figure 1). The peptides, recombinant E6 proteins and memory response mix (MRM), consisting of a mixture of tetanus toxoid, *Mycobacterium tuberculosis* sonicate and *Candida albicans* were produced and used as described previously [de Jong 2002a; de Jong 2004; Franken 2000; van der Burg 1999; van der Burg 2001; Welters 2003].

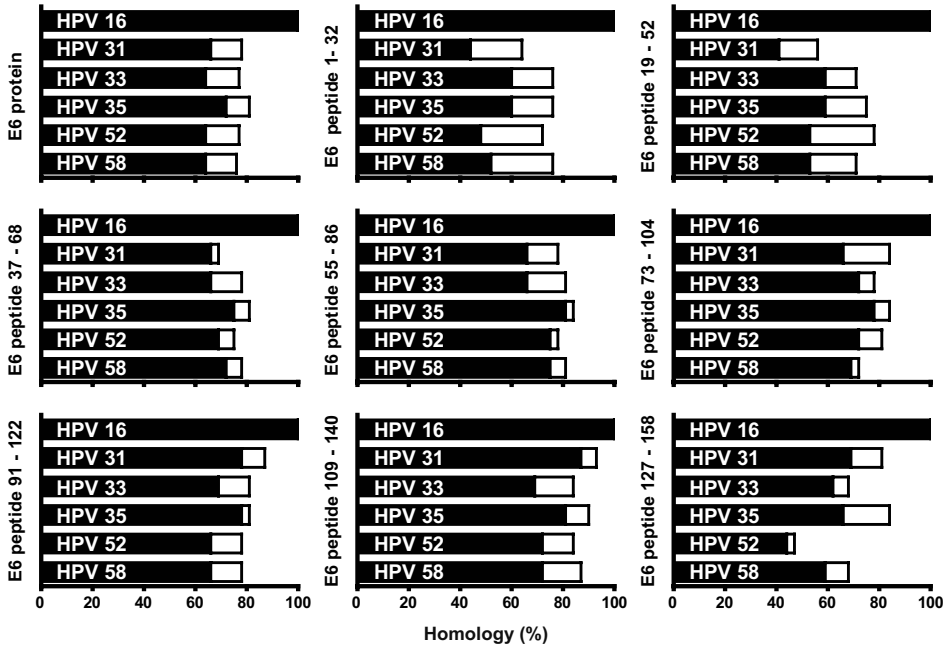

Figure 1. Sequence homology between E6 antigens of human papillomavirus (HPV) clade A9. Analysis of sequence homology of E6 protein and overlapping clade A9 peptides performed in SwissProt protein database using Basic Local Alignment Search Tool (BLAST) [Altschul 1997] showed >70% amino acid homology with HPV16E6. Both percentages of identical amino acids (black bars) and percentages of amino acids with similar properties (open bars) are depicted.

T-cell cultures and clones

Enriched HPV16E6 specific T-cell cultures were isolated by magnetic cell sorting (Miltenyi Biotech) based on IFNγ secretion after an extended period of in vitro stimulation as described by de Jong *et al.* [de Jong 2005]. T-cell clones were isolated from the enriched HPV16E6 specific T-cell cultures using limiting dilution [van der Burg 1999].

Analysis of T-cell responses

All analytical protocols used are part of standard operating procedures established in our laboratory, for which detailed descriptions have been published elsewhere. The presence of HPV clade A9 specific memory T cells in healthy donors and HPV16 positive VIN patients was analyzed by IFNγ enzyme-linked immunosorbent spot (ELISPOT) as described previously [van der Burg 2001; van Poelgeest 2006; Welters 2003; Welters 2006]. Antigen-specific T-cell responses were considered positive when T-cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background.

T-cell cultures and clones were tested for their specificity and function by IFNγELISPOT, intracellular cytokine staining (ICS) and in a 3-day proliferation assay. When analyzed by ICS [de Jong 2002a; de Jong 2005], positive response was defined as at least three times the percentage of IFNγ producing CD4+ T cells found with the medium-only control. When analyzed in 3-day proliferation assays [de Jong 2005; van der Burg 2001], responses were defined positive if the stimulation index (SI) ≥ 3. Antigen-specific IFNγ secretion into the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) or cytometric bead array (Becton Dickinson) as reported previously [de Jong 2004; van der Burg 1999].

Results

HPV clade A9 E6-specific T-cell memory in healthy subjects

Memory T-cell responses to the E6 antigens of HPV clade A9 members (types 16, 31, 33, 35, 52 and 58) were evaluated in primary PBMC cultures from 30 healthy donors. T cells responding to pools of overlapping peptides (Figure 1) were enumerated by means of IFNγELISPOT assays. We have previously shown that these assays primarily detect CD4+ memory T-cell responses [van der Burg 2001; van Poelgeest 2006; Welters 2003; Welters 2006]. In line with the notion that a vast majority of the population encounters a hrHPV type during life time (80-85% [Jenkins 1996]), immune responses to E6 of one or more clade A9 members were detected in 19 (63%) of 30 subjects (Table 1). Responses to HPV16 were observed in 40% of the healthy subjects (12 of 30), in accordance with our previous studies [de Jong 2002a; de Jong 2004; Welters 2003]. Peptides recognized differed between subjects, in line with natural human leukocyte antigen (HLA) variation in the human population. Responses against E6 of the other clade A9 members were found in a smaller fraction of donors (4-9 of 30 donors). In this latter group, responses to HPV58E6 were the most prevalent (found in 9 (30%) of 30), and their frequency approximated that of responses against HPV16. As indicated in bold in Table 1, 11 (58%) of the 19 responding subjects displayed T-cell responses against corresponding peptide pools from two or more clade A9 HPV types, which suggests immunological cross-reactivity. For instance, donor 15 shows reactivity against E6 peptide pool 3 of HPV16 and 33, while donor 26 reacts against pool 2 of HPV16 and 58. In all but one case (donor 28), reactivity against corresponding peptide pools was limited to two clade A9 members.

Table 1. HPV clade A9 specific CD4+ Th1 responses in healthy subjects

Donor[a]	HPV16 E6				HPV31 E6				HPV33 E6				HPV35 E6			
	1-50[c]	37-86	73-122	109-158	1-50	37-86	73-122	109-156	1-50	37-86	73-122	109-156	1-50	37-86	73-122	109-156
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	**11**[d]	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	**87**	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	**11**	-	-	-	-	-	**10**	-	**15**
6	-	-	-	-	-	-	-	*21*	-	-	-	-	-	-	-	-
7	-	-	-	*86*	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	*12*	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	*13*	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	**17**	-	-	-	-	-	-	-	**13**	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	**28**	-	-	-	-	-	-	-	**19**	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	**12**	-	-	-	-	-	-	-	**12**	-	-
19	-	-	*23*	-	-	-	-	-	-	-	-	-	-	*21*	-	-
20	-	-	*13*	-	-	-	-	-	-	-	-	-	-	-	-	*10*
21	-	-	**14**	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	25	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	**17**	-	-	-	-	-	-	-	**17**	-	-	-	-	-	-
26	-	**21**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	*13*	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	**20**	*73*	-	-	-	-	-	-	**18**	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Frequency[c] of responders	**40%** (12/30)				**17%** (5/30)				**17%** (5/30)				**13%** (4/30)			

[a] PBMC from 30 healthy blood donors were tested against E6 peptides of HPV clade A9 (HPV16, 31, 33, 35, 52 and 58) by IFNγ ELISPOT. [b] Encounter of HPV types. Defenite: showing ELISPOT response towards one or different peptide pools of one or more HPV types. Response can – with reasonable probality – be ascribed to infection with this particular HPV type. Ambigous: ELISPOT response towards similar peptide pool of 2 or more HPV types. Responses can reflect encounter of either one or both of the viruses. [c] Indicated are the first and last amino acid in the peptide pool used. [d] Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in

	HPV52 E6				HPV58 E6					Encounter of HPV type(s)[b]	
	1-50	37-86	73-122	109-155	1-50	37-86	73-122	109-156	MRM	definite	ambigous
	-	-	-	-	-	-	-	-	**408**	-	-
	-	-	-	-	-	-	**12**	-	**150**	-	31/58
	-	-	-	-	-	-	-	-	**35**	-	-
	-	-	**102**	-	-	-	-	-	**215**	-	31/52
	-	-	-	-	-	**41**	-	-	**19**	-	31/35/52
	-	-	-	-	-	-	-	-	**139**	31	-
	-	-	-	-	-	*21*	*20*	-	**177**	16; 58	-
	-	-	-	-	-	-	-	-	**107**	16	-
	-	-	-	-	-	-	-	-	**251**	-	-
	-	-	-	-	-	-	-	-	**81**	-	-
	-	-	-	-	-	-	-	-	**21**	33	-
	-	-	-	-	-	-	-	-	**50**	-	-
	-	**11**	-	-	-	**19**	-	-	**114**	-	16/33; 52/58
	-	-	-	-	-	-	-	-	**34**	-	-
	-	-	-	-	-	-	-	-	**83**	-	16/33
	-	-	-	-	-	-	-	-	**54**	-	-
	-	-	-	-	-	-	*12*	-	**75**	58	-
	-	-	-	-	-	-	-	-	**95**	-	31/35
	-	-	-	-	-	-	-	-	**132**	16; 35	-
	-	-	-	-	-	-	-	-	**230**	16; 35	-
	-	-	**21**	-	-	*15*	-	-	**57**	58	16/52
	-	-	-	-	-	-	-	-	**125**	-	-
	-	-	-	-	-	-	-	-	**135**	-	-
	-	**47**	-	-	-	**35**	-	-	**107**	16	52/58
	-	-	-	-	-	-	-	-	**198**	-	16/33
	-	-	-	-	-	**81**	-	-	**27**	-	16/58
	-	*17*	-	-	-	-	-	-	**209**	16; 52	-
	100	-	-	-	-	-	**34**	-	**147**	52	16/33/58
	-	-	-	-	-	-	-	-	**134**	-	-
	-	-	-	-	-	-	-	-	**24**	-	-
		20% (6/30)				**30%** (9/30)					

experimental wells. The number of spots per 100.000 PBMC are given. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg SH 2001]. These values are indicated: coinciding responses against corresponding E6 peptide pools are depicted in **bold** (possible crossreactivity) and responses not matched by coinciding reactivity against corresponding peptide pools are depicted in *italics*. A dash indicates no measurable specific T-cell reactivity and memory recall mix (MRM) was used as a positive control. [e] The total number of donors responding per HPV type are depicted.

The patterns of HPVE6-specific responses as collected in Table 1 were used to reconstruct (with reasonable probability) the HPV clade A9 infection history for each of the donors. This was possible for 8 of 19 subjects (Table 1): 4 donors (6, 8, 11 and 17) whose responses are limited to E6 of a single HPV type, and 4 donors (7, 19, 20 and 27) who responded against non-matching E6 peptide pools from 2 different HPV types. For instance, donor 7 responds to peptide pool 4 of HPV16 and peptide pools 2 and 3 of HPV58, arguing that this donor has encountered both HPV types and that the resulting E6-specific T-cell responses are not cross-reactive. In the remaining 11 cases, coinciding responses against corresponding peptide pools from different HPV types caused ambiguity with respect to the HPV types encountered. For instance, the reactivity by donor 4 against peptide pool 3 of HPV 31 and 52 could reflect encounter of either one or both of these viruses. Our data demonstrate that ELISPOT data shown in Table 1 cannot be readily used for retrospective evaluation of the infection history of subjects.

HPV clade A9 cross-reactivity in cultures enriched for HPV16E6-specific T cells

We conducted a more detailed analysis with cultures enriched for HPV16E6-specific T cells by means of magnetic cell sorting (MACS). The resulting 14 cultures (from donors 31-44) contained 2.3% (range 0.4-6.9%) HPV16E6 specific T cells before and 45.6% (range 16.5-65.3%) HPV16E6 specific T cells after MACS, representing a 9-60 fold enrichment. The first 10 enriched cultures were analyzed for immune responses against the different E6 antigens by IFNγELISPOT. The use of single peptides, rather than peptide pools, increased the resolution of the analysis. As expected on basis of enrichment, all cultures displayed a HPV16 specific immune response against one of more peptides. Importantly, 8 of 10 cultures also responded against one or more non-HPV16 peptides (Table 2), which supports the notion that coincidence of T-cell responses against related HPV types is a frequent event. In total, 34 HPV16E6 peptide-specific responses were detected. Because we have used

Table 2. Screening of HPV clade A9 cross reactive immune responses in HPV16E6 enriched T cell cultures by Elispot

	HPV16 Elispot			Potential cross-reactivity				
Donor[a]	E6 peptide		response	HPV31	HPV33	HPV35	HPV52	HPV58
31	37-68[b]	}	**33**[c]	-	-	-	-	-
	55-86	}	**32**	-	-	**62**	-	-
	73-104	} [d]	**359**	-	**33**	-	**142**	**46**
	109-140	}	**38**	-	-	-	-	-
	127-158	} •	**63**	-	-	-	-	-
32	55-86	}	**158**	-	-	-	-	**168**
	73-104	}	**209**	-	-	-	**264**	-

	HPV16 Elispot			Potential cross-reactivity				
Donor[a]	E6 peptide		response	HPV31	HPV33	HPV35	HPV52	HPV58
33	73-104	}	**151**	-	-	-	-	**91**
	109-140	}	**117**	-	-	-	-	-
	127-158	} ●	**291**	-	-	-	-	-
34	37-68	}	**235**	-	-	-	-	-
	55-86	}	**239**	-	-	**175**	-	-
	73-104	}	**225**	-	-	-	-	-
	109-140	}	**140**	-	-	-	-	-
	127-158	} ●	**329**	-	-	-	-	-
35	73-104	}	**160**	-	-	-	-	**84**
	91-122	}	**130**	-	-	-	-	-
	109-140	} ●	**66**	-	-	-	-	-
36	37-68	●	**120**	-	-	-	-	-
	109-140	}	**122**	-	-	-	-	-
	127-158	} ●	**290**	-	-	-	-	-
37	37-68	}	**17**	-	-	-	-	-
	55-86	}	**42**	-	-	**19**	-	-
	73-104	}	**17**	-	-	-	-	-
	109-140	}	**21**	-	-	-	-	-
	127-158	}	**69**	**32**	-	**41**	-	-
38	55-86	}	**169**	-	-	**10**	-	-
	73-104	}	**65**	-	-	-	-	-
	109-140	}	**511**	-	-	-	-	-
	127-158	} ●	**354**	-	-	-	-	-
39	127-158		**215**	-	-	**226**	-	-
40	55-86	} ●	**111**	-	-	-	-	-
	73-104	}	**160**	-	-	-	-	-
	91-122	} ●	**197**	-	-	-	-	-

[a] Enriched HPV16E6 specific T cell cultures of 10 healthy donors evaluated by IFNγ ELISPOT for HPV clade A9 cross reactive immune responses. [b] All peptides (32 mer) of the E6 protein of HPV16, 31, 33, 35, 52 and 58 were tested (single peptide, no pool). Only peptides showing a response are depicted, indicated by the first and last amino acid. [c] Specific spots were calculated by subtracting the mean number of spots + 2xSD of the background from the mean number of spots in experimental wells. The number of spots per 100.000 PBMC are given. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg SH 2001]. These values are indicated in **bold**. All donors show a HPV16E6 specific response. In 8 out of 10 donors, this response is matched by a response to one or more of the other clade members. [d] Brackets are placed over potentially overlapping epitopes and HPV16E6 specific responses not matched by reactivity against matching E6 peptides of the other clade members are indicated with a dot (●).

overlapping peptides, it is conceivable that many of these responses are directed against the same (overlapping) epitope (Table 2). In view of the latter, we can discern at least 21 distinct responses. Of these, 43% (9 of 21) are not paralleled by a response against a corresponding peptide of one of the other clade A9 members tested, strengthening the notion that these responses were primed by encounter of HPV16 (Table 2). In the other 57% cases (12 of 21), reactivity against a corresponding peptide from another HPV type was detected. In all but 2 cases (donor 31, peptide 73-104; donor 37, peptide 127-158), reactivity was limited to the corresponding peptides of HPV16 and one other clade A9 member. This indicates that, if these coinciding responses are due to cross-reactivity between HPV types, this cross-reactivity is not broad (see also Table 1).

To assess whether the coincidence of T-cell responses against E6 of different HPV types reflects the reactivity of highly potent T cells, we measured the response of the 4 remaining enriched T-cell cultures in the presence of peptide arrays as well as full-length E6 proteins. Our prior studies have shown that the repertoire of antigen-specific T cells responding to peptide-pulsed antigen-presenting cells (APCs) is generally larger than that responding to protein-pulsed APCs. We use this comparison to distinguish between highly potent T cells, capable of responding against limiting quantities of naturally processed antigen, and T cells that require greater quantities of cognate antigen [de Jong 2002a; van der Burg 2001; van der Burg 2007]. Although T cells that are capable of responding against peptide-pulsed (but not protein-pulsed) APCs may reflect physiologically relevant responses, these are less likely to play a prominent role in the immune defense in case antigen presentation is suboptimal, due to either limitations in quantity or differences in antigen structure. Analysis of the responses by ICS allowed more accurate comparison of the T-cell fractions responding to protein and peptide antigens and, in addition, discrimination between CD4+ and CD8+ T-cell responders.

In accordance with our prior studies [de Jong 2002a; Welters 2003; Welters 2008], the latter discrimination showed that all IFNγ producing T cells were detected in the CD4+ T-cell gate, confirming that our methodology using long peptide or protein pulsed APCs is geared towards detection of CD4+ T-cell responses. All 4 T-cell cultures responded not only to HPV16E6 peptide but also to HPV16E6 protein (Figure 2), arguing that at least a major fraction of the HPV16E6 peptide-reactive T cells reflect a potent immune response to naturally processed antigen. As expected on basis of the data in Tables 1 and 2, the 4 cultures also responded to E6 peptides of related HPV types. However, only one of the enriched T-cell cultures responded to the matching E6 proteins (Figure 2B), which suggests that this culture represents a T-cell response capable of reacting against HPV types 16, 31 and 35 with similar efficiency. On basis of this criterion, the HPV16-enriched cultures of the other three donors, which display strong reactivity towards HPV16E6 protein only, represent T-cell responses that will readily react when encountering HPV16, but much less

efficient against the closely related HPV types. Our findings indicate that cross-reactivity of HPV16E6-specific T cells to one or two other HPV types, even though commonly detected in peptide pulsed cultures, occurs less frequent in the presence of limiting quantities of naturally processed peptide-epitopes such as achieved when the HPV proteins are used to stimulate the T cells.

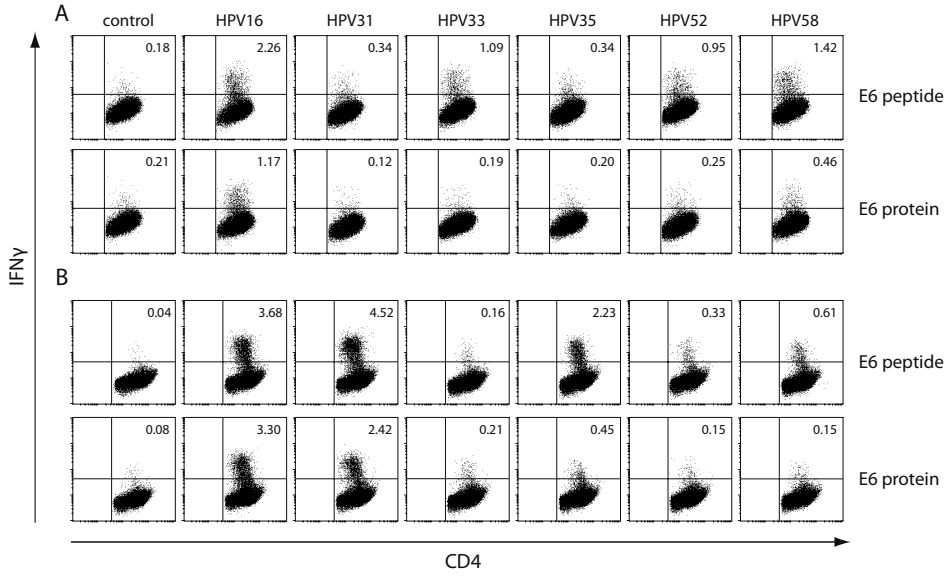

Figure 2. Human papillomavirus (HPV) clade A9 cross-reactivity in HPV16E6 enriched T-cell cultures. Peptide- and protein-specific recognition was analysed in 4 HPV16E6 enriched T-cell cultures (donors 41-44) by ICS. The percentage of IFNγ producing cells in the CD4+ fraction is depicted in the upper right corner. A positive response was defined as at least three times the percentage of IFNγ producing CD4+ T cells as in the medium control. Data shown are for donors 44 (A) and 42 (B).

T-cell cross-reactivity at the clonal level

The polyclonal T-cell cultures studied so far leave the possibility that reactivity against E6 antigens of more than one HPV type is due to distinct subpopulations of T cells within these cultures. We therefore examined the aspect of cross-reactivity by means of HPV specific T-cell clones isolated from the aforementioned enriched T-cell culture that was reactive to E6 of HPV types 16, 31 and 35 (donor 42; Figure 2B). Furthermore, we used shorter overlapping 10-mer peptides to minimize the possibility that reactivity would be due to distinct, partially overlapping epitopes encompassed by a single peptide.

In total, 73 different HPV16 peptide-reactive clones were isolated by limiting dilution, 14 of which were responsive to both HPV16 peptide and protein. In view of the likelihood to find cross-reactivity against proteins of other HPV types, we focused our further analyses on the latter 14 clones, which were able to recognize antigen when present at limiting quantity. Clonality was confirmed by V-beta staining (data not shown). Clone 105 (Figure 3A) is an example of 1 of 7 clones that exclusively responded against peptide and protein of HPV16. Clone 20 (Figure 3A) is 1 of 6 clones that responded to HPV16 (peptide and protein) as well as to other clade A9 peptides, but not to the other clade A9 proteins. Only one of the clones isolated (Figure 3A, Clone 8) responded to E6 of HPV types 16, 31 and 35 at both the peptide and protein level which suggests that efficient cross-reactivity is not only a rare event at the population level but also at the clonal level within the T-cell response of a single individual.

Additional analysis of clone 8 with major histocompatibility complex class II-blocking antibodies revealed that this response was restricted by HLA-DP (data not shown). Fine mapping of the HPV16 specific immune response by means of HPV16E6 10-mer peptides identified peptide LKFYSKISEY as the minimal epitope recognized by the T-cell clone (Figure 3B). This epitope sequence is largely conserved between the three E6 proteins recognized, whereas the non-recognized E6 proteins share a prominent tyrosine to leucine substitution at position 77 (Figure 3C).

HPV clade A9 cross-reactivity in HPV16 E6/E7-vaccinated patients

A clear disadvantage of charting hrHPV-specific T-cell responses in healthy donors is that the HPV infection history of these subjects is unknown. As illustrated in Table 1, this makes it difficult to causally link detection of memory T-cell responses to encounter of specific HPV types. Our laboratory recently performed a phase II therapeutic vaccination study in which patients who received a diagnosis of HPV16+ vulvar intraepithelial neoplasia grade 3 (VIN-III) received 3-4 consecutive doses of a synthetic peptide vaccine encompassing the complete amino acid sequences of HPV16E6 and E7 [Kenter 2009]. This study offered the unique opportunity to monitor the CD4+ T-cell reactivity against the E6 antigen of different clade A9 members in subjects known to have encountered both HPV16 and multiple doses of the HPV16E6 antigen. Before vaccination, none of the patients displayed IFNγ responses against E6/E7 peptides of HPV16, in accordance with failure of this immune response as documented in our prior studies [de Jong 2004; Welters 2003]. In contrast, the majority of the patients displayed HPV16-specific T-cell responses, predominantly reacting against E6, after the last vaccination [Kenter 2009].

For 10 of these vaccinated patients, who displayed strong HPV16-specific reactivity after vaccination, we charted T-cell reactivity against E6 peptide pools from the different clade A9 members (Table 3). In 5 patients, the vaccine-induced response against HPV16E6 was accompanied by reactivity to E6 peptides of one or more other clade A9 members (e.g. patient 6; Figure 4). For the other 5 patients, the post-vaccination E6-specific response was restricted to HPV16 (e.g. patient 6; Figure 4). Interestingly,

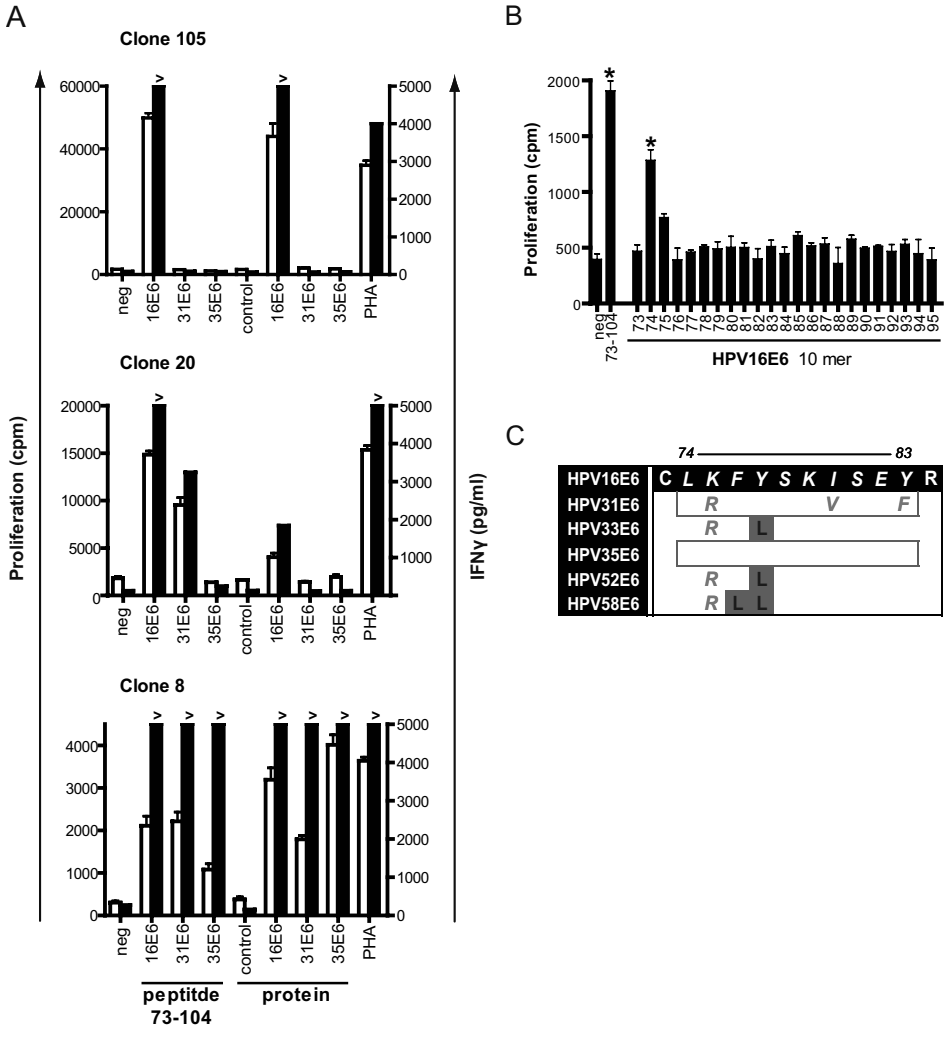

Figure 3. Clade A9 cross-reactivity in human papillomavirus (HPV) 16E6 specific T-cell clones. A. T-cell clones (n = 73) isolated by limiting dilution from the enriched HPV16, 31 and 35-reactive T-cell culture of donor 42 (Figure 2) were tested for their capacity to proliferate upon stimulation with monocytes pulsed with peptide (5 µg/ml) or protein (10 µg/ml). Phytohemagglutinine (PHA) served as a positive control. Proliferation was measured by [^{3}H] thymidine incorporation and was defined specific if stimulation index (SI) ≥ 3 (open bars). Supernatants were analysed for IFNγ by ELISA (black bars). Clones representative of three reactivity patterns found are shown. B. Fine mapping of the HPV16E6-specific immune response of clone 8 by means of HPV16E6 10-mer peptides in a 3-day proliferation assay. Specific responses (SI ≥ 3) are marked with an asterisk. C. The identified minimal epitope (74-83, LKFYSKISEY) is largely conserved between the three clade A9 types recognized by this T-cell clone (HPV16, 31 and 35). Depicted are the minimal epitope ( ), amino acids with similar properties ( ) and amino acid mismatches ( ).

Table 3. HPV clade A9 specific CD4+ Th1 responses in HPV16+ VIN patients before and after HPV16 peptide vaccination

Patient[a]		HPV16E6		HPV31E6		HPV33E6		HPV35E6		HPV52E6		HPV58E6		
	Vaccination	1-86[b]	73-158	1-86	73-156	1-86	73-156	1-86	73-156	1-86	73-155	1-86	73-156	MRM
01	pre	-	-	-	**11**	-	**11**	**10**	**10**	-	-	**15**	**13**	**73**
	post	**90**[c]	**167**	-	**120**	**39**	**116**	**87**	**121**	**69**	**65**	**66**	**89**	**78**
02	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	*30*	-	-	-	-	-	-	-	-	-	-	*10*	-
03	pre	-	-	-	-	-	-	-	-	-	-	-	-	**26**
	post	*53*	**50**	-	-	-	-	-	-	-	-	-	**10**	-
04	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	**84**	**42**	**13**	-	-	**19**	-	**24**	-	-	-	-	-
06	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	*62*	*128*	-	-	-	-	-	-	-	-	-	-	-
07	pre	-	-	*11*	-	-	-	-	-	-	-	-	-	**15**
	post	*82*	**74**	-	-	-	**13**	-	-	-	**14**	-	**26**	**60**
08	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	**73**	*51*	-	-	-	-	-	-	-	-	**10**	-	**17**
13	pre	-	-	-	**10**	-	-	-	-	-	**15**	-	**11**	**13**
	post	*37*	*23*	-	-	-	-	-	-	-	-	-	-	-
16	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	*79*	-	-	-	-	-	-	-	-	-	-	-	-
30	pre	-	-	-	-	-	-	-	-	-	-	*20*	-	**24**
	post	*79*	*37*	-	-	-	-	-	-	-	-	-	-	-

[a] Clade A9 cross reactive T cell responses evaluated by IFNγ ELISPOT in PBMC from 10 HPV16+ VIN patients before and after HPV16 E6/E7 peptide vaccination. [b] Indicated are the first and last amino acid in the peptide pool used. [c] Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in experimental wells. The number of spots per 100.000 PBMC are given. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg SH 2001]. These values are indicated: coinciding responses against corresponding E6 peptide pools are depicted in **bold** (possible crossreactivity) and responses not matched by coinciding reactivity against corresponding peptide pools are depicted in *italics*. A dash indicates no measurable specific T-cell reactivity and memory recall mix (MRM) was used as a positive control.

the incidence by which primary PBMC cultures display reactivity against E6 peptides of both HPV16 and one or more other clade A9 members is very similar for the HPV16-vaccinated VIN patients (5 [50%] of 10) and the healthy donors with unknown HPV infection history (5 [42%] of 12; Table 1). In 4 patients (patients 1, 7, 13, 30), modest pre-vaccination responses were detected against E6 of HPV 31, 33, 35, 52 and/or 58, but not against HPV16. Notably, the detection of these pre-vaccination responses was not correlated with post-vaccination responses (Table 3). Taken together, these data show that HPV16 vaccination of patients can induce IFNγ-responses against E6 of HPV16 and related clade A9 members independent of pre-existing immunity, and that the detection of post-vaccination IFNγ responses against E6 of the related clade A9 members varies greatly between patients.

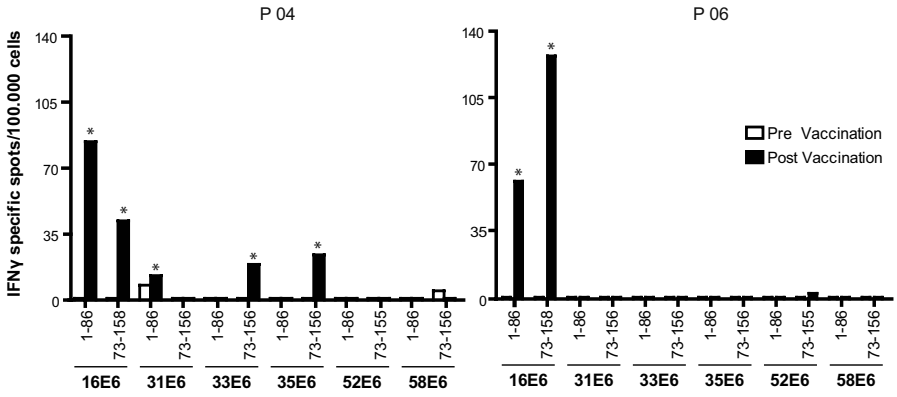

Figure 4. Human papillomavirus (HPV) clade A9 immune responses in HPV16E6/E7 vaccinated patients with vulvar intraepithelial neoplasia (VIN). Peripheral blood mononuclear cell (PBMC) samples of HPV16-positive patients with VIN who participated in an HPV16E6/E7 vaccination study were isolated before the first and after the fourth vaccination. IFNγELISPOT analysis was performed against peptide pools specified. Responses were considered positive when T-cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x medium control value (marked with an asterisk). All patients tested (n = 10) displayed strong HPV16E6-specific reactivity after vaccination (Table 3). In half of the patients, this vaccine-induced response was also accompanied by reactivity to E6 peptides of one or more other clade A9 members (patient 4 is shown as a representative example). For the other five patients, the post-vaccination response was restricted to HPV16E6 only (patient 6 is shown as a representative example).

Discussion

Charting of the CD4+ memory T-cell responses in healthy subjects against the E6 antigen of HPV16 and 5 closely related hrHPV types by means of overlapping peptide arrays revealed that responders frequently display reactivity against corresponding E6 peptides from two HPV types. This initially suggested immunological cross-reactivity between the E6 antigens. However, further dissection of these responses by means of HPV16-enriched and clonal T-cell cultures, as well as by using whole protein antigens,

indicated that CD4+ T cells capable of reacting efficiently with limiting quantities of naturally processed E6 antigens from two or more HPV types are only rarely observed. This finding implies that natural CD4+ T-cell immunity elicited through encounter of a given hrHPV type is unlikely to provide efficient cross-protection against closely related hrHPV types. In accordance with our data, analysis of sequential acquisition of HPV types indicated that risk of acquiring a new HPV type was not decreased among those with prior infection by a related type [Thomas 2000; Woo 2009

Our findings furthermore demonstrate that detection of memory CD4+ T-cell responses against a given HPV type cannot be readily used as an indicator that the subject concerned has encountered this particular HPV type. For 11 of 19 HPV-specific responders tested, the detection of responses against corresponding E6 peptides from two or more HPV types resulted in ambiguity with respect to number and identity of HPV types encountered (Table 1). In 6 cases (donors 13, 15, 21, 25, 26, 28), HPV16 was among the HPV types recognized. In view of the highest prevalence of HPV16, these 'overlapping' responses are most likely primed through encounter of HPV16. This notion is supported by our data showing that about half of the T-cell clones reacting to peptides of HPV16 and other clade A9 members respond to HPV16-derived E6 protein only. Our data also imply that detection of CD4+ memory T-cell responses by means of peptide arrays cannot readily be used for determining the prevalence of HPV types. This is expected to result in an overestimation of the prevalence of HPV types closely related to HPV16. For instance, we found HPV58 E6-specific T-cell reactivity in 9 (30%) of 30 subjects (Table 1), a frequency approximating that found for HPV16 (12 (40%) of 30 subjects). However, HPV58 prevalence on basis of DNA testing was found to be 5-6 fold lower than that of HPV16 [Clifford 2005].

With respect to our methodology of analysis, the following can be noted. First, HPV types included in our screening share more sequence homology with each other than with any of the other types. Therefore, we deem it unlikely that cross-reactivity against HPV types excluded from our study would play a significant role. Accordingly, we did not find indications for immunological cross-reactivity between E6 of HPV16 and 18 [Welters 2006]. Second, it is conceivable that charting of HPV-specific immunity with single and/or shorter peptides, instead of pools comprising two 32-mer peptides, would result in fewer coinciding responses, and will thereby allow for greater resolution with respect to the infection history of subjects concerned. Notably, the larger numbers of PBMC required for this fine mapping can generally not be obtained in the context of cohort studies.

The availability of PBMCs from HPV16E6/E7 vaccinated patients allowed analysis of the E6-specific reactivity in subjects known to have encountered the HPV16E6 antigen. In addition to the vaccine-induced HPV16E6-specific T-cell responses, these patients showed post-vaccination reactivity to E6 peptides from the other clade A9 members. Importantly, the overall frequency and breadth of these additional responses were not greater than those detected alongside naturally induced HPV16E6-specific responses

in healthy subjects. Our findings in vaccinated patients strengthen the notion that the majority of coinciding responses against corresponding E6 peptides of HPV16 and one or more other clade A9 members as found in healthy subjects have been induced by HPV16 encounter, and do not bear witness to encounter of the less prevalent HPV types. Consequently, screening for CD4+ memory T-cell responses with peptide arrays against, for example, HPV31 or HPV58 in the absence of parallel screening with a HPV16 peptide array could readily lead to misinterpretation of the resulting data. Furthermore, the resemblance between the clade A9 response pattern in healthy controls and HPV16 vaccinated patients suggests that the vaccine-induced CD4+ T-cell responses are unlikely to have therapeutic impact against neoplastic lesions positive for HPV types other than HPV16. More definitive information on the cross-reactivity of the vaccine-induced responses would require measurements of reactivity against full-length antigens. Unfortunately, insufficient PBMCs were available from the vaccinated patients to perform analysis on enriched T-cell cultures, while primary PBMC cultures are not suitable for measuring specific T-cell responses against recombinant proteins due to the high background levels associated with the particular experimental setting [Britten 2008]. We were therefore not able to assess whether responses against the non-HPV16E6 peptides as recorded in the HPV16E6/E7 vaccinated patients reflect functional, protective immunity against clade A9 members other than HPV16. However, based on our current data on T-cell responses from healthy subjects we deem this unlikely.

In conclusion, in spite of considerable sequence homology between the E6 antigens of clade A9 hrHPV types, E6-specific CD4+ T cells induced by HPV16 generally do not efficiently cross-react against the corresponding sequences of closely related hrHPV types. In view of our findings, and the similar, somewhat lower degree of sequence conservation between E7 proteins, the same is to be expected for E7-specific CD4+ T-cell responses. Therefore, the therapeutic efficacy of vaccines comprising E6 and E7 antigens of a given hrHPV type is likely to be limited to anogenital lesions positive for the specific hrHPV type concerned.

3

Skin reactions to Human Papillomavirus 16 specific antigens intradermally injected in healthy subjects and patients with cervical neoplasia

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Abstract

We have tested the safety and feasibility of a synthetic long peptide-based HPV16-specific skin test to detect cellular immune responses to HPV16 E2, E6 and E7 *in vivo*. Women with cervical neoplasia (n = 11) and healthy individuals (n = 19) were intradermally challenged with 8 different pools of HPV16 E2, E6 and E7 peptides. The skin test was safe as the injections were perceived as mildly painful and no adverse events were observed. The majority of skin reactions appeared significantly earlier in HPV16+ patients (<8 days) than in healthy subjects (8-25 days). The development of late skin reactions in healthy subjects was associated with the appearance of circulating HPV16-specific T cells and the infiltration of both HPV16-specific CD4+ Th1/Th2 cells and CD8+ T cells into the skin. These data show that the intradermal injection of pools of HPV16 synthetic long peptides is safe and results in the migration of HPV16-specific T cells into the skin as well as in an increase in the number of circulating HPV16-specific T cells. The use of this test to measure HPV16-specific immunity is currently tested in a low resource setting for the measurement of spontaneously induced T-cell responses as well as in our HPV16 vaccination trials for the detection of vaccine-induced immunity.

Introduction

The main cause of cervical carcinoma and cervical intraepithelial neoplasia (CIN) is a persistent infection with one of the high-risk, oncogenic human papilloma viruses [Bosch 2002; Munoz 2003]. Anogenital infection with a high-risk HPV type is very common [Burk 1996; Karlsson 1995; Koutsky 1997]. The rate of incidence of infection is estimated to be 80 to 85% worldwide [Jenkins 1996]. Fortunately, the majority of infected subjects will clear the virus infection within a year [Evander 1995; Ho 1998] and only a small proportion of women will become persistently infected and are at risk to develop HPV related malignancies [Kjaer 2002; Remmink 1995]. Cell mediated immune responses play an important role in controlling HPV infections [Ozsaran 1999; Sun 1997].

Delayed type IV hypersensitivity (DTH) reactions are used as a general measure of cell-mediated immunity *in vivo* [Turk 1975]. A local inflammatory reaction (induration and erythema), orchestrated by activated cytokine releasing CD4+ memory T cells, usually appears within 24 to 72 hours after intradermal injection of antigen [Black 1999; Poulter 1982; Vukmanovic-Stejic 2006]. Delayed type hypersensitivity reactions have been used to demonstrate an encounter with pathogens (*e.g.* Mantoux Test), vaccine-induced immune responses [Huebner 1993; Jaeger 1996; Rieser 1999; Thomas-Kaskel 2006], and in particular to show HPV-specific immune responses in various animal models [Chambers 1994; Höpfl 1993; Vambutas 2005]. Höpfl et al. were the first to study HPV16-specific cellular immunity by skin test in humans [Höpfl 1991; Höpfl 2000] and showed that a skin reaction, appearing within 2 to 6 days after intradermal injection with HPV16 E7, was correlated with regression of HPV induced CIN lesions.

We have previously studied the HPV16-specific T-cell responses in great detail *in vitro*, and the results of these studies suggested that the HPV16 E2, E6 and E7 specific type 1 and type 2 T-cell response was associated with the control of HPV16 induced disease [de Jong 2002a; de Jong 2004; van Poelgeest 2006; Welters 2003]. Based on these results we designed a HPV16-specific skin test, consisting of the most immunogenic regions of the early proteins E2, E6 and E7 [de Jong 2002a; Welters 2003], that might be used to screen spontaneously- and vaccine-induced immune responses to HPV in large groups of individuals and in areas where the access to specialized laboratories is limited. In this study, we have tested the safety and feasibility of a synthetic long peptide-based HPV16-specific skin test to detect cellular immune responses to HPV16 E2, E6 and E7 *in vivo.*

Materials and methods

Study design

A cross-sectional study to analyse HPV16 E2-, E6-, and E7-specific T-cell responses as measured by intradermal injection of pools of clinical grade HPV16 peptides in the upper arm was performed in patients with HPV16-related disorders of the cervix

and in healthy individuals. Since a delayed type hypersensitivity reaction represents a memory T-cell response, there was no prerequisite for HPV16-positivity at the time of analysis. The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center.

Subjects

Eleven women (P) with a history of HPV16 positive cervical carcinoma (n = 8) or CIN (n = 3) and a group of nineteen healthy individuals (HS) participated in this study after providing written informed consent. The clinical characteristics of the patients are summarized in Table 1. The age of the patients ranged from 29-72 years (median age, 46 years). The healthy volunteers displayed a median age of 31 years old (range, 20-51 years) and comprised of 80% women and 20% males. Peripheral blood mononuclear cells (PBMCs) were obtained from all subjects immediately before administration of the skin test. The late appearance of positive skin tests in healthy individuals made us decide to isolate a second blood sample, which could be drawn from 11 of 19 healthy volunteers.

DTH Skin test

Skin tests, based on Delayed Type Hypersensitivity reactions (DTH), can be used as a sensitive and simple method for in vivo measurement of HPV-specific cellular immune responses [Höpfl 1991; Höpfl 2000]. The skin test preparations consisted of 8 pools of long clinical-grade synthetic peptides spanning the whole HPV16 E6 and E7 protein and the most immunogenic regions of HPV16 E2 protein [de Jong 2004]. These clinical

Table 1. Patient characteristics.

Patient	Age (yrs)	Diagnosis	Grade/stage	Treatment	Time (months)[a]	HPV[b]
1	72	CxCa	IB[c]	radical hysterectomy	9	16
2	57	CxCa	IA	radical hysterectomy	18	16
3	50	CxCa	IB	radical hysterectomy	6	16
4	44	CxCa	IA	hysterectomy	36	16
5	34	CxCa	IB	radical hysterectomy	48	16
6	44	CxCa	IB	radical hysterectomy	7	16
7	43	CxCa	IB	radical hysterectomy	39	16
8	44	CxCa	IIA	radical hysterectomy	10	16
9	29	CIN	III	LEEP[d]	2	16
10	42	CIN	II	LEEP	3	16
11	44	CIN	III	LEEP	12	16

[a] Time of treatment before skin tests were performed. [b] HPV detection by polymerase chain reaction in paraffin embedded tissue. [c] Cervical cancer stage according to FIGO. [d] Loop electrosurgical excision procedure.

grade peptides were produced in the interdivisional GMP-Facility of the LUMC. Each pool of the skin test consisted of 2 or 3 synthetic peptides, indicated by the first and last amino acid of the region in the protein covered by the peptides. *Pool 1*: $E2_{31-60}$, $E2_{46-75}$, *Pool 2*: $E2_{301-330}$, $E2_{316-345}$, *Pool 3*: $E6_{1-31}$, $E6_{19-50}$, *Pool 4*: $E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$, *Pool 5*: $E6_{85-109}$, $E6_{91-122}$, *Pool 6*: $E6_{109-140}$, $E6_{127-158}$, *Pool 7*: $E7_{1-35}$, $E7_{22-56}$, *Pool 8*: $E7_{43-77}$, $E7_{64-98}$. Per peptide pool 0.05 ml of 0.2 mg/ml peptides in 16% DMSO in 20 mM isotonic phosphate buffer (10 μg/peptide) was injected intracutaneously. The pools of peptides and a negative control (vehicle of solvent only) were injected separately at individual skin test sites of the upper arm. Skin test sites were inspected at least three times, at 72 hours and 7 days after injection of the peptides [Höpfl 1991; Höpfl 2000] and at 3 weeks following the first report of a very late skin reaction in one of the first healthy subjects. Reactions were considered positive when papules greater than 2 mm in diameter arose no less than 2 days after injection. Healthy individuals with a positive skin reaction were asked to consent to the collection of a punch biopsy (4 mm). When consent was given, the punch biopsies were cut in small pieces and cultured in IMDM (Cambrex Bio Science, Verviers, Belgium) containing 10% human AB serum, 10% TCGF and 5 ng/ml interleukin 7 (IL 7) and IL15 to allow the emigration of lymphocytes out of the skin tissue [Piersma 2008]. After 2 to 4 weeks of culture the expanded T cells were harvested and tested for their HPV-specific reactivity.

Antigen for in vitro immune assays

A set of peptides, similar to the peptides used in the skin test, were used for T-cell stimulation assays and IFNγ ELISPOT assays. The four HPV16 E2 peptides consisted of 30-mer peptides overlapping 15 residues, HPV16 E6 consisted of 32-mers and HPV16 E7 of 35-mers, both overlapping 14 residues. The peptides were synthesized and dissolved as previously described [van der Burg 1999]. Notably, in the IFNγ ELISPOT assays peptide pool 4 and 5 slightly differed from the peptide pools used in the skin test, pool 4 contained peptides $E6_{37-68}$, $E6_{55-86}$, $E6_{73-104}$ and pool 5 comprised peptides $E6_{73-104}$, $E6_{91-122}$.

Memory response mix (MRM 50x), consisting of a mixture of tetanus toxoid (0.75 *Limus flocculentius*/ml; National Institute of Public Health and Environment, Bilthoven, The Netherlands), *Mycobacterium tuberculosis* sonicate (5 μg/ml; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands), and *Candida albicans* (0.15 mg/ml, HAL Allergenen Lab., Haarlem, The Netherlands) was used as a positive control. Recombinant HPV16 E2, E6 and E7 proteins were produced in recombinant *Escherichia coli* as described previously [van der Burg 2001].

Analysis of Antigen-specific Th Cells by IFNγ ELISPOT

The presence of HPV16-specific Th cells was analyzed by ELISPOT as described previously [van der Burg 2001]. Briefly, fresh PBMCs were seeded at a density of 2 x 10^6 cells/well of a 24-well plate (Costar, Cambridge, MA) in 1 ml of IMDM (Cambrex Bio Science) enriched with 10% human AB serum, in the presence or absence of the

indicated HPV16 E2, E6 and E7 peptide pools. Peptides were used at a concentration of 5 µg/ml/peptide. After 4 days of incubation at 37°C, PBMCs were harvested, washed, and seeded in four replicate wells at a density of 10^5 cells per well in 100µl IMDM enriched with 10% FCS in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT was performed according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted-video-imaging analysis system (Bio Sys, Karben, Germany). Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in experimental wells [van der Burg 2001].

T-cell proliferation assay

T-cell cultures of the skin biopsies were tested for recognition of the specific peptides and protein in a 3-day proliferation assay [van der Burg 2001]. Briefly, autologous monocytes were isolated from PBMCs by adherence to a flat-bottom 96-well plate during 2 h in X-vivo 15 medium (Cambrex Bio Science) at 37°C using $2x10^5$ PBMC per well. The monocytes were used as APCs, loaded overnight with 10 µg/ml peptide and 20 µg/ml protein. Skin test infiltrating lymphocytes were seeded at a density of 2-5 x 10^4 cells/well in IMDM supplemented with 10% AB serum. Medium alone was taken along as a negative control, phytohemagglutinine (0.5 µg/ml) served as a positive control. Proliferation was measured by [^{3}H]thymidine (5 µCi/mmol) incorporation. A proliferative response was defined specific as the stimulation index (SI) ≥ 3. Supernatants of the proliferation assays were harvested 48 hours after incubation for the analysis of antigen-specific cytokine production.

Analysis of cytokines associated with HPV16-specific proliferative responses

The simultaneous detection of six different Th1 and Th2 cytokines: IFNγ, tumor necrosis factor α, IL2, IL4, IL5 and IL10 was performed using the cytometric bead array (Becton Dickinson, Erebodegem-Aalst, Belgium) according to the manufacturer's instructions. Cut-off values were based on the standard curves of the different cytokines (100 pg/ml IFNγ and 20 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and >2x the concentration of the medium control [de Jong 2004].

Intracellular Cytokine Staining (ICS)

The specificity and character of the T-cell cultures derived from positive skin reaction sites was tested by ICS as reported previously [de Jong 2005]. Briefly, skin test infiltrating lymphocytes were harvested, washed and suspended in IMDM + 10% AB serum and 2-5 x 10^4 cells were added to autologous monocytes that were pulsed overnight with 50 µl peptide (10 µg/ml) or protein (20 µg/ml) in X-vivo medium. Medium alone was taken along as a negative control, phytohemagglutinine (0.5 µg/ml) served as a positive control. Samples were simultaneously stained with FITC-labelled anti-IFNγ (clone

4S.B3, BD PharMingen, San Diego, CA), PE-labelled anti-IL5 (clone JES1-39D10, BD PharMingen), APC-labelled anti-CD4 (clone SK3, BD PharMingen) and PerCP-labelled anti-CD8 (clone SK1, BD PharMingen). After incubation at 4°C, the cells were washed, fixed with 1% paraformaldehyde and analyzed by flow cytometry.

Statistical Analysis

Fisher's Exact test (2-tailed) was used to analyze the relationship between the detection of IFNγ-producing HPV-specific T cells in PBMC, the presence of a skin test reaction or the presence of HPV-specific T cells in skin biopsies, as well as differences between patients and healthy controls with respect to the size or the number of the skin reactions within these groups. Statistical analyzes were performed using Graphpad Instat Software (version 3.0) and Graphpad Prism 4.

Results

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

We studied skin reactions in both healthy subjects and patients with a history of HPV16 induced disease after intracutaneous injection with HPV16 E2, E6 and E7 peptides. The injections were perceived as mildly painful and no adverse events were observed, indicating that the use of this skin test was safe. Positive skin reactions appeared as flat reddish papules of 2 to 20 mm of diameter, arising within 2 to 25 days after injection. A positive skin reaction was detected in 46 of the 152 test sites in the control group and in 22 out of 88 test sites in the patient group.

A classical DTH reaction, within 24 to 72 hours after injection, was only observed in 1 patient (P4, Figure 1). The time for the other skin reactions to appear, differed considerably between the groups of patients and healthy volunteers. Significantly more patients (6 out of 11) developed a positive skin reaction within 8 days as compared to healthy subjects (2 out of 19, p = 0,03 two tailed Fisher's exact test; Figure 1). Whereas the chance to detect a later skin reaction was significantly higher in the healthy control group (p = 0,007, two tailed Fisher's exact test; Figure 1). More than 90% of the positive skin reactions (43 out of 46) in this control group were detected at 8 or more days after injection. In 3 of the patients and 1 healthy control (P3, P10, P11 and HS5), both early and late positive skin reactions were observed within the same subject. Over all, each peptide-pool present in the skin test was able to induce a positive skin reaction in one or more subjects. Noteworthy, the majority of early positive skin reactions in the patients was directed against one or more of the E6 peptide pools (10 out of 14 test sites), or to E2 in combination with an E6 response. Only one patient (P7) developed an early positive skin reaction not accompanied by an E6 immune response (Figure 1). In the control group the majority of reactions were against $E2_{31-75}$ (10 out of 19 subjects) and $E6_{41-95}$ (9 of 19). This reaction pattern resembles that of the responses previously found in PBMC [de Jong 2002a; Welters 2003], as well as resembles the skin reaction pattern observed in the patient group (Figure 1). The size of the skin reactions did not differ between the two groups.

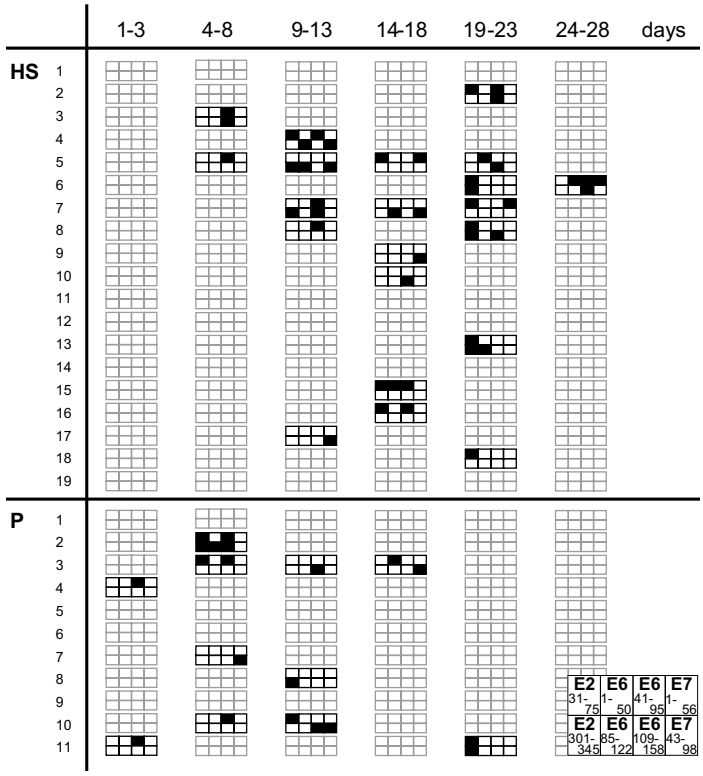

Figure 1. Summary of skin reactions in patients and healthy subjects. An overview of the number, day of appearance and injected antigen that induced a positive skin reaction in the group of 19 healthy subjects (HS) and 11 patients (P) with a history of HPV16 related cervical neoplasia. Skin reactions were considered positive when papules greater then 2 mm in diameter arose no less then 2 days after injection. The indicated layout is used for the 8 peptide pools, the first and last amino acid in the protein of the peptide pool used is indicated. The layout printed in bold indicates at least one positive reaction within this timeframe; a filled square represents a new developed, positive skin reaction to the indicated peptide pool. Significantly more patients developed a positive skin reaction within 8 days as compared to healthy subjects ($p = 0.03$ two tailed Fisher's exact test). Whereas the chance to detect a later skin reaction was significantly higher in the healthy control group ($p = 0.007$, two tailed Fisher's exact test).

Late skin reactions in healthy subjects are associated with an increase of HPV16-specific T cells in the peripheral blood.

To compare the results of the skin test with the presence of circulating HPV16-specific type 1 T cells, an IFNγ ELISPOT assay was performed with PBMC's collected before the intradermal peptide-challenge was given. In the 2 healthy subjects with an early skin reaction we were able to detect a HPV16-specific immune response to the corresponding peptides by IFNγ ELISPOT. In contrast, HPV16-specific IFNγ producing T cells were not detectable in the pre-challenge blood sample of 5 of the 6 patients displaying an early positive skin reaction, which is consistent with the findings of our

previous studies on HPV16-specific immunity in patients with HPV16+ lesions [de Jong 2004; Welters 2003]. In addition, in the pre-challenge blood sample of 3 patients and 3 healthy controls we detected small numbers of IFNγ producing T cells against peptides that were not positive in the subsequent skin test (not shown). Interestingly, the late positive skin reactions detected in healthy controls were not associated with the detection of HPV16-specific type 1 T cells in the pre-challenge blood sample.

To assess the frequency of HPV-specific T cells at the time that a late skin reaction appeared, additional blood samples from 11 of the 13 healthy volunteers, displaying a late positive skin reaction, were collected. In these individuals 39 out of 88 test sites were positive. In 25 of the 39 positive skin sites these reactions coincided with the detection of an HPV 16-specific T-cell response to the same peptides in the post-challenge blood sample. However, we also observed an HPV16-specific T-cell response in the post-challenge blood sample to peptides injected in 10 of the 49 negative skin test sites (Figure 2A). The detection of circulating HPV-specific IFNγ-producing T cells in the post-challenged blood sample and the presence of a positive skin reaction was significantly correlated ($p < 0.0001$, Fisher's exact test; Figure 2A). This shows that the frequency of HPV16-specific T cells in the blood of healthy volunteers is significantly higher following an intradermal challenge with HPV16 peptide and indicates that intracutaneous injection of peptide antigens enhances the number of HPV16-specific T cells in the blood of healthy volunteers.

Positive skin reaction sites are infiltrated by both HPV16-specific CD4+ Th1/Th2 cells and CD8+ T cells.

Approximately 25% of the late positive skin reactions of healthy volunteers were not associated with the detection of HPV16-specific IFNγ-producing T cells in the blood,

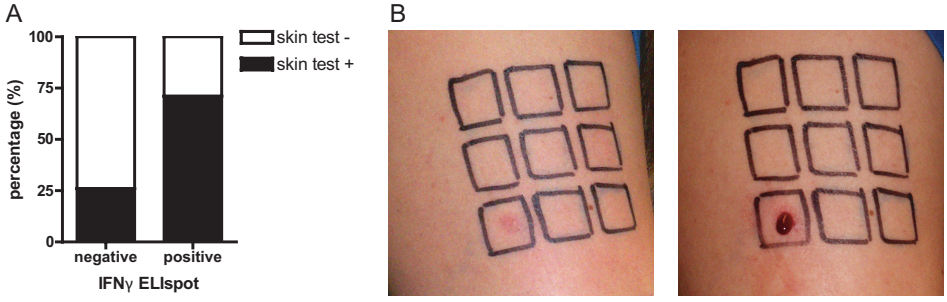

Figure 2. Relation between a positive skin test and the presence of circulating HPV16-specific T cells in the post-challenge blood sample. A. Association between the appearance of a positive skin reaction and the simultaneous detection (IFNγ ELISPOT) of circulating HPV16-specific T cells in the post-challenge blood sample of healthy subjects ($p < 0.0001$, two tailed Fisher's exact test). From a total of 88 skin tests, 39 were positive. Twenty-five of these 39 reactions were associated with a positive reaction in ELISPOT (T-cell frequency ≥ 5 in 100.000 PBMCs). Of the 49 skin test sites that did not show a skin reaction, 10 were associated with a positive ELISPOT. B. Example of a healthy subject (HS10) displaying a positive skin reaction at day 14 to peptide pool 6 ($E6_{109-140}$, $E6_{127-158}$) (left panel). Punch biopsy of the positive skin reaction site (right panel).

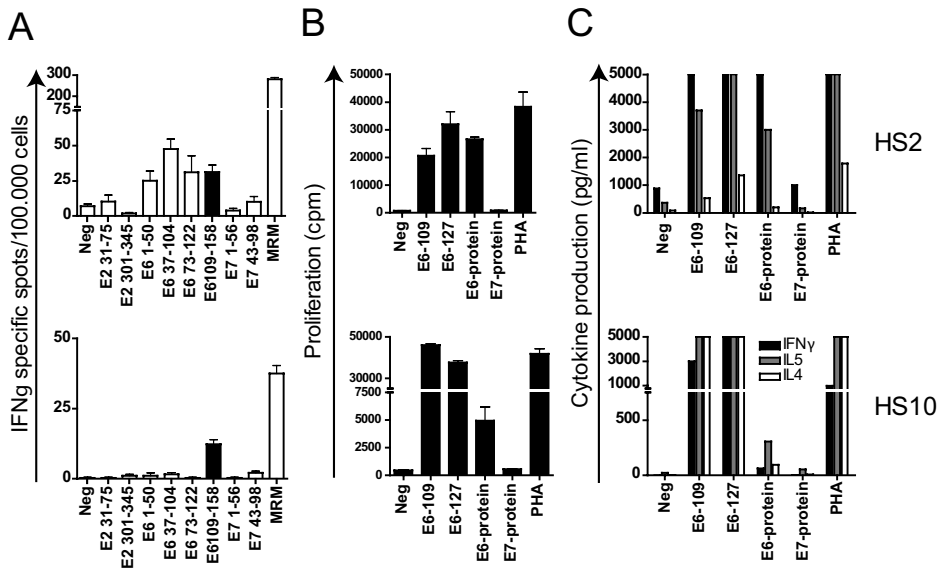

Figure 3. Infiltrating HPV16-specific T cells produce type 1 and type 2 cytokines. A. Two representative examples of HPV16 specific T-cell responses detected by IFNγ ELISPOT in the post-challenge blood sample of healthy subjects displaying a positive skin reaction. The mean number of spots per 100.000 PBMCs are depicted. Memory response mix (MRM) was used as a positive control. The filled bar indicates the positive skin reaction site of which a punch biopsy was taken and put in to culture. B. T lymphocytes ex-filtrating from punch biopsies were, after a 14 to 28 day period of cytokine driven expansion, tested for their capacity to proliferate upon stimulation with monocytes pulsed with peptides (10 μg/ml) – as injected in the skin test – or with protein (20 μg/ml). Phytohemagglutinine (PHA) served as a positive control. Proliferation was measured by [^{3}H]thymidine incorporation and a proliferative response was defined specific as the stimulation index (SI) ≥ 3. Both healthy subjects 2 and 10 (HS2, HS10) are examples for skin reaction sites comprising HPV-specific T cells. C. Supernatants of the proliferative responses in B were analyzed for the presence of IFNγ, interleukin 4 (IL4), IL5 and tumor necrosis factor α, IL2, IL10 (not shown) by cytometric bead array. Cut-off values were based on the standard curves of the different cytokines (100 pg/ml IFNγ and 20 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cut off level and >2x the concentration of the medium control.

suggesting that other, non IFNγ-producing types of T cells may infiltrate the skin after intradermal injection of HPV16 peptides. In order to characterize the cells that infiltrated a positive skin reaction site, punch biopsies were taken from consenting individuals (Figure 2B). In total, 8 biopsies were taken from different positive skin reaction sites of 7 healthy controls (HS2 (2x), 10, 13, 15, 16, 17, 18) and cultured with a cocktail of cytokines that allowed the outgrowth of T cells *in vitro* in the absence of any additional antigenic stimulants [Piersma 2007]. T cells ex-filtrated the tissue, expanded within 3-4 weeks and were tested for their specificity in a short-term proliferation assay. In 4 cases we were able to detect HPV16-specific T cells in the cultures (HS2, 10, 15, 16), in 3 cases the T cells did not respond (HS2, 17,18; not shown) and in

one case no T cells could be cultured from the biopsy (HS13; not shown). Figure 3 shows examples of T-cell cultures that specifically proliferated upon stimulation with autologous monocytes pulsed with the pool of peptides, which was also injected in this site during the skin test (HS2, HS10), as well as with monocytes pulsed with HPV16 E6 protein (Figure 3B). This indicates that these T cells were capable of recognizing their cognate HLA-peptide complexes after the antigen was naturally processed and presented. Analysis of the supernatants of these proliferative T-cell cultures revealed a mixed Th1/Th2 cytokine profile in that the HPV16-specific T cells produced IFNγ, IL-4 and IL-5 (Figure 3C). The detection of HPV 16-specific T cells in the biopsy culture coincided with the detection of HPV16-specific IFNγ-producing T cells the post-challenge blood sample (Figure 3).

Co-staining of the biopsy-derived T cells by CD4 and CD8 cell surface markers showed that not only HPV16-specific CD4+ but also HPV16-specific CD8+ T cells infiltrated the skin site upon intradermal challenge with HPV16 peptide (Figure 4). Overall, in 3 out of 4 biopsies infiltrated by HPV16-specific T cells; we were able to detect HPV16-specific CD8+ T cells. The CD8+ T cells isolated from the biopsy (pool 6) of HS2 responded to both overlapping peptides of the injected skin test: HPV16 $E6_{109-140}$ and $E6_{127-158}$ (not shown). While the CD8+ T cells of both subjects HS15 and HS16 responded to HPV16 $E6_{37-68}$ (see example for HS15 Figure 4).

Taken together, the population of immune cells migrating into the skin upon an intradermal challenge with HPV16 peptides comprises HPV16-specific CD4+ Th1 and Th2 cells and CD8+ T cells. This infiltration is paralleled by the appearance of circulating HPV16-specific IFNγ-producing T cells in the blood.

Discussion

Skin tests are commonly used as a simple assay for *in vivo* measurement of cell-mediated immunity. We have tested the safety and feasibility of the skin test assay for the *in vivo* detection of HPV16-specific cellular immune responses directed against the early antigens E2, E6 and E7 and compared the outcome to parallel measurements of T-cell reactivity by *in vitro* assays. Our study reveals that this skin test is safe and that the majority of responding HPV16+ individuals develop a positive skin reaction within 8 days after injection, suggesting that the test can be used to detect HPV-specific immune responses. For instance, as a quick and easy applicable screening method to monitor the induction of specific cellular immunity in vaccination trials.

A classical DTH reaction to the HPV16-specific skin test was only observed in one patient. However, similar to the observations of Höpfl et al. [Höpfl 2000], the majority of patients with HPV16 related neoplasia develop a positive skin reaction within 8 days after intradermal antigen challenge. In fact, this study shows that patients develop significantly more early positive skin reactions than the healthy volunteers ($p = 0.03$, Figure 1) and the majority of the reactions is directed against one or more of the E6 peptide pools. In order

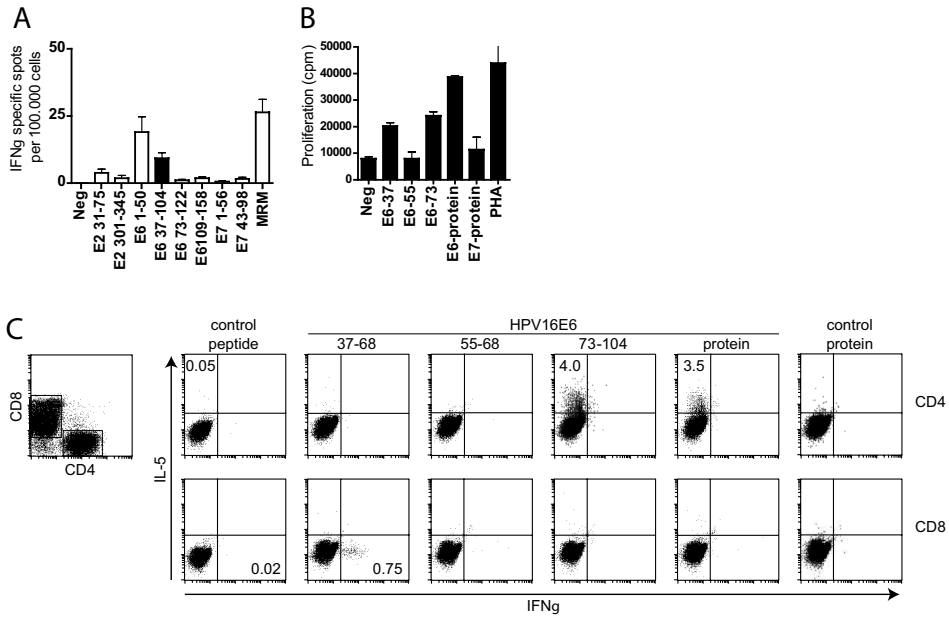

Figure 4. Positive skin test sites are infiltrated by both CD4+ and CD8+ HPV16-specific T cells. A. HPV16-specific T-cell response detected by IFNγ ELISPOT in the post-challenge blood sample of healthy subject 15 (HS15). A punch biopsy was taken from the positive skin reaction site of pool 4 ($E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$), indicated by the filled bar. B. T lymphocytes ex-filtrating the punch biopsy of pool 4 ($E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$) of healthy subject 15 (HS15) were tested for their capacity to proliferate upon stimulation with monocytes pulsed with peptides (10 μg/ml) – as injected in the skin test – or with protein (20 μg/ml). Phytohemagglutinine (PHA) served as a positive control. Proliferation was measured by [^{3}H]thymidine incorporation. C. T-cell culture of the skin biopsy of pool 4 ($E6_{41-65}$, $E6_{55-80}$, $E6_{71-95}$) of healthy subject 15 (HS15) consists of both HPV16-specific CD4+ and CD8+ T cells. The specificity of the culture was tested in an intracellular cytokine staining (ICS) against the protein (20 μg/ml) and the peptides (10 μg/ml) corresponding with the injected skin test.

to characterize the immune responses as measured *in vivo*, the results were compared with the detection of HPV-specific T cells by *in vitro* immunological assays. Only a small number of healthy volunteers displayed early positive skin reactions. In this group, known to display HPV16-specific type 1 T-cell responses in vitro [de Jong 2002a; Welters 2003], the appearance of an early skin reaction (within 8 days) was associated with the detection of IFNγ-producing HPV16-specific T cells by ELISPOT, at a frequency of at least 1 per 20.000 PBMC. The same cut-off criteria for a positive reaction in the IFNγ ELISPOT assay are recommended by Jeffries et al [Jeffries 2006], who used mathematical tools to define the appropriate cut-off of the ELISPOT in relation to Mantoux-tests. The low number of circulating memory T cells may explain why the skin reactions appear somewhat delayed compared to classical DTH tests. The T cells need to be boosted or reactivated and start to divide before enough cells are produced to cause a local inflammatory reaction: the positive skin test.

Despite the continuous presence of antigen, 4 out of 11 patients did not respond to the skin test (early or late). This fits with our earlier observation that almost 50% of all cervical cancer patients do not display detectable numbers of proliferating E2, E6, and/or E7-specific T cells in their blood [de Jong 2002a; de Jong 2004; van Poelgeest 2006; Welters 2003]. The absence of these circulating HPV-specific T cells may explain the failure to develop a skin reaction. In addition, we recently reported the involvement of regulatory T cells in cervical cancer [Piersma 2007; van der Burg 2007] and one could argue that their presence may prevent the development of early or late positive skin tests. Although we can not formally exclude this it should be noted that also 5 out of 19 healthy controls did not develop a skin reaction suggesting that the development of late skin reactions does not have to occur *per se* following an intradermal peptide challenge. The early positive skin reactions which did appear in the patient population were not associated with circulating HPV16-specific type 1 T cells as measured by IFNγ ELISPOT, suggesting that HPV16-specific T cells producing other cytokines infiltrated the skin test site in these patients. Historically it has been postulated that IFNγ-producing Th1 cells induce DTH responses [Black 1999], however, several studies have now shown that both Th1 and Th2 cells are associated with DTH responses and infiltrate the skin test sites [Wang 1999; Woodfolk 2001]. Also in our study, the skin test sites of the healthy subjects were infiltrated by HPV16-specific CD4+ Th1 and Th2 cells as well as CD8+ T cells (Figure 3 and 4), suggesting that the positive skin reactions in cancer patients are the result of circulating HPV16-specific non-Th1 cells.

Unexpectedly, we observed the majority of skin reactions in healthy individuals to appear 2 to 3 weeks after intradermal injection of the antigen. We were not able to detect HPV-specific CD4+ Th1 cells in the pre-challenge blood sample to the peptides causing these late positive skin reactions. However, in the post-challenge blood sample we detected circulating HPV16-specific IFNγ-producing T cells and their appearance was significantly correlated with the presence of late skin reactions to the corresponding peptides ($p < 0.0001$, Fisher's exact test; Figure 2A). In a number of cases HPV16-specific circulating IFNγ-producing T cells were detected in the post-challenge blood samples but without a concomitant skin reaction (Figure 2A), it is unclear what this means. We hypothesize that the presence of HPV16-specific type 1 T cells in the post-challenge blood sample might reflect a vaccination-induced type of T-cell response. This has also been noted in 29% of patients who underwent a 2-step tuberculin skin testing protocol and who were positive only at the second test round [Akcay 2003]. In general, vaccine-induced T-cell responses peak at 10 to 14 days after vaccination and not at three weeks. However, one should bear in mind that in most vaccine protocols both a higher antigen dose and strong adjuvants are injected. It is therefore reasonable to assume that the T-cell responses induced by intradermal challenge develop slower and peak at a later period. Since the intradermal peptide challenge in healthy volunteers results in the induction of both HPV16-specific CD4+ and CD8+ T cells it could, therefore, be considered as a single, low dose vaccination.

In conclusion, the use of synthetic long peptides in a skin test for the detection of cellular immune response to HPV16 E2, E6 and E7 *in vivo* is safe and feasible. The use of this test to measure spontaneously induced HPV16-specific immune response requires a follow-up of 8 days, because skin reactions to these antigens develop slower (this study and [Höpfl 2000]). Notably, our results indicate that this test does not distinguish between Th1, Th2 or other types of T cells and this should be taken into consideration when the results of this test are interpreted. Currently, this test is used to measure the spontaneously induced HPV16-specific immune response in a large group of HPV-typed patients and healthy individuals in Indonesia. The use of this test to measure vaccine-induced T-cell responses is expected to follow the classical kinetics of a DTH response. In order to study this, we have included the skin test in two of our HPV16 vaccination studies in patients with low and high-grade precancerous lesions of the cervix. Our first preliminary data indicate that skin reactions are strong and appear within 48 hours after application.

4

Evaluation of HPV E6-specific T-cell immunity in Haitian and South African women in relation to clearance or persistence of cervical HPV infections

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Submitted

Abstract

Purpose: Systemic T-cell memory against high-risk HPV (hrHPV) early antigens, bearing witness to past HPV encounters, is frequently detected in healthy subjects. This suggests that T-cell dependent immunity is commonly involved in control and clearance of anogenital HPV infections. Formal proof of this causal relationship can only be obtained in women with a documented history of cervical HPV infections.

Experimental design: E6-specific T-cell immunity for 5 prevalent hrHPV types (Clade A9 HPV16, 35, 52, 58 and Clade A7 HPV18) was charted by IFNγ ELISPOT in women enrolled in prospective cervical screening programs in Haiti and South Africa.

Results: Frequencies of HPV E6-specific T-cell responses were similar as found in our prior studies. Women with documented history of transient HPV infection were occasionally found to display T-cell immunity matching the HPV type cleared. However, in most of these women, the E6-specific immunity detected was directed against one of the other 4 HPV types tested, pointing at T-cell memory induced by an earlier HPV infection.

Conclusion: Our data demonstrate that encounter of hrHPV can elicit systemic E6-specific T-cell memory, but also suggest that local adaptive and/or innate immunity, not associated with establishment of detectable systemic T-cell memory, suffices in clearing the majority of cervical hrHPV infections.

Introduction

Cervical neoplasia is still a frequent cause of cancer-related death among women, in particular in developing countries [Ferlay 2010; Jemal 2011]. Infection with high-risk, oncogenic human papilloma viruses (hrHPV) is the key etiological factor in this respect [Bosch 2002; Jenkins 1996; Munoz 2003]. Several lines of evidence suggest that T-cell dependent immunity plays an important role in controlling and clearing cervical HPV infections, and thereby in preventing cervical cancer. One example is the apparent success of preventive vaccination against oncogenic HPV types, which involves T-cell dependent immunoglobulin responses. Vaccine-induced immunity was shown to strongly reduce the incidence of persisting HPV infections and ensuing anogenital lesions ([Munoz 2010], and references therein). Although evidence for a direct role for T-cell immunity in the protective effect of prophylactic vaccines is so far lacking [Pinto 2003], this role is evident from studies in which we evaluated HPV-specific T-cell immunity in subjects with or without HPV-positive cervical disease [Stanley 2010]. For instance, systemic CD4+ T-cell responses against HPV early antigens E6 and E2, associated with production of the Th1 cytokine IFNγ, were frequently detected in healthy subjects, but rarely in subjects with HPV-positive cervical neoplasia ([de Jong 2004] and references therein). Furthermore, we have recently shown that the induction of a strong HPV16-specific Th1 response by a therapeutic HPV16 vaccine comprising the E6 and E7 antigens correlated with complete regression of HPV16-induced disease in approximately 50% of chronically infected patients [Kenter 2009; Welters 2010].

Nevertheless, a causative role of natural CD4+ T-cell immunity in control/clearance of cervical HPV infections and associated epithelial transformation still remains to be substantiated. A major hurdle in this respect is the immunological cross-reactivity between related hrHPV types, which causes the complication that detection of memory CD4+ T-cell responses against a given HPV type cannot be readily used as an indicator that the subject concerned has encountered this particular HPV type. This was demonstrated in a recent study in which we charted the E6-specific CD4+ T-cell responses in healthy subjects for clade A9 HPV types 16, 31, 33, 35, 52 and 58 [van den Hende 2010]. Our data clearly indicated that investigation of the causative relationship between T-cell immunity and outcome of cervical HPV infection requires cohorts of women with a documented history of HPV infection. The availability of two cohorts of women already enrolled in prospective cervical screening studies, in Haiti and South Africa respectively, offered a unique opportunity to evaluate HPV-specific T-cell responses in the context of known virological status. PBMC samples were analysed by IFNγ ELISPOT for reactivity against E6 antigen of clade A9 HPV types 16, 35, 52, 58 and clade A7 HPV type 18. Our approach was based on the prevalence of these HPV types in the populations concerned [Munoz 2003; WHO/ICO information Centre 2011], as well as on the high frequency by which we have observed E6-specific CD4+ T-cell immunity in our previous cohort studies [de Jong 2004; van den Hende 2010; Welters 2003; Welters 2010].

Materials and Methods

Cohorts: selection of subjects for immune analysis on basis of HPV typing

To investigate the relationship between HPV type-specific T cell memory and persistence or clearance of cervical HPV infections we initially focused on HPV clade A9 types 16, 31, 33, 35, 52, 58 and HPV18 (clade A7), which at the time of study planning were known to be the most frequently detected HPV types in infected women with normal cytology and low grade cervical lesions [Munoz 2003]. Evaluation of the HPV prevalence in our pilot study (Haiti) revealed that HPV types 31 and 33 were detected considerably less frequent than the other types. Since the numbers of PBMCs isolated from 50 ml of blood are limited, these two types were not included in the immune analyses. HIV-positive subjects were also excluded. In view of the aim of our study, we evaluated HPV status and immunity for the aforementioned HPV types in three subgroups: women consistently HPV-negative at all visits (controls), women found HPV-positive at a prior visit who became HPV DNA negative (transient infection), and women found HPV-positive at both prior visits and their last visit (persistent infection).

Patient data and samples

A pilot study was conducted in the context of a cervical cancer screening project that ran between May 2003 and September 2004 in the Zanmi Lasante health clinic in Cange, Haiti. After obtaining written informed consent, 73 women with an average age of 37 years (30-50 years) were followed up to 16 months and underwent 3 separate cervical examinations including HPV testing. PBMC samples were collected at the final (third) visit. On basis of HPV typing data, samples from 46 of the 73 subjects were selected for full immunological analysis, as these fell into one of the three pre-defined categories (Figure 1A). Reasons for exclusion were cervical positivity for a selected HPV type at 3rd visit but not at both prior visits (14), subjects exhibiting a transient infection pattern who – upon further data analysis – were found to be treated for their cervical lesions at one of the prior visits (8), and insufficient PBMC counts (5).

The second study was staged as part of a randomized clinical trial evaluating two different screen and treat approaches for cervical cancer prevention in Khalyelitsha, South Africa [Denny 2005; Denny 2010]. In total, 113 women with an average age of 43 years (35-65 years) were followed – with prior written consent – over 44-55 months (June 2000-May 2006). Cervical examinations and HPV typing were performed at 6 consecutive times and blood samples were collected at the final (6th) visit. Of these samples, 84 were from subjects that fell into one of the three pre-defined categories (Figure 1B). Reasons for exclusion were cervical positivity for HPV31 or 33, but not any of the selected HPV types (10), a longitudinal HPV infection pattern that did not fit into one of the three subject groups of interest (15), subjects exhibiting a transient infection pattern that – upon further data analysis – were found to be treated at one of the prior visits (2), and insufficient PBMC counts (2).

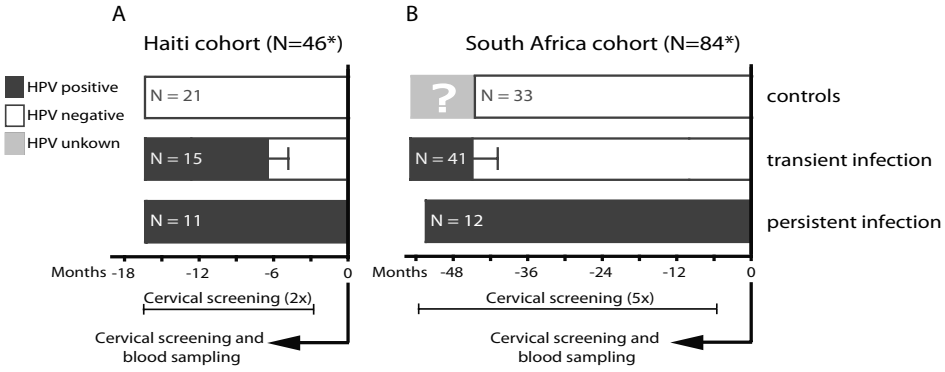

Figure 1. Study cohort: time frame of clinical examinations and collection of samples. Schematic overview of the Haitian (A) en South African (B) cohorts. Histograms depict the distribution of the subjects over the three subgroups: women consistently HPV negative at all visits (controls), women found HPV-positive at a prior visit who became HPV DNA negative (transient infection), and women found HPV-positive at both prior and their last visit (persistent infection). Notably, numbers in categories do not match up with total subjects per cohort (*), because 1 subject in the Haitian cohort (H-60) and 2 in the South African cohort (SA-99, SA-112) were registered under both transient and persistent infection due to the fact that they displayed each of these infection patterns for different HPV types of interest. Time lines at the bottom of each graph show period of prior screening in relation to final visit during which PBMC samples were collected. Grey and white areas in 'transient infection' histograms depict the average point in time (+/- SD) at which HPV infections were considered as cleared on basis of cervical screening.

Gynecologic exams routinely included a visual inspection of the cervix with acetic acid (VIA) and collection of cervical specimens for HPV DNA testing and liquid based cervical cytology. Women underwent colposcopy with endocervical curettage and/or biopsy of all cervical abnormalities. All women with CIN grade ≥2 lesions were offered treatment. PBMC were isolated on site from heparinized blood samples by Ficoll (Sigma) density centrifugation, followed by freezing, storage and shipment on dry ice.

Laboratory testing of cervical specimen

Cervical samples were tested at Columbia University for the presence of high-risk HPV DNA with the use of the Hybrid Capture 2 assay according to the manufacturer's instructions (Qiagen, Gaithersburg, MD, USA), which detects infection with one or more of 13 hrHPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The cutoff for a positive HPV test was 1.0 relative light unit (RUL) [Castle 2004]. Specific HPV typing was performed on all hc2 positive samples using the Roche Linear Array HPV test (LA) according to the manufacturer's instructions (Roche Molecular Systems, Inc., Branchburg, NJ, USA). This test is able to genotype 37 HPV types: 6, 11,16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108 [Gravitt 2000]. Liquid-based cytology samples were processed at Health Networks Laboratory, Allentown, PA. Results were reported according to the 2001 Bethesda terminology [Solomon 2002] and all abnormal cytology

specimens were evaluated by a single senior cytopathologist. Cervical biopsies and endocervical curettages were processed at Columbia University, evaluated by a single pathologist and reported using CIN terminology [Wright 2007].

Antigens and immunomonitoring

All immunological assays were performed according to validated standard operating procedures that have been previously applied for the evaluation of clinical vaccination studies [Kenter 2009; Welters 2010]. HPV specific immune responses were analyzed by Interferon-γ enzyme-linked immunospot (IFNγ-ELISPOT) using pools of overlapping peptides as described previously [van der Burg 1999; van den Hende 2010]. A standardized mixture of recall antigens (memory response mix or MRM: 0,75/ml *Limus flocculentius* tetanus toxoid, 5 μg/ml *Mycobacterium tuberculosis* and 0.15mg/ml *Candida albicans)* served as a positive control. IFNγ producing HPV-specific T cells were quantified using ELISPOT assays as described previously [de Jong 2004; van der Burg 2001; Welters 2003]. Specific spots were calculated by subtracting the mean number of spots + 2xSD of medium control from the mean number of spots in experimental wells. Antigen-specific T-cell responses were considered to be positive when T-cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg 2001].

Results

HPV distribution and frequency of HPV-specific T-cell immunity

The relative incidence of the selected 5 HPV types (HPV 16, 35, 52, 58, 18) as detected in the participants from the Haitian (n = 73) and South African (n = 113) studies is shown in Figure 2A. In both cohorts HPV16 is frequently detected (Haiti 28%, South Africa 25%). However, the relative predominance of HPV16 over the other 4 types was less prominent than reported in other cohorts of infected women with normal cytology and/or low-grade lesions [Munoz 2003; WHO/ICO information Centre 2011].

Based on pre-defined inclusion criteria (see detailed description in materials & methods), PBMC samples from 46/73 and 84/113 of the women in the Haitian and South African cohorts were used for full immunological analysis. The relative incidence of the selected 5 HPV types within these sub-cohorts did not differ significantly from that in the entire cohorts (Figure 2B). Overall evaluation of the IFNγ-ELISPOT analyses revealed that the cell viability of PBMC samples, isolated in a low-resource setting and shipped intercontinentally on dry ice, was excellent and comparable to samples in our previous in-house studies. The vast majority (98%) of the samples, exhibited strong responses against a standardized mixture of recall antigens (MRM). Furthermore, an immune response to one or more of the HPV types was detected in 16/46 PBMC samples (35%) in Haiti and in 37/84 samples (44%) in the South African cohort. Although T-cell responses against HPV16 were frequently detected, the incidence of T-cell responses against several of the other HPV types was very similar (Figure 2C; HPV58 and 18 in the Haitian cohort, HPV52 and 58 in the South African

cohort). Furthermore, there was no overt correlation between relative frequencies of infections and T-cell response detected for the 5 HPV types studied. (Figures 2B and 2C). For instance, responses against HPV58 peptides were more frequent that would be expected on basis of relative incidence of this HPV type.

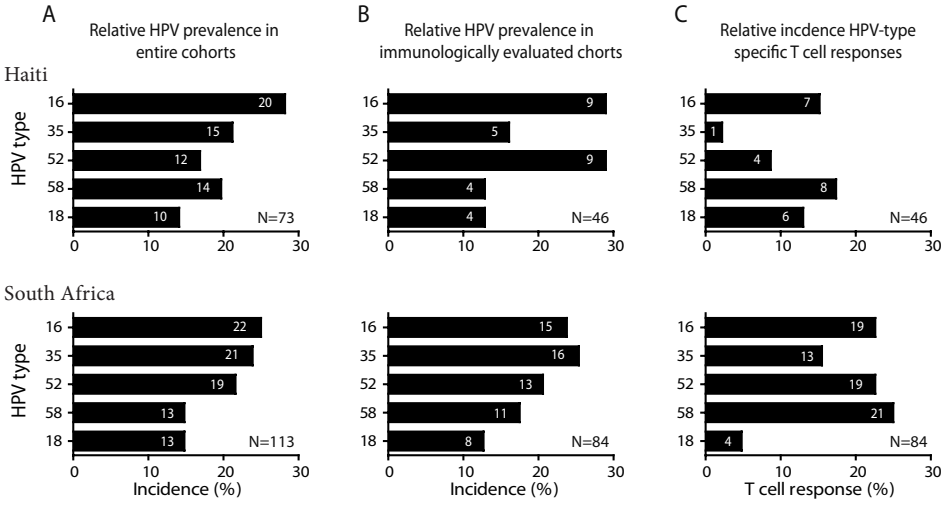

Figure 2. Relative frequency of infection and specific immune responses for selected HPV types in Haiti and South Africa cohorts. A. Relative prevalence of HPV types of interest (HPV 16, 35, 52, 58, 18) in the Haitian (upper, n = 73) and South African (lower; n = 113) study cohort. B. Relative prevalence of HPV types of interest (HPV 16, 35, 52, 58, 18) in the sub-cohorts for which full immunological evaluation was performed (n = 46 and 84 respectively). C. Relative incidence of HPV type-specific T-cell responses as detected by IFNγ-ELISPOT in aforementioned sub-cohorts.

The HPV-specific T-cell responses recorded were further evaluated in the context of the longitudinal HPV infection profile of three pre-defined subgroups (Figure 1): women consistently HPV negative at all visits (controls), women found HPV-positive at a prior visit who became HPV DNA negative (transient infection), and women found HPV-positive at prior visits and their last visit (persistent infection).

Women with no documented history of HPV infection

In the Haitian cohort, 21/46 of women selected for full immune analysis (see materials and methods for inclusion criteria) were found HPV negative during 3 subsequent visits over a period of 16 months (Figure 1A). For the South African cohort, this was the case for 33/84 subjects (Figure 1B). The latter women were found negative during 6 visits over a period of 42 to 47 months (average 3.7 years). In line with our prior findings for HPV-negative subjects in The Netherlands, a considerable fraction of these women displayed HPV E6 specific immunity: 5/21 (24%) and 14/33 (42%) for the Haitian and South African cohorts

respectively (see Tables 1 and 2 for data summary). In view of the absence of detectable cervical HPV in these women, it is conceivable that these T-cell responses represent immunological memory induced by viral encounter prior to the time frame of our studies.

Table 1. Distribution of HPV infections and E6 peptide-specific T-cell responses in Haiti cohort

	HPV16	HPV18	HPV35	HPV52	HPV58	Total
Women with no documented history of infection *(N = 21, mean age 36 [30-48])*						
Infection *(%)*	-	-	-	-	-	**0**
Responder *(%)*	*1 (5%)*	*1 (5%)*	-	*2 (10%)*	*3 (14%)*	**5/21**[b] *(24%)*
Women with documented history of infection *(N = 15, mean age 36 [30-48])*						
Infection *(%)*	*2 (12%)*	*2 (12%)*	*5 (29%)*	*6 (35%)*	*2 (12%)*	**17**[a]
Responder *(%)*	*6 (40%)*	*4 (27%)*	-	*1 (7%)*	*3 (20%)*	**8/15**[b] *(53%)*
Women with persistent infection *(N = 11, mean age 42 [30-50])*						
Infection *(%)*	*7 (64%)*	*2 (18%)*	-	*2 (18%)*	*3 (21%)*	**14**[a]
Responder *(%)*	-	*1 (9%)*	*1 (9%)*	*1 (9%)*	*3 (27%)*	**4/11**[b] *(36%)*

[a] Total numbers and percentages (rounded to full decimals) do not add up correctly, since some subjects are infected with 2 different HPV types. [b] Total numbers and percentages (rounded to full decimals) do not add up correctly, since some subjects respond to multiple HPV types.

Table 2. Distribution of HPV infections and E6 peptide-specific T-cell responses in South Africa cohort

	HPV16	HPV18	HPV35	HPV52	HPV58	Total
Women with no documented history of infection *(N = 33, mean age 43 [35-56])*						
Infection *(%)*	-	-	-	-	-	0
Responder *(%)*	*9 (27%)*	*2 (6%)*	*5 (15%)*	*8 (24%)*	*9 (27%)*	**14/33**[b] *(42%)*
Women with documented history of infection *(N = 41, mean age 43 [35-60])*						
Infection *(%)*	*11 (22%)*	*7 (14%)*	*11 (22%)*	*12 (24%)*	*8 (16%)*	**49**[a]
Responder *(%)*	*8 (20%)*	*2 (5%)*	*7 (17%)*	*11 (27%)*	*9 (22%)*	**19/41**[b] *(46%)*
Women with persistent infection *(N = 12, mean age 47 [36-64])*						
Infection *(%)*	*4 (25%)*	*2 (13%)*	*5 (31%)*	*2 (13%)*	*3 (19%)*	*16*[a]
Responder *(%)*	*2 (17%)*	-	*1 (8%)*	*1 (8%)*	*5 (42%)*	**6/12**[b] *(50%)*

[a] Total numbers and percentages (rounded to full decimals) do not add up correctly, since some subjects are infected with 2 different HPV types. [b] Total numbers and percentages (rounded to full decimals) do not add up correctly, since some subjects respond to multiple HPV types.

Women with documented history of transient HPV infection

The primary goal of our study was to assess whether clearance/control of cervical HPV infections would be associated with the detection of T-cell immunity against the HPV type concerned. Women found positive for one of the 5 HPV types of interest at a prior visit, who became HPV DNA negative within the time frame of our study, accounted for 15/46 and 41/84 of the selected Haitian and South African subjects respectively (Figure 1). Within this category, the fraction of women displaying significant PBMC reactivity against E6 peptides of any of the 5 HPV types was 8/15 (53%) and 19/41 (46%) for Haiti and South Africa respectively (see Tables 1 and 2 for underlying data). In the Haiti cohort, the specificity of these responses matched with the HPV type cleared in only one case: subject H-60 who cleared HPV58 (Figure 3A). In addition, clearance of HPV35 in subject H-45 (Figure 3B) was mirrored by a weak HPV35-specific T-cell response that did not significantly exceed control values. Notably, the picture is rather complex in both cases. Subject H-60, while showing control of HPV58, displays persisting HPV16 (Figure 3A). Subject H-45, while showing a weak response to E6 peptides of HPV35, also displays clear cut reactivity against the corresponding peptide pools of the 4 other HPV types (Figure 3B). Therefore, the data from these two subjects do not constitute a firm basis for conclusions about the causal relation between T-cell immunity and HPV infection control.

Importantly, the data from the South African cohort are more compelling: viral clearance is correlated with the detection of a matching immune response in 8/19 of the cases. For representative examples, featuring clearance of HPV types 16, 58 and 35 respectively, see the data for subjects SA-36, SA-39 and SA-41 in Figure 4A, B and C. In the other 11/19 cases, the detected T-cell immunity did not match the HPV type cleared (e.g. subjects SA-61 and SA-4 in Figure 4D and E). The HPV-specific immunity detected in the majority of Haitian subjects similarly did not match the cleared HPV type (7/8; e.g. subject H-68 in Figure 3C). Taken together, our data lead us to conclude the following. First, that systemic E6-specific T-cell immunity against the HPV type concerned can be observed in conjunction with clearance of cervical hrHPV infections (see overview in Figure 5: 'transient HPV' histograms, black areas), suggesting that these responses have been induced during – and may have been instrumental in controlling – the infection. This is in accordance with our prior studies in which we detected such memory responses in a major fraction of healthy subjects [Welters 2003; de 2004; van den Hende 2010; Welters 2010]. However, in the majority of cases (14/15 for Haiti, 33/41 for South Africa) immunity matching the cleared HPV type was not observed (Figure 5: 'transient HPV' histograms, white and grey areas). The second conclusion for our study therefore is that establishment of systemic HPVE6-specific T-cell immunity, as detectable by IFNγ ELISPOT, is not a mandatory aspect of clearance of cervical HPV infections. Importantly, the lack of T-cell responses matching the HPV type cleared does not appear to reflect the failure of the subjects concerned to raise systemic, HPVE6-specific T-cell immunity, because a considerable number of women

in both cohorts do display such responses against the other HPV types included in our study (Figure 5: 'transient HPV' histograms, grey areas). Implications of these findings will be addressed in the discussion of our manuscript.

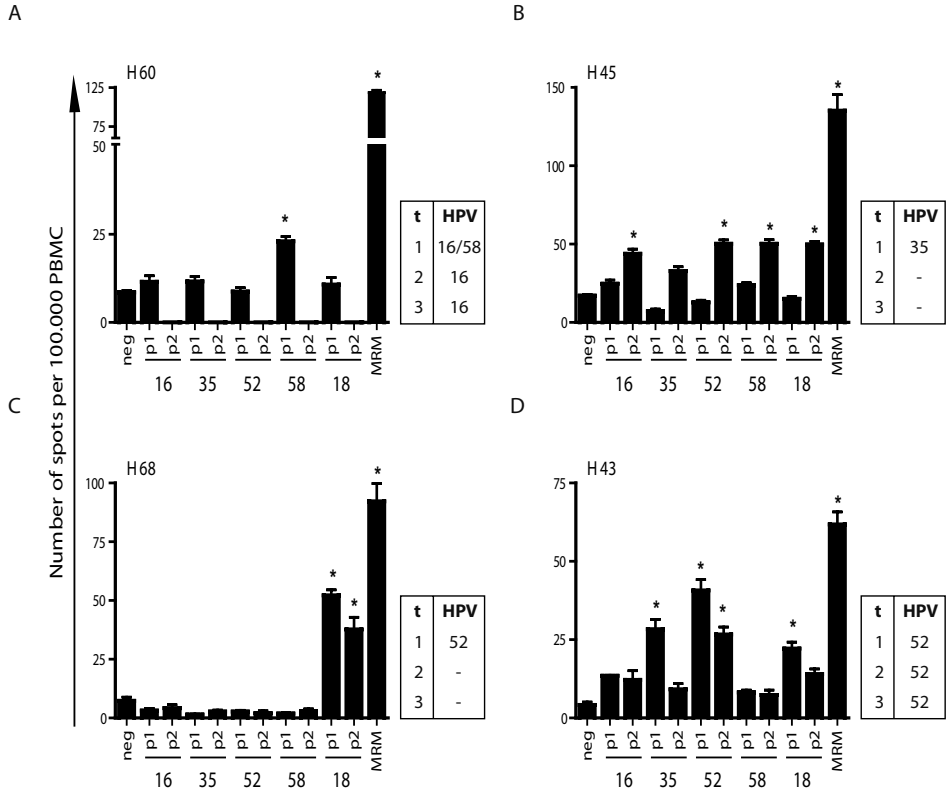

Figure 3. Examples of HPV E6-specific response patterns as measured by IFNγ-ELISPOT in HPV-positive subjects of the Haitian cohort. Responses as detected by IFNγ-ELISPOT analysis performed against specified peptide pools (p1, p2) per HPV type. MRM was used as a positive control. The mean number of spots per 100.000 PBMC in quadruplicate readings are depicted. Responses that significantly exceeded the medium control (see material and methods section for criteria) are marked with an asterisk. Detection of cervical HPV (types 16, 35, 52, 58 and/or 18) at each of the monitoring time points is shown at the right of each figure. A. Subject H-60, shown to clear HPV58, displays a matching T-cell response against peptides from HPV58. Notably, this subject was also persistently infected with HPV16. B. Subject H-45, shown to clear HPV35, displays significant reactivity against matching peptide pools from HPV types 16, 52, 58 and 18, as well as a weaker HPV35-specific response that does not significantly exceeds control values. C. PBMC cultures of subject H-68, shown to clear HPV52, display significant reactivity against HPV18 peptides, but not against any of the other HPV types. D. Subject H-43, in spite of showing significant immunity against peptides from HPV52 and 2 other HPV types, displayed a persistent infection by HPV52.

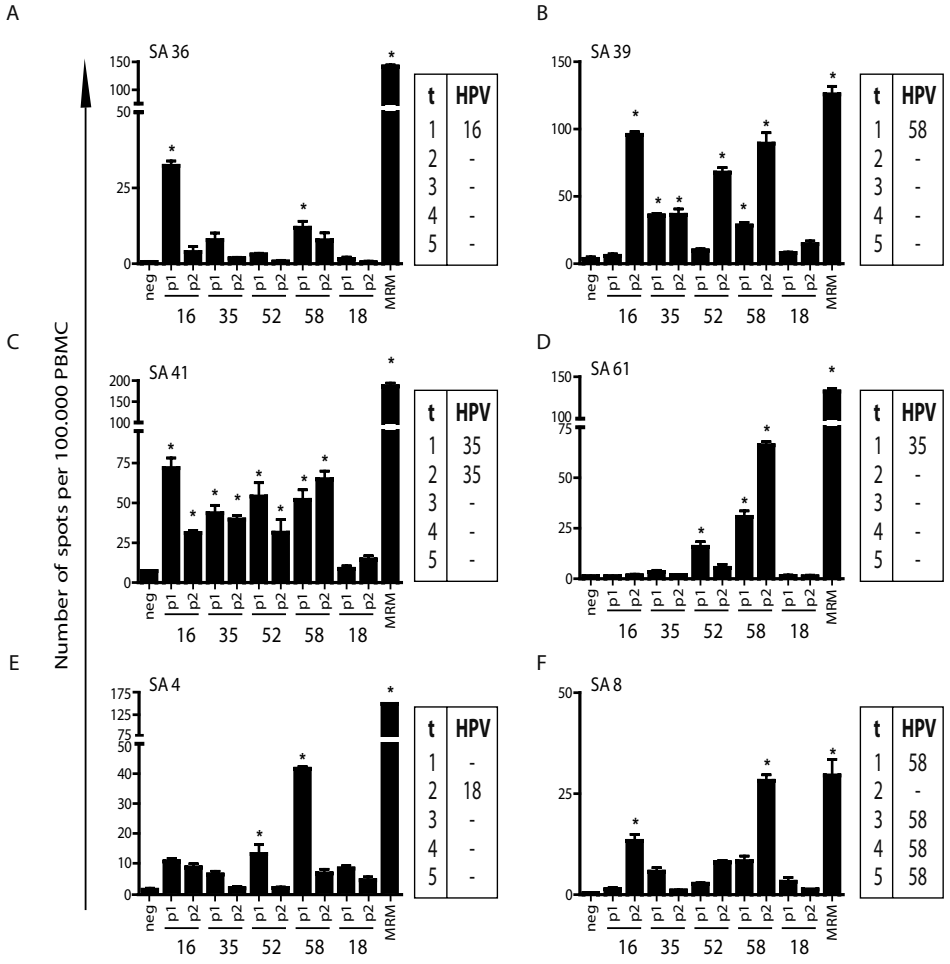

Figure 4. Examples of HPV E6-specific response patterns as measured by IFNγ-ELISPOT in subjects of the South African cohort. Responses as detected by IFNγ-ELISPOT analysis performed against specified peptide pools (p1, p2) per HPV type. MRM was used as a positive control. The mean numbers of spots per 100.000 PBMC in quadruplicate readings are depicted. Responses that significantly exceeded the medium control (see material and methods section for criteria) each of the monitoring time points is shown at the right of each figure. A. Subject SA-36, shown to clear HPV16, showing a matching HPV16-response and well as a response against the corresponding peptide pool of HPV58. B and C. Subjects SA-39 and SA-41, two cases in which broadly reactive T-cell responses against multiple corresponding peptide pools of different HPV types showed a match with the HPV type cleared (respectively HPV 58 en 35). D and E. PBMC of subjects SA-61 and SA-4 displaying E6-specific T-cell immunity not matching the HPV types cleared. F. Subject SA-8, persistently infected with HPV58, displaying a T-cell response against HPV16.

Women with persistent HPV infection

The number of women showing persistence of a cervical HPV infection over the duration of our study was relatively small, 11 for Haiti and 12 for South Africa (Figure 1), because the majority of these displayed a premalignant lesion and were offered treatment. One subject in the Haitian cohort (H-60) and two in the South African cohort (SA-99, SA-112) were evaluated in both the 'transient HPV' and 'persistent HPV' subgroups, because they featured these two distinct infection outcomes for different HPV types. Within the 'persistent HPV' subgroups, the fraction of women displaying a significant immune response to any of the 5 HPV types was 4/11 (36%) and 6/12 (50%) for Haiti and South Africa respectively (see Tables 1 and 2 for underlying data). The frequency by which T-cell responses are detected is, thereby, not significantly different between the 'persistent HPV' and 'transient HPV' categories (Figure 5). In line with our expectations for the persistently infected subjects, the specificity of the T-cell responses detected did not match the persistently infecting HPV type in the majority of cases (Figure 5: 'persistent HPV' histograms, grey areas; example SA-85 in Figure 4F). Notably, these non-matching E6-specific T-cell responses against closely related HPV types, which must represent T-cell memory induced by earlier HPV encounters, were apparently ineffective in mediating clearance

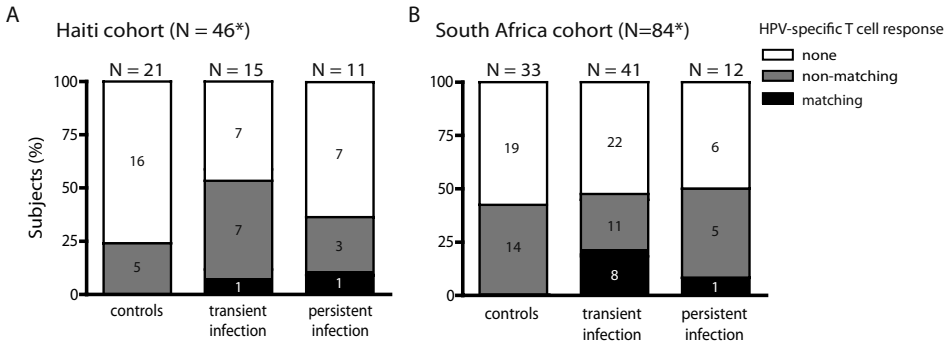

Figure 5. HPV E6-specific immunity in relation to virological status. Overview of the coincidence between HPV E6-specific immunity detected in PBMC samples collected at the final visit, and development of cervical HPV infections as observed through longitudinal cervical screening. For each of the cohorts, the subgroups of women negative for HPV, found to clear HPV and showing persistent HPV infection are represented by separate histograms. Notably, 1 Haitian subject and 2 South African subjects were classified under both 'transient HPV' and 'persistent HPV' categories (see legend to Figure 1). Black areas in the 'transient HPV' and 'persistent HPV' histograms represent the fraction of subjects displaying HPV-specific immunity matching the cleared or persistently infecting HPV type respectively. Grey areas represent subjects showing immunity against any of the other 4 HPV types, and white areas the fraction of women lacking detectable immunity against any of the 5 HPV types examined. Absolute numbers of subjects are shown in each of the areas. Statistical evaluation did not reveal significant differences in incidence of either matched or non-matched HPV-specific T-cell responses between the three subgroups in each cohort.

of the current infection. In rare cases, such as HPV52-positive Haitian subject H-43 (Figure 3D) and South African subject SA-8 (Figure 4F), even E6-specific immunity matching the infecting HPV types seems ineffective.

Discussion

The main objective of the exploratory study described in the present paper was to evaluate whether the presence or absence of hrHPV-specific responses, as measured by IFNγ ELISPOT against peptide pools encompassing the E6 antigen, would correlate with, respectively, clearance or persistence of cervical HPV infection. Crucial in this respect, was the availability of cohorts of women in Haiti and South Africa with documented cervical HPV status. In spite of the resulting a low-resource setting, the majority of the PBMC samples isolated were of excellent quality, as witnessed by good cell viability and strong responses against our standardized mixture of common recall antigens (MRM). The validity of the immune analyses is further supported by the notion that E6-specific responses were found in 40% (53/130) of the PBMC samples analysed, and that approximately half of these responses (28/53) involved reactivity to E6 peptides of 2 or more of the HPV types analysed. These responder frequencies resemble that of HPV specific immunity as detected in our previous studies [de Jong 2004; van den Hende 2010; Welters 2003; Welters 2010].

The hypothesis that clearance of cervical HPV infection may be associated with systemic HPV-specific T-cell immunity was based on our prior findings that a majority of healthy subjects displayed CD4+ T-cell immunity against HPV early antigens, as measured by IFNγ ELISPOT against peptide arrays for E6 and E2, bearing witness to prior encounter and control of HPV16 and 18 infections [de Jong 2004; van den Hende 2010; Welters 2003; Welters 2010]. Moreover, vaccination of chronically infected patients with a peptide-based vaccine comprising the E6 and E7 antigens was shown to induce complete regression of HPV16-induced lesions [Kenter 2009; Welters 2010]. In line with our previously published data, such immunity against E6 was found frequently (overall 40%) in the newly tested cohorts. Furthermore, in subjects displaying a persistent HPV infection this immunity rarely matched the infecting HPV types (Figure 5: 'persistent HPV' histograms, black areas), as expected on basis of their failure to clear these infections. However, also in the sub-group of women that showed cervical HPV infection followed by clearance, only a modest fraction displayed E6 peptide-specific T-cell immunity matching the HPV type cleared (Figure 5: 'transient HPV' histograms, black areas). Thus, even though clearance of cervical HPV infections can be observed in conjunction with matching E6-specific immunity, this is evidently not a common theme. It is unlikely that non-matching T-cell responses mediate HPV clearance, because we have found previously that the in vitro assays used can readily detect cross-reactivity of T-cell immunity between related hrHPV types [van den Hende 2010]. As such, we would expect cross-protection to reveal itself by cross-reactivity to the cleared HPV type in the in vitro assays.

With respect to the biology of cervical HPV infections, there are several potential explanations for the absence of E6-specific T-cell immunity matching the cleared HPV type in the majority of cases. For instance, local adaptive immune responses could suffice in clearing of the majority of cervical infections. Alternatively, many infections may be cleared by innate immunity before the adaptive response kicks in. Similar explanations were proposed in a recently published study in which authors also did not find a correlation between detection of systemic T-cell responses to HPV16 E6 and E7 antigens and regression of HPV16+ CIN lesions [Trimble 2010a]. Others have postulated that transient HPV infections, as detected by exfoliated cell scrapes, are rapidly cleared by (local) innate immune factors before infecting the basal cell layer of the epithelium, thereby preventing antigenic stimulation of the adaptive immune system [Einstein 2009a; Woodworth 2002]. Thus, the HPV-specific CD4+ T-cell responses detected so frequently in healthy women may merely bear witness to the tip of the iceberg of transient cervical HPV infections, in that many of these do not trigger long-lasting, systemic T-cell immunity. Notably, the current sensitivity of the HPV DNA assays allows detection of transient hrHPV infections that are not associated with virus-induced cytologic manifestations [Cuzick 2008].

We could also have missed immunity associated with HPV clearance due to aspects of our study design. For instance, the time between viral clearance and PBMC sampling for immune monitoring in the South African cohort is relatively long (Figure 1B; 3.7 years at average). Others have reported that patients with a recently cleared HPV16 infection (i.e. within 4-8 months) tend to show more HPV16E6 specific immune responses than patients who have cleared their infection further in the past (> 20 months) (13). This suggests that the number of circulating memory cells among those who cleared an infection in the past are lower, which is in line with the expected contraction of the T-cell response after viral clearance. This notion seems paradoxical to the frequent detection of CD4+ T-cell memory in our present cohorts against HPV types that were not detected within the time frame of the study, arguing that such memory responses are long lasting. However, since levels of memory T cells are generally related to the magnitude of the effector responses [Kalia 2006], it is very well possible that the CD4+ T-cell memory routinely detected in our PBMC ELISPOT assays is a reflection of the strongest clashes between HPV and immune system, and that – as already proposed above – the majority of cervical HPV encounters pass without eliciting a powerful, systemic T-cell response. Alternatively, we could have missed many protective immune responses due to our focus on the E6 antigen. A broader survey of CD4+ T-cell responses, including other antigens for which T-cell memory has frequently been observed (E2, L1 and L2), could reveal a stronger association between HPV clearance and matching T-cell responses. This may especially be the case for subclinical infections, in which expression of late antigens predominates over that of early antigens, whereas screening of T-cell immunity with early antigens could be most suitable for subjects with premalignant lesions. In view of the latter consideration, we re-evaluated our cohorts by focusing on E6-specific T-cell immunity in relation to regression versus persistence/

progression of cervical dysplasia. Even though this analysis revealed that E6 responses matching the cleared HPV type were only found in subjects with regressing lesions, and not in any of the subjects with persistent or progressing lesions, the numbers were not sufficiently large to achieve statistical significance (data not shown).

With respect to the design and feasibility of further studies, the following can be said. Focusing immune analysis on the key infecting HPV type will permit inclusion of a broader array of HPV antigens with the limited number of PBMC that can routinely be obtained in cohort studies. Inclusion of additional hrHPV types will increase the number of subjects that can be included for immune analysis. Shortening of the time between detection of HPV clearance and blood sampling can be achieved by enabling blood sampling from women as soon as possible after their cervical samples indicated clearance of HPV and/or regression of lesions. In practical sense, this means that blood sampling should be incorporated as an integral part of the cohort study, rather than a one-time event as was the case in our pilot studies.

In conclusion, our cohort studies in Haiti and South Africa revealed that clearance of cervical hrHPV infections was occasionally associated with systemic CD4+ T-cell responses against the E6 antigen of the HPV type concerned. This finding is in line with the therapeutic efficacy of our peptide-based HPV16 E6/E7 vaccine in chronically HPV16-infected patients [Kenter 2009], and supports the notion that inclusion of early antigens in prophylactic vaccines will extend their protective capacity to subjects with pre-existing HPV infections. Notably, in a majority of cases, clearance of a given hrHPV type was not associated with a matching E6-specific T-cell response, suggesting that natural clearance of the majority of hrHPV infections may be mediated by innate immunity and/or local adaptive responses that do not evolve into potent systemic T-cell memory. Absence of T-cell responses matching the cleared HPV type is unlikely the result of failure of the in vitro assay, or of the subject's immune system to respond to HPV, because strong CD4+ memory responses against one or more other HPV types were commonly detected. The latter observation indicates that such non-matching T-cell responses, even though directed against closely related hrHPV types, do not provide cross-protective immunity, which has further implications for vaccine design.

5

A prospective study on the natural course of low-grade squamous intraepithelial lesions and the presence of HPV16 E2-, E6- and E7-specific T-cell responses

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Abstract

This study investigates the clinical course of low grade squamous intraepithelial lesions (LSIL), HPV status and HPV16-specific immune response in a large prospective study of 125 women with LSIL followed cytologically, virologically and histologically. Women with low-grade abnormal smears were recruited and followed-up for one year. Colposcopy, cervical biopsy for histology and brushings for HPV typing was performed at recruitment, 6 months (no biopsy) and upon completion of the study at one year. HPV16-specific T-cell responses were analysed interferon-γ ELISPOT at entry, 6 and 12 months.

Infection with multiple HPV types was detected in 70% of all patients, HPV16 was found in 42% of the patients. LSIL lesions progressed to HSIL in 24%, persisted in 60% and regressed to normal in 16% of the patients. No difference was observed in the clearance rate of infections with single or multiple HPV types among the groups with a different histological outcome. HPV16-specific type 1 T-cell responses were detected in only half of the patients with an HPV16+ LSIL, and predominantly reactive to HPV16 E2 and E6. Interestingly, the presence of HPV16 E2-specific T-cell responses correlated with absence of progression of HPV16+ lesions ($p = 0.005$) while the detection of HPV16 E6 specific reactivity was associated with persistence ($p = 0.05$). This large prospective study showed that the majority of LSIL persisted or progressed within the first year. This was paralleled by immune failure as most of the patients with an HPV16+ LSIL failed to react to peptides of HPV16 E2, E6 or E7.

Introduction

Cervical cancer is preceded by a spectrum of epithelial atypia known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL) characterised by disturbances of cellular maturation, stratification and cytological atypia of increasing severity. CIN/SIL are caused by persistent infection with one of a subset of genital human papillomaviruses (HPV), in particular HPV type 16. Natural history studies [Moscicki 2006] show that most (90%) low grade cervical intraepithelial neoplasia (CIN 1) regress spontaneously and this is attributed to the development of HPV antigen specific cellular immune responses [Stanley 2006a] but 30-40% of CIN3 progress to invasive cervical cancer [McCredie 2008] and spontaneous regression is relatively uncommon.

The key role of the adaptive cellular immune system in protection against HPV-induced lesions is indicated by the high incidence of persistent HPV-infections and subsequent HPV-related malignancies in immunosuppressed individuals [Arends 1997] and the observation that only a small fraction of infected non-immunosuppressed subjects develop progressing epithelial lesions or cancer [zur Hausen 2002]. Composite data indicate the importance of CD4+ T-cells in the control of HPV-induced diseases as more severe lesions are observed in patients with low numbers of circulating CD4+ cells [Fakhry 2006; Stetson 2002] and the increase in CD4 count, after anti-retroviral treatment, correlates with the regression of HPV-induced CIN lesions in HIV + patients [Ahdieh-Grant 2004]. At the time of spontaneous regression of HPV infected genital warts, the lesions are infiltrated with CD8+ cytotoxic T-cells (CTL), $CD4^+$ T-cells and macrophages [Coleman 1994]. HPV16 E2- and E6-specific Th1- and Th2-type CD4+ T-cell responses and HPV16 E6- and E7-specific CTL responses are frequently detected in peripheral blood mononuclear cell (PBMC) cultures of healthy individuals [de Jong 2002a; de Jong 2004; Nakagawa 1997; van Poelgeest 2006; Welters 2003]. Moreover, circulating HPV16-specific CD4+ and CD8+ T cells are able to migrate from the circulation to the epithelium upon antigenic challenge in healthy subjects [van den Hende 2008]. In contrast, similar analyses of HPV16-specific immunity in patients with HSIL or cervical cancer reveal that the occurrence of HPV-induced cancer is strongly associated with failure to mount a strong HPV-specific type 1 T-helper and CTL response [Nakagawa 2000; van Poelgeest 2006; Welters 2003] indicated by the lack of $CD8^+$ T-cells migrating into the lesion, the induction of HPV16-specific regulatory T-cells and the influx of regulatory T-cells into the lesion [Piersma 2007; Piersma 2008; van der Burg 2007; de Vos van Steenwijk PJ 2008]. All together, these studies suggest that successful defence against HPV16-induced progressive infections is commonly associated with the induction of a systemic effector T-cell response against the HPV16 E2, E6 and possibly E7 viral antigens.

Here we present one of the largest prospective studies that has followed women with LSIL cytologically, virologically, histologically and immunologically for one year. The

majority of lesions persisted or progressed, and this was paralleled by immune failure as most of the patients with an HPV16+ LSIL failed to mount HPV16-specific immunity.

Material and Methods

Study group

A total of 125 women, aged 20-30 (average 24.6) years old, with low grade cytological abnormalities, referred to Addenbrooke's Hospital for colposcopy were recruited for this prospective study investigating the immunological responses in women with low-grade cervical intra-epithelial neoplasia. Forty-four (35%) of these women were smokers and 93 (74.4%) used hormonal contraception. The exclusion criteria were pregnancy, immunosuppression or active viral disease. Only three of the 125 women had previous cervical treatments but all more than 3 years prior to recruitment into this study.

All colposcopic assessment and follow-up were performed by a single colposcopist (YLW) throughout the length of the study. As part of the study, each patient had a colposcopic assessment, cervical biopsy and cervical brushings taken for HPV detection and typing at recruitment and upon completion of the study. During the first follow-up visit at the 6 months, colposcopy was performed and cervical brushings taken. All histology was reviewed by a single consultant histopathologist (NC) blinded to all other clinical information as well as to the base-line pathology report. Cases were classified as normal, LSIL or HSIL. In total, 65 patients completed the study, providing entry and exit cervical biopsies and at least two cervical brushings for HPV typing over the one year period (with the exception of four patients who developed progressive disease and underwent definitive treatment in the form of loop excision at 6 months). The other recruited patients were excluded for the following reasons: biopsy proven HSIL or normal at recruitment, pregnancy or loss of follow-up. PBMC were isolated from 50 ml of blood taken at entry, 6 months and 12 months for the analyses of HPV16-specific T-cell responses. This study was approved by the Local Research Ethics Committee, Addenbrooke's Hospital, Cambridge, United Kingdom.

HPV detection and typing

The Linear Array HPV Genotyping Test (LA) is a qualitative *in vitro* testing kit for detecting HPV in clinical specimen. The test utilizes amplification of target DNA by PCR and nucleic acid hybridization and detects 37 anogenital HPV types. LA is based on four major processes that include specimen preparation utilizing the Amplitude Liquid Media Extraction Kit (EXTRN), PCR amplification of target DNA, hybridization of amplified products to oligonucleotide probes utilizing the Linear Array HPV Genotyping test kit (LA HPV GT) and finally, detection of the amplified products by colorimetric determination using the Linear Array Detection Kit (LA DK). In this study, DNA extraction was performed using phenol/chloroform extraction method as described previously [Woo 2007]. For the LA test, target DNA was measured

spectrophotometrically to a concentration of 2-4ng/µl. All other steps were as per instructions by Roche Diagnostics. Briefly, the first step involved PCR amplification made up of 50µl working master mix containing MgCl2, KCL, Amplitaq Gold DNA polymerase, uracil-N-glycosylase, deoxynucleotides (dNTPs) and biotinylated PGMY and b-globin primers together with 50µl of DNA sample. This was followed by the addition of 100µl of denaturing solution (DS) to the PCR product. The denatured amplicons were hybridized on to the strip containing specific probes for 37 HPV genotypes and b-globin reference lines before undergoing stringent washes. The colourimetric change reaction utilized streptavidin-horseradish peroxidase mediated precipitation of working substrate. Positive reactions appeared as blue lines of different intensities on the strip. The strips were interpreted using the HPV reference guide provided.

Interferon-γ ELISPOT

The pools of peptides used to determine HPV16-specific T-cell reactivity spanned the entire HPV16 E2, E6 and E7 proteins [de Jong 2002a; de Jong 2002b; Welters 2003;]. Twelve peptide pools of two overlapping HPV16 E2 peptides (30 mer), 4 peptide pools of two overlapping HPV16 E6 peptides (32 mer) and 2 peptide pools of two overlapping HPV16 E7 peptides (35 mer). 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 and 16-45). Phytohaemgglutinin (PHA, 0.5 µg/ml, Sigma, UK) served as positive control for T-cell reactivity.

Interferon-γ (IFNγ) producing HPV-specific T-cells were quantified using ELISPOT that was performed as described previously [Baldwin 2003; de Jong 2002b; Smyth 2004; van der Burg 2001]. Briefly, peripheral blood mononuclear cells (PBMC) were seeded at a density of 2 x 10^6 cells/well in a 24-well plate (Costar, Cambridge, MA) in 1 ml of ISCOVE's medium (Bio-Whittaker, Belgium) enriched with 10% human AB serum (Sigma, UK), in the presence or absence of the indicated HPV16 E2, E6 and E7 peptide pools. Peptides were used at a concentration of 5 µg/ml/peptide. As a positive control, PBMC were cultured in the presence of PHA. Following four days of incubation at 37°C, PBMC were harvested, washed and seeded in four replicate wells at a density of 105 cells/well in a Multiscreen 96-well plate (Millipore, The Netherlands) coated with an IFNγ catching antibody (Mabtech, Sweden). Further antibody incubations and development of the ELISPOT was performed according to the manufacturer's instructions (Mabtech). Spots were counted with a fully automated computer-assisted-video-imaging analysis system (BioSys 4000). Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be increased compared to non-responders when specific T-cell frequencies were ^{3} 1/10,000 [Baldwin 2003; de Jong 2002b; Smyth 2004; van der Burg 2001; van Poelgeest 2005]. ELISPOT results were excluded from analyses when the medium control displayed a high background, when it was not possible to test the samples in triplicate wells or when the positive (PHA) control was negative.

Statistics

The relationship between histological outcome and clearance rate of an infection with a single HPV type *versus* that of an infection with multiple HPV types was tested at each time point by grouping the patients according to histological outcome and then comparing the number of patients that did or did not clear an infection with a single HPV type *versus* the number of patients clearing (or not) an infection with multiple HPV types using a 2 by 2 table and a 2-sided Fishers Exact Test. The relationship between the presence or absence of HPV16-specific immunity and the clearance or persistence of HPV16 was tested by 2-sided Fishers Exact Test. The relationship between HPV16-specific immunity and the transition of lesions from LSIL to normal, LSIL or HSIL were tested by a Chi-Squared Test for Trend analysis, as this allows low numbers in each category, by dividing the patients into two categories (absence or presence of immunity) and arranging them according histological outcome (normal, LSIL and HSIL). All comparisons were performed using GraphPad InStat version 3.00, GraphPad Software (San Diego California USA). P values £ 0.05 were considered significant.

Results

Histological and virological course of disease

Of the 125 patients referred with low grade cytological abnormalities, 74 were confirmed to have LSIL by histology. 39/125 patients were found to have HSIL at recruitment and were offered treatment while 12 patients did not show a histological abnormality. Of the 74 LSIL patients, 65 completed the follow-up but only 60 patients were suitable for histological analysis. Others were excluded due to protocol violation. Patients who completed follow-up were classified as progressors (exit histology HSIL or worse), persistors (LSIL at exit) and regressors (exit histology normal). In total, 14 histological progressors, 37 histological persistors and 9 histological regressors were identified over the study period indicating that over 80% (51/60) of the group of LSIL patients were unable to clear their lesion in one year.

At recruitment, 56 of the 60 (93%) patients with LSIL were HPV positive and most of them displayed an infection with multiple HPV types. The prevalence of infection with multiple HPV types was high (Table 1) but did not differ too much between the histological progressors (11/14; 78%), persistors (28/37; 76%) and regressors (6/9; 67%). Notably, HPV16-infection was found in 25 of the 60 (42%) patients, most often in patients with multiple HPV infection; but was not overrepresented in any one of the patient groups as defined by histology (Table 1).

At the end of the study, the prevalence of infection with multiple HPV types among the three groups was 57% (8/14) among histological progressors, 46% (17/37) in persistors and 22% (2/9) in the histological regressors. The overall clearance rate of HPV16 was 48% (12/25) at 12 months (Table 2). Since most of the HPV16 infections were among the group of patients with multiple HPV infection, we compared the clearance rate of HPV16 with

Table 1. HPV status as defined by Linear Array among the patients who completed the study

HPV outcome	Progressors (N = 14)			Persistors (N = 37)			Regressors (N = 9)		
	0 months	6 months	12 months	0 months	6 months	12 months	0 months	6 months	12 months
Not tested	**0**	**0**	**2**	**0**	**2**	**2**	**0**	**1**	**1**
No HPV detected	**0**	**0**	**1**	**2**	**3**	**11**	**2**	**3**	**2**
Single HPV infection	**3**	**3**	**3**	**7**	**4**	**7**	**1**	**2**	**4**
HPV16	1	1	0	1	2	1	0	1	1
Other HPV types	2	2	3	6	2	6	1	1	3
Multiple HPV infection	**11**	**11**	**8**	**28**	**28**	**17**	**6**	**3**	**2**
HPV16	8	8	5	12	12	6	3	1	0
Other HPV types	3	3	3	16	16	11	3	2	2
2-3 HPV types	6	7	6	11	13	10	3	3	2
4-5 HPV types	4	3	1	13	12	5	2	0	0
≥ 6 HPV types	1	1	1	4	3	2	1	0	0
Total HPV positive cases	**14**	**14**	**11**	**35**	**32**	**24**	**7**	**5**	**6**

Table 2. Clearance rate of HPV16 and non-HPV16 versus multiple HPV types

Group	0 months	6 months	12 months
Histological Progressors (HPV16; mHPV[a])	9; 11	9; 11	5; 8
Clearance rate (HPV16; mHPV)		0%; 0%	44%; 27%
Histological Persistors (HPV16; mHPV)	13; 28	14; 28	7; 17
Clearance rate (HPV16; mHPV)		0%; 0%	46%; 39%
Histological Regressors (HPV16; mHPV)	3; 6	2; 3	1; 2
Clearance rate (HPV16; mHPV)		33%; 50%	67%; 67%
All patients (HPV16; mHPV)	25; 45	25; 42	13; 27
Overall clearance rates (HPV16; mHPV)		0%; 6%	48%; 40%

Group	0 months	6 months	12 months
Histological Progressors (non-HPV16; mHPV)	5; 11	5; 11	6; 8
Clearance rate (non-HPV16; mHPV)		0%; 0%	0%; 27%
Histological Persistors (non-HPV16; mHPV)	22; 28	18; 28	17;17
Clearance rate (non-HPV16; mHPV)		18%; 0%	23%; 39%
Histological Regressors (non-HPV16; mHPV)	4; 6	3; 3	5; 2
Clearance rate (non-HPV16; mHPV)		25%; 50%	0%; 66%
All patients (non-HPV16; mHPV)	31; 45	26; 42	28; 27
Overall clearance rates (non-HPV16; mHPV)		16%; 6%	10%; 40%

[a] mHPV: number or percentage of patients with multiple HPV infection

that of multiple infections (including HPV16) and did not observe a difference (Table 2). Such a correlation was not observed when the clearance rate of multiple HPV types and the clearance of other specific (non 16) HPV types was assessed (Table 2). In addition, no differences were found in the clearance rate of HPV16 compared to co-infecting HPV types among the histological progressors, persistors and regressors.

HPV16-specific immune response rate and specificity at study entry and exit

At recruitment, there were 54 LSIL and 16 HSIL patients of whom sufficient numbers of PBMC were isolated to analyse the presence of HPV16 E2-, E6 and/or E7-specific T-cells by IFNγ-ELISPOT. Similar to our earlier observations [de Jong 2004; de Vos

van Steenwijk PJ 2008] most of the patients (14/16; 88%) diagnosed with HSIL (80% of which were HPV16+) failed to respond to HPV16 (Fig. 1A, B). In the group of patients with LSIL (n = 54), only 10 (19%) displayed HPV16-specific reactivity (Fig. 1A). This response rate was not altered when only HPV16+ LSIL were analysed, 5 out of 22 (23%)

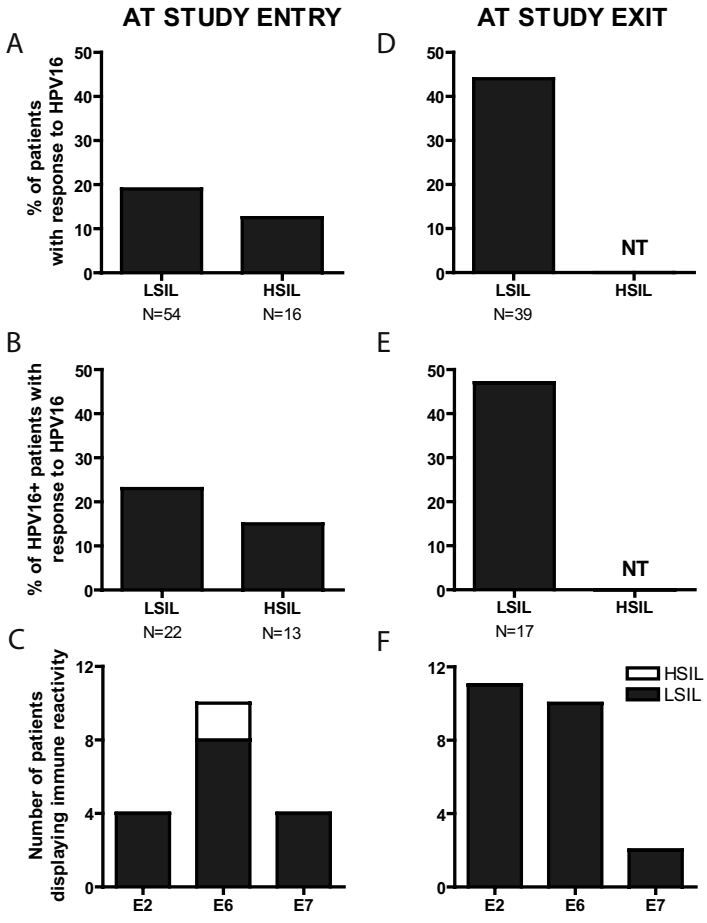


Figure 1. Overview of HPV16 specific immune responses at study entry and exit. A. At study entry, both LSIL (N = 54) and HSIL (N = 16) patients display low HPV16 specific reactivity: LSIL patients 19% and HSIL patients 13%. B. The response rate of HPV16+ LSIL patients (23%) and HPV16+ HSIL patients (15%) at study entry. Responses do not differ from the response rates in all patients (see A). C. In total, 10 LSIL- and 2 HSIL patients display a HPV16 specific immune response at recruitment. All three antigens (E2, E6 and E7) are recognized by LSIL patients (black bar), whereas the 2 HSIL patients (stacked open section on bar) only respond to E6. D. At study exit, HPV16 specific immune responses were evaluable in 39 of the 54 LSIL patients. Showing an increased response rate of 44%. E. At study exit, HPV16+ LSIL patients show a response rate of 47% (8/17). HSIL patients were not tested (NT) during follow up. F. Seventeen LSIL patients display a HPV16 specific immune response at study exit. All three antigens (E2, E6, E7) were recognized, but the patients show a remarkable increase in E2 responses.

displayed HPV16-specific T-cell immunity (Fig. 1B). Analysis of the specificity of the observed responses revealed that all three antigens were recognized by the group of patients with LSIL, predominantly HPV16 E6 (Fig. 1C).

From 39 of the 54 LSIL patients we were able to determine HPV16-specific responses during follow-up (at 6 or 12 months). Interestingly, the response rate increased to 44% (17/39) of all patients (Fig. 1D) mostly due to an increase in the HPV16 E2-specific responses (Fig. 1F). Importantly, some of the immune responses observed at recruitment could not be detected during follow-up, suggesting that some cases may represent false negatives during follow-up. The response rate of the LSIL group positive for HPV16 at recruitment differed neither in the follow-up samples (Fig. 1E) nor when compensated for potential false negatives, by the analysis of the number of patients tested both at entry and follow-up. As a consequence of the study design, patients with HSIL at recruitment were not tested during follow-up.

HPV16-specific immunity in relation to histological outcome in HPV16+ LSIL patients

To analyse the potential role of HPV16-specific immune responses on the clinical outcome of patients with LSIL, all HPV16-negative subjects were excluded from the groups of patients classified as histological regressors, persistors or progressors. Each category was then divided according to the presence or absence of an HPV16-specific immune response. Presence of an immune response was based on the detection of HPV16-specific immunity in at least one of the entry or follow-up samples, whereas the absence of an immune response was based on a failure to detect HPV16-specific immunity in two consecutive samples. Within the group of histological regressors, 5 patients were HPV16+, 4 of whom showed reactivity to E2, 2 to E7 and 1 to E6 (Table 3). The group of histological persistors comprised of 20 evaluable HPV16+ patients. About half of the patients (9/20) responded to E6, 30% (6/20) to E2 and 20% (4/20) to E7. The progressor group consisted of 6 evaluable HPV16+ patients of whom none responded to E2, 3 to E6 and none to E7 (Table 3). A significant correlation was found between the presence of HPV16 E2-specific T-cell responses in patients with regressing LSIL lesions and the absence of this type of immune responses in patients with progressing LSIL lesions ($p = 0.005$; Chi-Squared test for trend; Table 3).

HPV16-specific immunity in relation to virological status of the patients

All patients were HPV typed at study entry, at 6 months and at the end of the study (12 months). In 33 patients HPV16 was detected at least once. Grouping the patients according to their HPV16 status at each occasion resulted in 7 different HPV16 trends which could be classified into: HPV16 clearers, possible HPV16 clearers, HPV16 persistors and unclassified HPV16 status. This led to the identification of 7 indisputable HPV16 clearers and 12 patients with HPV16 persistence. Four of the seven clearers and 7 out of 10 evaluable persistors displayed HPV16-specific immunity. Stratification according to HPV16 status and HPV16 immunity revealed no

Table 3. Immune responses and histological outcome in HPV16+ LSIL patients

		From LSIL to			
		Normal (N = 5)	LSIL (N = 20)	HSIL (N = 6)	p-value[a]
HPV16 spec IR -		1	8	3	0.317
HPV16 spec IR +		4	12	3	
HPV16E2	negative	1	14	6	**0.005**
	positive	4	6	0	
HPV16E6	negative	4	11	3	0.333
	positive	1	9	3	
HPV16E7	negative	3	16	6	0.094
	positive	2	4	0	

[a] The low numbers in some of the categories does not allow Chi-square calculations, the indicated p-values are therefore based on Chi-squared test for trend.

Table 4. Immune responses versus viral status in HPV16+ patients

		HPV16 viral status		
		Clearance (N = 7)	Persistence (N = 10)	p-value[a]
HPV16 spec IR -		3	3	0.64
HPV16 spec IR +		4	7	
HPV16E2	negative	4	7	0.64
	positive	3	3	
HPV16E6	negative	6	3	**0.05**
	positive	1	7	
HPV16E7	negative	7	9	1.00
	positive	0	1	

[a] Fishers Exact Test, 2 sided.

significant difference between patients with or without a detectable HPV16-specific T-cell response ($p > 0.05$; Fishers Exact test; Table 4). Of note, HPV16 E6-specific immunity was more prominent in the group of HPV16 persistors than in patients who cleared HPV16 ($p = 0.05$; 2-sided Fishers Exact test; Table 4;). The enlargement of the group of viral clearers by including the possible HPV16 clearers revealed the same picture (not shown).

HPV16 status and histological outcome

Intriguingly, HPV16-specific immunity, histological outcome and viral status were not clearly related and therefore the relationship between these parameters was analysed in the groups of patients for which all three sets of data were available.

Three out of four HPV16+ patients who showed a clear regression of their lesion displayed a concomitant loss of HPV16. All three displayed immunity to HPV16 E2. In the one patient who did not show an HPV16-specific immune response, HPV16 was detected only at 12 months while there was a strong signal for HPV33 at entry. It seems probable that the occurrence and subsequent regression was not HPV16-related, suggesting that this patient may have been tested too early in the natural history to see an immune response to HPV16.

In only 6 of the 19 evaluable patients could the persistence of a lesion be attributed to the consistent presence of HPV16. Five patients were HPV16+ only at 6 months and one only at 12 months. In 4 cases clearance of HPV16 was observed, and in 3 cases potential clearance of HPV16. Persistence of the lesion in all these latter cases was due to other HPV types (8 cases) or non-HPV related reasons (5 cases). In 3 of the 6 patients with a persisting HPV16 infection an E2-specific response was observed while 5 subjects responded to E6. One patient did not show a response at all. Of the 13 subjects who cleared or potentially cleared HPV16 or in whom HPV16 was only detected at 6 months of follow-up, 6 displayed HPV16-specfic immunity, 3 of which were to E2 and 4 to E6.

In the group of 6 histological progressors, two patients were able to clear HPV16 but in another a co-infecting HPV type persisted during the whole period. Three of the patients displayed an HPV 16 E6-specific immune response.

When HPV16-specific immunity was analysed only in those patients who showed clear regression of an HPV16-attributable lesion ($n = 4$), persistence of an HPV16-attributable ($n = 6$) or progression of an HPV16-induced lesion ($n = 4$), could a significant trend be observed in that E2-specific immune responses were more frequently present in patients with regressing HPV16+ lesions (4/4) than in patients with persistent (3/6) or progressing (0/4) HPV16-induced lesions ($p = 0.005$, Chi-Squared for trend). Notably, a correlation between histological outcome and HPV16-specific immunity was not observed in the group of evaluable patients ($n = 16$) with HVP16-negative lesions ($p > 0.7$; Chi-Squared for trend; not shown).

Discussion

This study is one of the largest prospective studies that has followed women with LSIL cytologically, virologically, histologically and immunologically. Spontaneous regression was found in less than 20% of all patients, in the others the lesions persisted or progressed. These histological results were paralleled by the immunological outcomes as most of the patients, with an HPV16+ LSIL at recruitment or during follow-up, failed to mount HPV16-specific immunity during the course of their disease. In those who developed an HPV16-specific response the HPV16 E2 and E6 proteins formed the major target antigens when compared to HPV16 E7 (Figure 1), similar to earlier observations in healthy subjects [de Jong 2002a; de Jong 2004; Welters 2003]. Our data suggest that the immune response to HPV16 E2 is beneficial. Trend analysis showed a highly significant correlation between the presence of HPV16 E2-specific T-cell responses in patients with regressing LSIL lesions which was absent in patients with LSIL that progressed (Table 3). Importantly, such a trend was not observed in patients with HPV16-negative LSIL lesions. Although the final number of patients with regressing LSIL examined is too low to make firm conclusions the outcome of this data are supported by studies in the canine oral papilloma virus (COPV) model and in the cotton tail rabbit papilloma virus (CRPV) model. As early as 2-3 weeks after COPV-infection E2-, E6- and E7-specific T-cell responses were detectable but the strongest responses were detected against E2 and maximal responses coincided with wart regression [Stanley 2006b]. In addition, CRPV E2-specific T-cell reactivity was regularly detected in rabbits with regressing CRPV-induced papillomas while responses to E6 and E7 were infrequently observed [Selvakumar 1995]. Furthermore, HPV16-E2-specific responses are frequently observed in healthy subjects [de Jong 2002a; de Jong 2004] as well as at the time of viral clearance in patients with HPV16+ cytological abnormalities [Bontkes 1999].

The presence of an HPV16 E2-specific T-cell response *per se* is not correlated with regression as a number of HPV16+ patients with persistent lesions also display HPV16 E2-specific reactivity (Table 3). This notion is sustained by the analysis of the immune response in patients who most likely cleared their HPV16 infection and in those who failed to do so; these revealed no difference with respect to the presence or absence of E2-specific reactivity (Table 4). However, it must be remembered that in the present study HPV16-specific immunity was measured on the basis of a single parameter that is the specific production of IFNγ. Recently it has been shown that there is substantial heterogeneity in T-cell cytokine responses, with T cells able to produce only INFg after stimulation whereas other T cell populations produce multiple cytokines such as IFNg, IL-2 and TNFa following cognate interaction with their specific peptide-epitope. Both the protection against Leishmania major infection in mice [Darrah 2007] as non-progressive HIV-2 infection [Duvall 2008] was associated with increased frequencies of CD4+ T cells simultaneously producing IFNγ, IL-2 and TNFα. Although it would be tempting to speculate that patients with persistent HPV16 infection who display

E2-specific immunity may clear the virus in the end, it is more probable that functional differences exist between the IFNγ-producing HPV16-specific (E2 and/or E6) T cells in the groups of clearers and persistors and these are responsible for the difference in clinical outcome.

The natural history of HPV infections is not synonymous with natural history of cervical intraepithelial neoplasia. Large scale natural history studies on cervical intraepithelial neoplasia are mainly based on cytological and colposcopic assessment [Holowaty 1999; Pretorius 2006; Woodman 2001]. When compared to other studies, the strength of this study lies in the fact that this was a prospective longitudinal study, with the clinical management of all patients undertaken by a single colposcopist and the histology was based on the two-tier reporting system reviewed by an experienced consultant histopathologist in cervical pathology. Most published studies are either retrospective, covered a broader age range, were purely based on cytology, were subject to inter-observer variations and/or involved small cohorts. In this study, 62 patients were referred with a borderline or mildly dyskaryotic smear. With cytology as the starting reference point and using biopsy proven histology as an end point, 15/62 (24%) patients with these low-grade smear abnormalities progressed to HG-CIN while 37/62 (60%) persisted and 10/62 (16%) regressed to normal within 1 year. This regression rate is similar to the earlier reported regression of 65% after 3 years of follow up in a retrospective longitudinal study [Cuschieri 2007] and fits with the annual rate of transition probability from LSIL to normal of 0.23 [Cantor 2005]. The progression rate from borderline and mildly abnormal smears to HSIL by histology within 1 year was about 25% which is somewhat higher than previously reported studies in which biopsy based progression rates of LSIL to HSIL was reported to be 12 to 26% within 2 years [Campion 1986].

In this cohort, 93% of patients with histological LSIL were positive for HPV and 60-80% displayed an infection with multiple HPV types. Consistent with studies investigating females who are <30 years of age, infection with multiple HPV types is a common finding [Brown 2005; Sargent 2008]. In this study no difference could be observed in the rates of infection with multiple HPV types among the three clinical groups (histological regressors, persistors and progressors). Furthermore, the findings are in line with other studies suggesting that the presence of one HPV type is permissive for co-infection with other HPV types, without necessarily increasing the risk of developing high grade CIN [Liaw 2001; Wheeler 2006]. Although HPV16 has been clearly associated with poorer clinical outcomes [Kovacic 2006; Winer 2005], such a relationship was not observed in this study. Overall, about half of the patients were HPV16+ at recruitment, with no observable difference in prevalence among the different clinical groups. This is probably a reflection of the relatively short length of observation. Interestingly, the clearance rate of HPV16 was comparable to the clearance of multiple infections whereas this was not the case in patients with persistent non type 16 HPV infections.

At recruitment only 23% of the patients with an HPV16+ LSIL and 15% of the patients with an HPV16+ HSIL displayed a detectable response to HPV16. This was also found in a previous study in a group of 38 patients with HPV16+ HSIL, in which 16% of the patients displayed an IFNg-associated immune response to HPV16 [de Vos van Steenwijk PJ 2008]. Therefore, the present study supports the observation that patients with HPV16+ HSIL fail to induce HPV16-specific immunity but it also shows that this failure to mount a response is evident early in HPV-induced intraepithelial disease. It is particularly interesting to see that LSIL patients who also displayed persistence of HPV16, developed HPV16 E6-specific responses more frequently than those who were able to clear the virus (Table 4) even though all were biopsied at study entry. A similar observation was made previously in that HPV16-specific T-cell reactivity in HPV 16+ HSIL patients was predominantly detected in those returning to the clinic for repetitive treatment of a persistent or recurrent HPV16+ HSIL lesion after initial destructive treatment [de Vos van Steenwijk PJ 2008]. All together, these studies show that biopsy or treatment of HPV16-induced lesions followed by persistence of the virus is related to the induction of E6-specific responses at a stage where infection has already led to the development of an intraepithelial neoplasm.

While there is an air of optimism about the prophylactic HPV vaccines and their potential in reducing the burden of cervical cancer [Charo 2007], there is still an urgent need for therapeutic vaccines that treat infection and disease. The current worldwide prevalence of HPV infection is high [Dunne 2007] and there is evidence that this continues to rise [Dillner 2000]. Over the last two decades many types of therapeutic vaccines have been developed to combat HPV-induced neoplasia [Frazer 2004; Melief 2008], the great majority of which aim at inducing T-cell immunity to HPV16 E7 and/or E6. A highly immunogenic synthetic long peptide vaccine which was able to provoke the eradication of established tumors in mice as well as to significantly control wart growth and to abrogate latent CRPV infection compared to controls has been developed [Hung 2008]. Recently, it was reported that vaccination of cervical cancer patients with HPV16 E6 and E7 long peptides in Montanide ISA 51 induces a systemic HPV16-specific type 1 and type 2 CD4+ and CD8+ T-cell response [Welters 2008]. Moreover, therapeutic vaccination of patients diagnosed with HPV16+ high grade lesions of the vulva resulted in the complete regressions of lesions in a substantial number of patients [Kenter 2009], suggesting that vaccination with such E6 and E7 peptide antigens may well overcome the defective anti-HPV16 immune response in patients persistently infected with HPV. Therapeutic vaccination studies in the physiologically relevant CRPV animal model of high-risk HPV infection [Brandsma 2004; Brandsma 2007; Han 2000] and in the COPV model [Moore 2003] as well as the current study indicate that HPV16 E2 forms an additional important target antigen, which at least in patients with HPV16-induced LSIL, may support the eradication of established low grade lesions.

6

General discussion

Summary

The immune system plays an important role in the balance between viral clearance and viral persistence in HPV related (pre)malignant lesions. In this thesis, we analyzed HPV clade A9-specific T-cell responses in relation to virological and clinical outcome to gain further insight into HPV-specific cellular immunity in relation to the natural course of disease.

In depth analysis of cellular immune responses against the E6 antigen of HPV16 and the closely related members of clade A9 (HPV31, 33, 35, 52 and 58) showed us that HPV-specific cross-reactive CD4+ T cells are rare and unlikely to mediate cross protection (*chapter* 2). The clinical course of cervical HPV infection and HPV-specific immune responses in prospective studies are described in *chapter 4 and* 5. In those chapters, a strong correlation is observed between a persistent HPV infection or progressive disease and the lack or failure of a type-specific immune response (>90% of the cases). No correlation is detected between HPV type-specific cellular immune responses and virological clearance of the infection and HPV type-specific immunity may be associated with clearance of a cervical HPV induced lesion (*chapter 4 and 5*). Interestingly, a statistically significant trend could be detected between the presence of a type-specific immunity (HPV16E2) and regression of a low-grade lesion (*chapter 5*). A similar, but – due to small number of patients – not statistically significant trend was observed in *chapter 4.* Together, this suggests that the local innate immune system might play a role in the clearance of transient infections, whereas the cellular immune response can play a role in regression of histologically proven HPV induced lesions. Detection of HPV16 type-specific cellular immune responses in vivo, by the use of a DTH skin test, confirmed that cellular immune responses in healthy subjects consist of both HPV-specific CD4+ Th1/Th2 and CD8+ T cells (*chapter 3*).

Natural course of disease and immunity

As previously described, infection with HPV is very common (lifetime incidence ~80-85% [Baseman 2005; Rodriguez 2008]). In the majority of women however, this infection is transient and will be spontaneously cleared within 8 to 16 months [Moscicki 2001; Plummer 2007; Trottier 2006]. Natural history studies show that 85-90% of LSIL will spontaneously regress, however when progressed to HSIL and left untreated, 30% will further progress in to invasive cervical cancer [McCredie 2008; Stanley 2006a; Wheeler 2008]. Cellular immune responses in relation to progression and regression of HPV related disease have intensively been studied and seem to play an important role in control and clearance of anogenital HPV infections. HPV-positive cervical neoplasia is associated with failure of a systemic and local or intra-lesional CD4+ and CD8+ T-cell response [Bontkes 2000; de Gruijl 1998; de Jong 2004a; Monnier-Benoit 2006; Nakagawa 2000; Steele 2005; Trimble 2010a; de Vos van Steenwijk 2008; Welters 2006; Woo 2008; Youde 2000]. In contrast, healthy individuals and women with regression of their

cervical lesion commonly display strong CD4+ memory T-cell responses and infiltration of large numbers of CD4+ and CD8+ T cells into the regressing lesions [Bontkes 2000; Coleman 1994; Farhat 2009; de Jong 2002; de Jong 2004a; Nakagawa 1997; Nakagawa 2000; Seresini 2007; Welters 2003; Welters 2006; Woo 2008]. Formal proof of this causal relationship remains to be established. The three large prospective cohort studies, as described in *chapter 4 and 5* gave us the unique opportunity to test the hypothesis that clearance of cervical HPV infection may be associated with systemic HPV-specific T-cell immunity. Indeed, in line with our previously published data, a strong correlation between a persisting HPV-infection or low-grade lesion and failure of a type-specific immune response (90%) was observed, as expected based on their failure to clear these infections [de Jong 2004a; de Vos van Steenwijk 2008; Welters 2006]. No correlation between HPV type-specific immune responses and viral clearance could be observed. In only 10 to 18% of the individuals with a transient infection, clearance is matched by such a response. Immunity towards the other HPV types tested however is detected in 30-50% of the individuals (*chapter 4*), indicating that the lack of matching T-cell responses does not reflect overall immune failure against HPV. Interestingly, the analysis of immune responses and clinical (or histological) outcome, revealed a weak but statistically significant correlation between HPV16E2 specific immunity and regression of LSIL lesions (*chapter 5*). Such an observation, although not statistically significant, was also shown in *chapter 4*. We hypothesize that the lack of a positive correlation between viral clearance of transient infections (i.e. no cytopathological abnormalities) and a specific immune response might be subscribed to innate immunity and that cellular immune responses play a role in the control of histologically proven infections (LSIL lesions) – Figure 1. The innate immune system acts as a first line of defense as many infections are cleared by innate immunity before the adaptive response kicks in [Medzhitov 1997]. Others have postulated that transient HPV infections, as detected by exfoliated cell scrapes, are rapidly cleared by (local) innate immune factors (i.e. mucus, phagocytes and natural killer cells) before infecting the basal cell layer of the epithelium [Einstein 2009b; Woodworth 2002]. This may preclude antigen processing and presentation by local dendritic cells (DC) or Langerhans cells (LC) thereby preventing stimulation of the adaptive immune system. Consequently, these women will not show HPV-specific T-cell memory. Notably, the current sensitivity of HPV DNA assays allows detection of very low copies of the HR-HPV in subclinical or non-productive (harmless) infections that are not associated with virus-induced cytological manifestations [Cuzick 2008]. Literature reports of a correlation between HPV type-specific cellular immune responses and spontaneous clearance of cervical or vulvar lesions are rare [Bourgault, I 2004; Seresini 2007]. A small percentage of HPV16+ HSIL lesions spontaneously regress after a diagnostic biopsy, suggesting that local danger signals (i.e. biopsy) in combination with HPV16 antigens can activate HPV type specific T-cell responses [Trimble 2005]. As reported by Sarkar et al., recurrence of HSIL lesions after local treatment is associated with the lack of proliferative T-cell response whereas patients who remained disease-free after local treatment show a Th1 response [Sarkar 2005]. On the other hand, Trimble et

al. reported that spontaneous regression of HPV16+ HSIL lesions after biopsy (26%) is not correlated with systemic T-cell responses to HPV16E6 and E7 [Trimble 2010a]. One should bear in mind however, that HPV16 specific immunity was measured based on a single parameter that is the specific production of IFNγ. As reported, only in a limited number of patients HPV-specific T-cell responses are associated with the production of IFN [Heusinkveld 2011] and a stronger correlation is observed with proliferative T cells

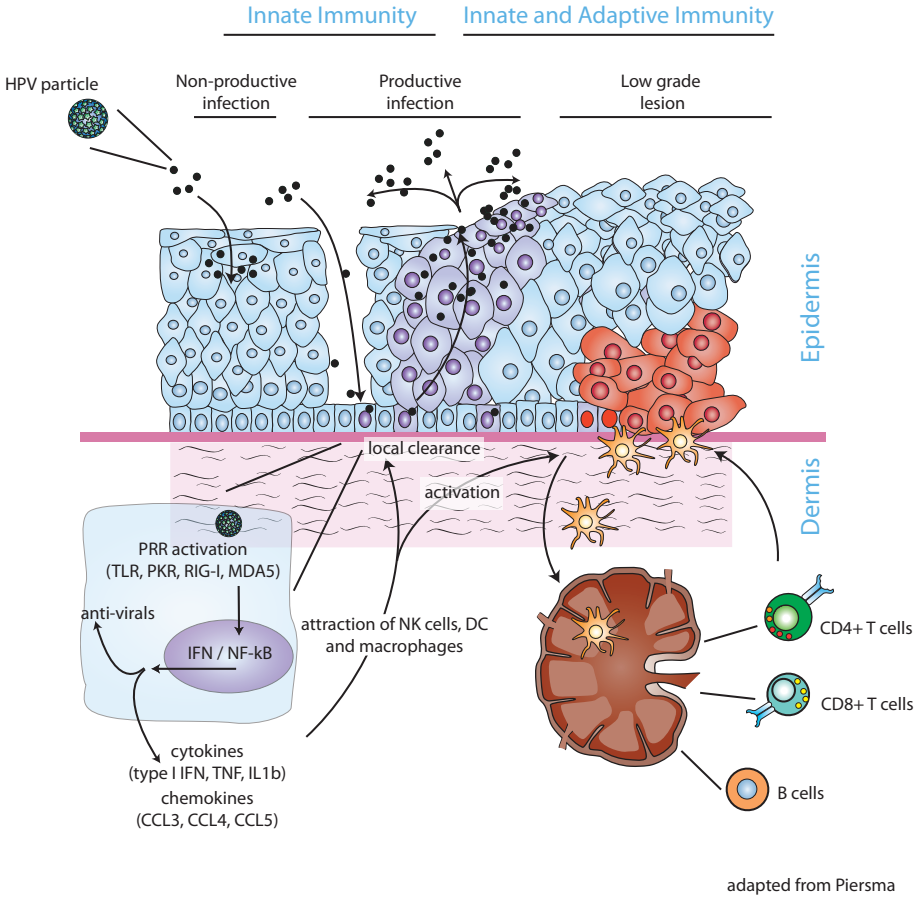


Figure 1. Proposed model of the role of innate and adaptive immune response in local viral clearance. A. Non-productive HPV infection or "contamination" (detected by very sensitive assay) cleared by a local innate immune response (i.e. mucus, macrophages and NK cells) before the virus infects the basal layer of the epithelium. No activation of dendritic cells (DCs). B. Productive infection without cellular abnormalities of the epithelium (i.e. cytopathological normal). Virus replicates in the basal layers without inducing cell death and danger signals. Activation of DCs and triggering the adaptive immune response does not occur. C. LSIL: mild dysplasia of the epithelium, leading to triggering of DCs and thereby activating the adaptive or cellular immune response.

and production of a mixture of Th1/Th2 cytokines (*chapter 3*) [Welters 2010]. Finally, the recent compelling results in HPV16-positive VIN3 patients of almost 50% complete clinical response after vaccination sustain the notion that the cellular (e.g. CD4 and CD8) immune response indeed play an important role [Kenter 2009; Welters 2010]. This all seems to fit with our data, as described in *chapter 4 and* 5, that the presence of HPV type-specific immunity was correlated with clinical (or histological) but not virological outcome and suggests that the cellular immune system might at least play an important role in the control of histologically proven HPV induced lesions, rather than transient or non-productive subclinical infections.

To gain further proof of this hypothesis, we would recommend a new prospective study with the focus on HPV16-type specific cellular immune responses in women with transient HPV16 infections (i.e. no cytopathological manifestation) compared to women with proven HPV16+ low-grade lesions. However, we do realize – based on the prevalence of HPV16 in AS-CUS and low-grade lesions, as experienced in our previous studies – that it will be difficult and time-consuming to include enough subjects. Based on the results in *chapter 4*, not only responses towards E6 and E7, but also HPV16E2 specific responses should be analyzed. Insight in this matter is important for the development of therapeutic vaccines as will be discussed later.

Cellular HPV type-specific immune responses: no cross protection

In natural infections, the humoral immune response to HPV (i.e. antibodies) is type-specific and do not appear to be cross-protective [Palmroth 2010]. However, VLP-vaccines are reported not only to induce type-specific but also cross-protective antibodies [Bonanni 2009; Brown 2009; Jenkins 2008; Kemp 2011; Smith 2007a; Wheeler 2009]. The exact mechanism is still unknown, but probably the highly immunogenic VLPs prime and boosts a large number of B cells thereby generating a broad and strong antibody response with cross-reactive properties. The affinity and duration of cross-protection against other HPV types however is currently unknown. In *chapter* 2, we are the first to describe the evaluation of cross-reactivity in HPV-specific cellular immune responses. Analysis of CD4+ memory T-cell responses against the E6 antigen of HPV16 and the closely related members of clade A9 (HPV31, 33, 35, 52 and 58) in healthy subjects by overlapping peptide arrays (ELISPOT assay) revealed that responders frequently display reactivity against corresponding E6 peptides from two HPV types. This initially suggested frequent immunological cross-reactivity between the different HPV types. Further dissection of the response by clonal T-cell cultures taught us that CD4+ T cells capable of efficiently reacting against E6 antigen form multiple HPV types are rare and only occur when epitope sequences are highly conserved. Although the members of clade A9 share up to 80% nucleotide homology, only three regions or stretches of possible minimal T-cell epitopes (minimal 10 aa) are present (Table 1). Natural CD4+

T-cell immunity, as initiated by encounter of a given HR-HPV type, is unlikely to provide effective cross-protection against infections with closely related HR-HPV types. Detection of non-matching epitope-specific immune responses to related clade A9 HPV types in women with persistent HPV infections (*chapter 4*) sustain this notion and suggest that HPV-specific immunity as detected is not likely to confer in vivo cross-protection. Furthermore, the lack of cross-reactivity in HPV16-vaccinated VIN patients sustains the notion that vaccine induced CD4+ T-cell responses are also unlikely to mediate cross-protection against other clade A9 members and indicate that therapeutic efficacy of vaccines is type-specific (*chapter 2*). As reported, the HPV16SLP vaccine is capable of inducing a broad and robust cellular immune response [Welters 2010], but in contrary to the strong VLP/prophylactic vaccine-induced humoral immune responses, it is not cross-reactive. Similar to the CD4 T-cell responses reported by us, others have shown CD8 T-cell mediated type-specific recognition of highly homologous HLA class I-restricted T-cell epitopes [Wang 2008].

Table 1. HPV16E6 – peptide sequence and homology with other clade A9 members

1-53	MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVYDFA
54-106	FRDLCIVYRDGNPYAVCDKCLKFYSKISEYRHYCYSLYGTTLEQQYNKPLCDL
HPV31	KCL+FYSK+SE+R
HPV35	KCLKFYSKISEYR
HPV58	IVYRDGNP+AVC
107-158	LIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQL
HPV31	CQ+PLCPEEKQRHLDKK+RFHNI
HPV33	CQ+PLCP+EK+RH+D
HPV35	EKQRHL++K+RFHNI
HPV52	PLCPEEK+RH++
HPV58	CQ+PLCP+EK+RH+D

Potential minimal T-cell epitopes of at least10 amino acids (aa) sharing sequence homology are indicated. Identical amino acids or non-identical amino acids with similar properties (+) compared to the HPV16 sequence are depicted.

The absence of major cross-reactivity at the T-cell level is best explained by the fact that CD4 and CD8 T-cell responses are, other than B-cell responses, human leukocyte antigen (HLA) – restricted and the peptide-epitopes recognized by these T cells require processing and presentation of the viral antigens within these HLA molecules. Small amino acid changes in the vicinity of the amino acid residues within the epitope that are recognized by the T-cell receptor may already affect processing, presentation and recognition. Similar changes in neighboring amino acids of residues recognized by antibodies are less likely – provided that they do not alter the protein quaternary structure – to change recognition. Recently, a HPV16+ VIN patient who has participated in our therapeutic vaccination trial and showed a complete response of the vulvar lesion did develop a new HPV31-positive CIN lesion (unpublished data), indicating that the therapeutic HPV16SLP vaccine is indeed type-specific, urging the need for development of other HPV type-specific therapeutic vaccines, like HPV18 or HPV31.

Additionally, our data as described in *chapter 2* demonstrates that detection of CD4+ memory T-cell responses by means of overlapping peptide arrays cannot be readily used for retrospective evaluation of HPV infection history other than the dominant HPV type 16 as it might result in an overestimation of the response to HPV types closely related to HPV16. Our prior studies have shown that the number of antigen-specific T cells responding to peptide-pulsed antigen-presenting cells (APCs) is generally larger than that responding to protein-pulsed APCs [van der Burg 2007; de Jong 2002; Welters 2003]. To distinguish between highly potent T cells (i.e. responding against limiting quantities of naturally processed antigen) and T cells that require greater quantities of antigen, responses can be analyzed by using APC pulsed with whole protein as antigen. It is our experience however that the use of recombinant proteins, produced and replicated in *E. Coli*, may result in the activation of non-HPV-specific immune responses. Dose-titration experiments in our laboratory confirmed that the use of 10 μg/ml of peptide is quite high and may stimulate low affinity T cells (unpublished observations). Therefore, in order to analyze HPV type-specific immune responses to other than HPV16 by IFNγ ELISPOT, we recommend to lower the dose of peptide-antigen to detect only highly potent, or type-specific, T cells.

Immunotherapeutic approaches to combat (persistent) HPV infections

Prophylactic vaccines designed to prevent HPV infection and development of HPV related (pre)malignant lesions – theoretically resulting in reduction of 70% of the cervical cancer burden – seem to have great potential ([Einstein 2009a; Kjaer 2009; Paavonen 2009; Villa 2006] and reviewed in [Stanley 2006a; Frazer 2011]), however it will take many years before the prevalence of HPV infections among the population will decrease. This is especially true since the vaccination coverage is lower as expected: 56,4% in the Netherlands and only up to 32% of the young women in the USA have

been fully vaccinated [Centers for Disease and Prevention 2010 and 2011; de Hoogh 2011]. HPV vaccination coverage was influenced by the complexity of multiple factors: both parental attitudes and local (political) policies regarding sexual activity of young adolescents. The current prophylactic vaccines are not therapeutic in women already infected with HPV and do not increase viral clearance [Hildesheim 2007]. The recent significant clinical success in VIN3 patients vaccinated with our HPV16 SLP vaccine (complete clinical response in 47% of the patients, paralleled by a strong systemic HPV-specific CD4+ and CD8+ T cell response – [Kenter 2009; Welters 2010]) tempted us to speculate about a potential additional benefit of immunotherapy in the treatment of pre-malignant low grade and high grade cervical lesions.

Since 85 to 90% of LSIL will regress spontaneously within 12 to 24 months [Moscicki 2001; Plummer 2007], there is no role for (surgical) treatment. Cytological screening and follow up (with or without HPV typing) is performed at 6, 12 and 24 months to identify the women with progressive disease and offer proper treatment [Wright 2007; Werkgroep Oncologische Gynaecologie 2004]. If left untreated, 30% of the women progressing to HSIL are at risk to develop invasive cervical carcinoma [McCredie 2008; Wheeler 2008]. The most common treatment techniques for HSIL are loop electrosurgical excision procedure (LEEP), cryotherapy, carbon dioxide laser abalation or conization [Cirisano 1999]. These techniques are effective and comparable, with a cure-rate of 90% and overall rate of recurrent or persistent disease of 5 to 17% [Martin-Hirsch 2010].

The local micro-environment in a persistent HPV infection and related (pre)malignant lesions is anti-inflammatory (i.e. inhibits/impaired innate immunity – reviewed by Bhat et al.[Bhat 2011]) and systemic and local HPV-specific cellular immune responses are lacking or impaired in women with persistent HPV infection or progressive disease (*chapter 4 and 5*) [Bontkes 2000; de Gruijl 1998; de Jong 2004a; Monnier-Benoit 2006; Nakagawa 2000; Steele 2005; Trimble 2010a; de Vos van Steenwijk 2008; Welters 2006; Woo 2008; Youde 2000]. Successful immunotherapy will at least require the generation of effector T cells (adaptive immunity) by vaccination, of which the effect may be enhanced through stimulation of local inflammation (innate immunity). For instance by pre-treatment with a TLR agonist (Imiquimod topical treatment [Garland 2003; Terlou 2010]) as recently shown by Daayana et al [Daayana 2010]. As a consequence, not only patients with a persisting HPV16+ LSIL (i.e. failure of immune response and at risk to develop progressive disease) but also HPV16+ HSIL patients after local treatment by LEEP or cryotherapy (i.e. failure of immune response and at risk to develop recurrent disease) might benefit from treatment with our highly immunogenic therapeutic HPV16 SLP vaccine [Kenter 2009; Welters 2010]. Since the therapeutic efficacy of the vaccine is highly likely to be type specific (*chapter 2*), the lesions have to be HPV16-positive. Biopsy or local treatment (LEEP) can be seen as a local or inflammatory stimulus, activating the innate immune system. However, as reported by de Vos van Steenwijk et al. and in *chapter 5*, the majority of these HPV16E6 specific

T-cell responses detected in recurrent lesions after local treatment are weak and do not secrete pro-inflammatory cytokines therefore failing to developing protective immunity [de Vos van Steenwijk 2008]. Simultaneous stimulation by the HPV16 SLP vaccine and local treatment however might lead to the development of a proper response [de Vos van Steenwijk 2010]. The E2 protein is highly expressed in low-grade lesions, whereas the oncoproteins E6 and E7 are primarily expressed in high levels in more advanced or HSIL lesions [Maitland 1998; Stevenson 2000; zur Hausen 1996]. Therefore, the E6 and E7 oncoproteins are considered major targets in immune intervention of HSIL and cervical carcinoma, while the E2 protein might be an attractive target in treatment of LSIL lesions. HPV16E2-specific memory T-cell responses are frequently detected in healthy subjects and in patients clearing HPV16+ cytological abnormalities [Bontkes 2000; de Jong 2002]. In *chapter 5* we report a weak but significant correlation between HPV16E2 specific T-cell responses and regression of LSIL lesions, suggesting the response to E2 is beneficial in clearance of low-grade lesions. The outcome of this data is supported by vaccination studies in rabbits and dogs (CRPV and COPV model), where strong E2 responses correlate with regression of warts and papilloma's [Brandsma 2004; Brandsma 2007; Han 2000; Moore 2003; Selvakumar 1995; Stanley 2006b]. In view of these considerations, it would be of interest to develop a vaccine consisting of E2, E6 and E7 peptides to support the eradication of both low-grade and high-grade lesions. The E2 protein is quite large and therefore this would translate in a substantial number of peptides. One may opt to focus on a number of highly immunogenic peptides from this protein, which are recognized by many individuals (*chapter 3*) [de 2002].

The HPV16 SLP vaccine contains nine HPV16E6 and four HPV16E7 synthetic peptides, dissolved in dimethyl sulfoxide, phosphate-buffered saline and Freund's adjuvant. One dose of each vaccine contains 0,3 mg of each peptide in a total volume of 2.8 ml [Kenter 2009]. This large volume is needed to inject the vaccine at that antigen-dose in patients with high-grade disease (~immune failure) to induce a clinical and cellular immune response. Unfortunately, this is generally accompanied by (long lasting and) large swelling of the vaccination site. In patients with low-grade lesions or persistent HPV infection, injection of a vaccine of almost 3 ml might cause disproportional and discomforting side effects (swelling), leading to rejection of this therapy. Since these individuals are (more) immunocompetent, we might not need such a high dose. As most vaccines, the HPV16 SLP vaccine is administered via subcutaneous injection. However, cutaneous (i.e. intradermal or epidermal) routes are a highly attractive alternative because of the immunological characteristics of the skin (reviewed in [Nicolas 2008]). The dermis is the major site of cellular and fluid exchanges between the skin and blood- and lymphatic vessels. These exchanges occur in the so-called "micro vascular dermal units", located in the papillary dermis near the dermal-epidermal junction. Both Langerhans cells (LC) and dermal dendritic cells (DC) reside in epidermis and dermis. After capture of the antigen and maturation of the APC, they can migrate through the lymphatic vessels into the draining lymph nodes and activate B and T cells. Free antigen (e.g. vaccine) can also directly migrate or diffuse into the draining lymph node since tissue fluid

is also absorbed directly into lymphatic capillaries. Thereby, intradermal vaccination triggers two synergistic pathways. Moreover, the number of circulating DCs in the subcutis is substantially lower and therefore subcutaneous injection is a less optimal route for antigen presentation and T cell triggering. Intradermal vaccination has been extensively tested in humans (Rabies, Influenza, HBV) and most studies show that only 10% of the dose is as immunogenic as intramuscular- or subcutaneous vaccination (see references in [Nicolas 2008]). Transcutaneous or epidermal immunization (DNA tattooing) in mice and non-human primates show similar results [Oosterhuis 2012]. In *chapter 3* we reported that intradermal injection of HPV16 synthetic long peptides (SLP) in healthy individuals results in the migration of both HPV16-specific CD4+ and CD8+ T cells into the skin and an increase in the number of circulating HPV16-specific T cells. Therefore, these injections could be considered as a single, low dose vaccination. The injections consisted of 0,01 mg of each peptide, compared to 0,3 mg in the HPV16 SLP vaccine: a 30 fold reduction. Intradermal vaccination with such a small volume might be an attractive alternative in the treatment of pre-malignant low-grade and high-grade cervical lesions as it is less likely to cause discomfort by swelling.

In view of these considerations and based on the results of the studies described in this thesis, it would be of interest to develop a therapeutic vaccine consisting of E2, E6 and E7 peptides and develop other HPV type-specific therapeutic vaccines, starting with HPV18. In order to lower the dose and reduce or minimize side effects, it might be worthwhile to explore alternative routes (e.g. intra- or epidermal) of vaccination.

7

Summary

Humaan papillomavirus (HPV) en (pre)maligne afwijkingen aan de baarmoederhals

Van alle vormen van kanker wereldwijd wordt 5% veroorzaakt door een infectie met het humaan papillomavirus (HPV). De meerderheid wordt gevormd door baarmoederhalskanker of het cervixcarcinoom en slechts in 0,7% van de gevallen betreft het een tumor van het hoofd/halsgebied, de schaamlippen, vagina of anus. Baarmoederhalskanker is na borst- en darmkanker de meest voorkomende vorm van kanker onder vrouwen, met 529.800 nieuwe gevallen in 2008. De hoogste incidentie (85%) wordt gezien in ontwikkelingslanden, met name door het gebrek aan screeningsprogramma's. In Nederland heeft het bevolkingsonderzoek een belangrijke rol gespeeld bij het terugdringen van baarmoederhalskanker. Vanaf het 30ste levensjaar worden vrouwen elke 5 jaar opgeroepen voor het uitstrijkje, gericht op de vroege detectie van voorstadia van baarmoederhalskanker. Toch worden in Nederland jaarlijks ongeveer 600 nieuwe gevallen van baarmoederhalskanker gediagnosticeerd, en sterven er ruim 200 vrouwen aan de gevolgen van de ziekte.

Een persisterende, langdurige infectie met HPV is door de Wereldgezondheidsorganisatie (WHO) aangeduid als de primaire oorzaak van baarmoederhalskanker. Het humaan papillomavirus behoort tot de DNA-virussen en de familie van de papillomaviridae. Op dit moment zijn al meer dan 100 verschillende HPV typen bekend, waarvan ongeveer 30 genitale HPV typen die de huid en slijmvliezen van de geslachtsorganen kunnen infecteren. Gebaseerd op epidemiologisch onderzoek wordt onderscheid gemaakt tussen zogenaamde laag risico- en hoog risico HPV (hrHPV) typen. Infecties met een laag risico HPV type veroorzaken goedaardige of benigne anogenitale wratten, terwijl infectie met een kankerverwekkend of oncogeen hrHPV type een causale rol speelt bij de ontwikkeling van premaligne en kwaadaardige of maligne afwijkingen aan de baarmoederhals.

Van de 15 oncogene of hoog risico HPV typen, zoals vastgesteld door de WHO, is HPV type 16 het meest voorkomende en meest oncogene type virus. In ongeveer 20 tot 44% van de premaligne afwijkingen (voorstadia) en bijna 55% van de gevallen van baarmoederhalskanker wordt dit HPV type aangetroffen. Genitale HPV typen behoren tot het genus van α-papillomavirussen, welke op basis van genetische kenmerken zijn gegroepeerd in 15 verschillende subgroepen of species. HPV16 behoort met HPV31, HPV33, HPV35, HPV52 en HPV58 tot de α9 species of "clade A9". Deze virussen zijn voor 71-89% genetisch identiek.

Genitale HPV infecties komen zeer veel voor, het is één van de meest voorkomende seksueel overdraagbare aandoeningen (SOA). Geschat wordt dat ongeveer 80% van alle mensen gedurende het leven geïnfecteerd raakt met een hrHPV type. De overgrote meerderheid van deze mensen is instaat om de virusinfectie binnen anderhalf tot twee jaar zelf onder controle te krijgen. Het immuunsysteem speelt hierbij een belangrijke rol. Echter, een hele kleine groep vrouwen (<1%) is hiertoe niet in staat. Deze groep

vrouwen blijft hierdoor langdurig met het virus geïnfecteerd en loopt hierbij het risico om baarmoederhalskanker te ontwikkelen. Tijdens een persisterende infectie integreert het virale DNA in het DNA van de gastheer- of geïnfecteerde cellen van de baarmoederhals. Dit leidt tot verhoogde productie of expressie van de (oncogene) eiwitten E6 en E7, die betrokken zijn bij de regulatie van de celcyclus, met als gevolg ongeremde groei en celdeling wat uiteindelijk kan leiden tot het ontstaan van (pre) maligne afwijkingen van de baarmoederhals.

Het afweersysteem

Het immuunsysteem of afweersysteem beschermt het lichaam tegen lichaamsvreemde en ziekmakende organismen, zoals onder andere virussen en kan ingedeeld worden in aangeboren immuniteit en verworven immuniteit.

De aangeboren of "innate" immuunrespons is aspecifiek en snel en werkt als eerste verdedigingsreactie tegen een infectie. Voorbeelden hiervan zijn de huid en slijmvliezen als natuurlijke barrière, een lokale ontstekingsreactie en macrofagen of fagocyten die het virus "opeten" (fagocyteren), in stukjes knippen en deze stukjes presenteren op de celmembraan (antigeen presentatie) en zo de "adaptieve" of verworven immuunrespons activeren.

Deze "adaptieve" of verworven, specifieke immuunrespons bestaat uit 2 componenten: een humorale en een cellulaire afweerreactie. Bij een humorale respons spelen de B cellen een belangrijke rol. Na activatie door een antigeen presenterende cel zullen deze B cellen antilichamen produceren die gericht zijn tegen het viruskapsel, met als doel het virus te neutraliseren en daarbij een infectie – het binnendringen van het virus in de cel – te voorkomen. Als het virus uiteindelijk toch de cel infecteert, is deze niet meer bereikbaar voor de antilichamen en verloopt de afweerreactie via T cellen, de cellulaire immuunrespons.

Cellulaire afweer is een immuunrespons gericht tegen intracellulaire micro-organismen, zoals virussen (HPV) en bacteriën, waarbij T cellen deze geïnfecteerde cellen kunnen herkennen en opruimen. Binnen de T cellen kunnen de volgende verschillende subtypes onderscheiden worden met elk een specifieke functie: CD8+ cytotoxische T cellen (CTL), CD4+ T helper (Th) cellen en CD4+ regulatoire T cellen (Treg). De CD4+ T cellen produceren verschillende signaalstoffen (cytokines) zoals interferon gamma (IFNγ) en interleukine (o.a. IL2, IL4, IL5, IL10) en hebben een regulerende functie in het opgang brengen en houden van zowel de cellulaire als humorale immuunrespons. De vele verschillende T helper cellen zijn elk verantwoordelijk voor een specifiek stukje van de afweerreactie, zoals activatie van B cellen, activatie van effector cellen van de "innate" immuunrespons (macrofagen) en activatie en "aantrekken" van de CD8+ cytotoxische T cellen welke in staat zijn de virus geïnfecteerde cellen en tumorcellen te herkennen en te doden. De regulatoire T cellen produceren ontstekingsonderdrukkende cytokinen en zijn daarmee in staat de andere cellen van het immuunsysteem te onderdrukken.

Zowel de B als T cellen vormen "geheugencellen" en daarom zal bij nieuw contact met dezelfde ziekteverwekker de specifieke afweer sneller en krachtiger reageren om zo infectie te voorkomen (het principe van vaccinatie).

Afweerreacties tijdens het natuurlijk beloop van een HPV infectie

Genitale HPV infecties komen zeer veel voor en de meerderheid van de vrouwen is in staat om de virus infectie onder controle te krijgen. Dit gaat gepaard met het verdwijnen van de afwijking aan de baarmoederhals. Intensief en jarenlang onderzoek van verschillende onderzoeksgroepen suggereerde dat de afweerreactie van T cellen hier een belangrijke rol in speelt. In *hoofdstuk 4* en *5* worden drie grote prospectieve cohort studies beschreven waarin wij de hypothese dat "klaring van een cervicale HPV infectie geassocieerd is met HPV specifieke T-cel immuniteit" hebben onderzocht. Hier wordt inderdaad bevestigd dat er een sterke correlatie (90%) bestaat tussen een persisterende HPV infectie of cervicale laesie en een falende HPV specifieke afweer: het lichaam is niet in staat deze infectie op te ruimen. Echter, anders dan verwacht, werd er geen positieve relatie aangetoond tussen het klaren van de virus infectie en de aanwezigheid van HPV specifieke immuun responsen. Analyse van immuun responsen en de klinische dan wel histologische uitkomst toonde interessant genoeg wel een zwak, maar statistisch significant verband tussen de HPV16 specifieke afweerreactie tegen het E2 eiwit en regressie van laaggradige afwijkingen aan de baarmoederhals (LSIL). Deze resultaten suggereren dat HPV specifieke cellulaire afweerreacties een belangrijke rol spelen bij het opruimen van histologisch bewezen afwijkingen van de baarmoederhals, maar niet noodzakelijk bij kortdurende of voorbijgaande infecties met HPV die geen afwijkingen van het epitheel van de baarmoederhals veroorzaken. Wij denken dat het aangeboren of "innate" immuunsysteem mogelijk deze infecties opruimt, voordat het virus de cellen van de basaal membraan kan infecteren en dus geen histologische afwijkingen kan veroorzaken. Hierdoor vindt er geen antigeen presentatie plaats aan de cellen van het adaptieve of cellulaire immuun systeem – het virus blijft "onzichtbaar" – en worden er geen T cellen geactiveerd.

HPV specifieke T cel immuniteit biedt geen kruisbescherming

Kruisbescherming of bescherming tegen infectie met nauw gerelateerde en genetisch verwante HPV typen is tot nu toe alleen beschreven voor humorale of B cel gemedieerde afweerreacties. Na vaccinatie met een profylactisch HPV vaccin (zowel Gardasil® als Cervarix®) worden kruis beschermende antilichamen gedetecteerd die zowel een infectie met HPV16 als HPV31 en HPV58 (clade A9) of HPV18 en HPV45

(clade A7) kunnen neutraliseren en voorkomen. Voor de T cel gemedieerde of cellulaire afweerreacties was dit tot nu toe niet bekend. Uitgebreide en gedetailleerde analyse van CD4+ "memory" T cel reacties tegen het E6 eiwit van HPV16 en de andere genetisch verwante HPV typen van clade A9 (HPV31, HPV33, HPV35, HPV52 en HPV58) in gezonde mensen heeft laten zien dat kruis reactieve T cellen bestaan, maar extreem zeldzaam zijn en alleen gevonden worden als het herkende stukje virus DNA nagenoeg identiek is (*hoofdstuk 2*). De immuun responsen tegen de verschillende HPV typen van clade A9 in patiënten met een HPV16 positieve afwijking van de schaamlippen (VIN3) die participeren in onze therapeutische vaccinatie trial met het HPV16SLP vaccin laten een zelfde patroon zien. Ter illustratie: één van de patiënten, met een complete remissie of verdwijnen van de HPV16 positieve afwijking aan de schaamlippen heeft na vaccinatie vervolgens een HPV31 positieve premaligne afwijking van de baarmoederhals ontwikkeld. Hieruit kan geconcludeerd worden HPV specifieke T cel responsen geïnduceerd door het therapeutisch HPV16SLP vaccin niet kruisreactief zijn en niet beschermen tegen een infectie met een ander, gerelateerd HPV type.

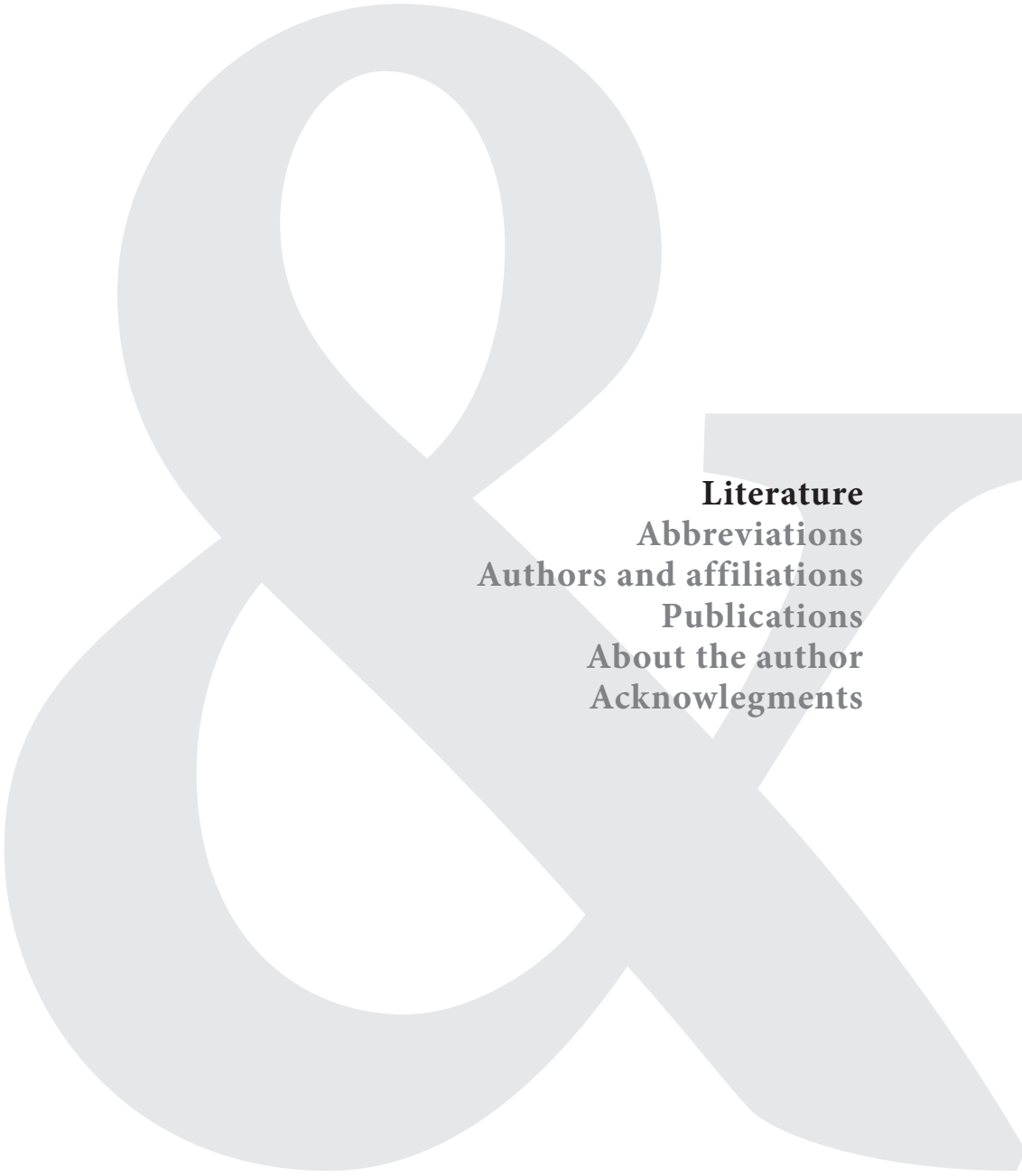
Immunologische behandeling van (persisterende) HPV infecties

Bij laaggradige of LSIL afwijkingen van de baarmoederhals is het beleid deze niet te behandelen, maar frequent cytologisch te controleren en te vervolgen. Een chirurgisch behandeling (lisexcisie) is wel geïndiceerd bij hooggradige afwijkingen (HSIL). De profylactische vaccins, gericht op het voorkomen van de HPV infectie, kunnen een bestaande virusinfectie niet opruimen of klaren en hebben geen therapeutisch effect. Gezien de goede klinische response in patiënten met voorstadia van schaamlipkanker (VIN3) na vaccinatie met het HPV16SLP vaccin, biedt dit wellicht in de toekomst mogelijkheden voor behandeling van voorstadia van baarmoederhalskanker.

De oncogene eiwitten E6 en E7 komen met name tot expressie bij HSIL laesies en worden gezien als "target" voor de immunotherapie. De significante correlatie tussen de HPV16E2 immuun respons en regressie van LSIL laesies (*hoofdstuk 5*), suggereert dat E2 een belangrijke rol speelt bij het opruimen van laaggradige afwijkingen.

Het te injecteren volume van het HPV16SLP vaccin, wat E6 en E7 peptiden bevat, is groot: bijna 3 ml, waarbij vele vervelende bijwerkingen van pijn en zwelling ontstaan. In het kader van de ontwikkeling van een huidtest om een HPV specifieke immuun respons aan te tonen is in *hoofdstuk* 3 beschreven dat intradermale injectie van HPV16 specifieke peptiden leidt tot de migratie en influx van CD4- en CD8 specifieke T cellen in de huid en toename van HPV specifieke T cellen in het bloed. Deze injecties zouden beschouwd kunnen worden als een "lowdose" of intradermale vaccinatie.

Gezien deze overwegingen en gebaseerd op de resultaten zoals beschreven in dit proefschrift, zou de ontwikkeling van een therapeutisch vaccin ter behandeling van LSIL en HSIL afwijkingen, bestaande uit HPV16E2, E6 en E7 peptiden overwogen kunnen worden. Om de dosering te kunnen verlagen is het de moeite waard om alternatieve routes van vaccinatie (intra- of epidermaal) te exploreren. Aangezien kruisbescherming niet bestaat, pleit dit voor de ontwikkeling van nieuwe therapeutische type-specifieke vaccins, allereerst HPV18.



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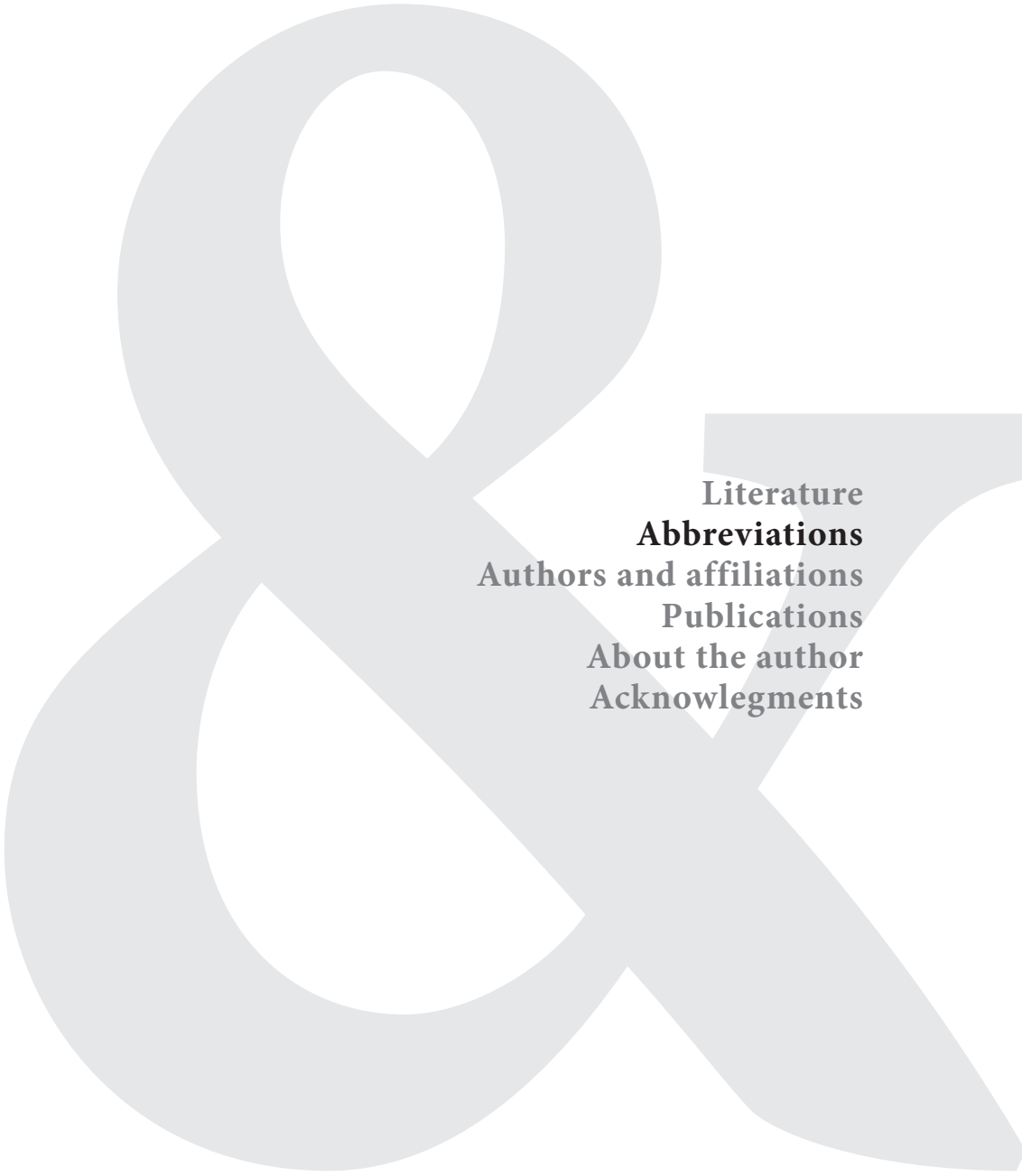
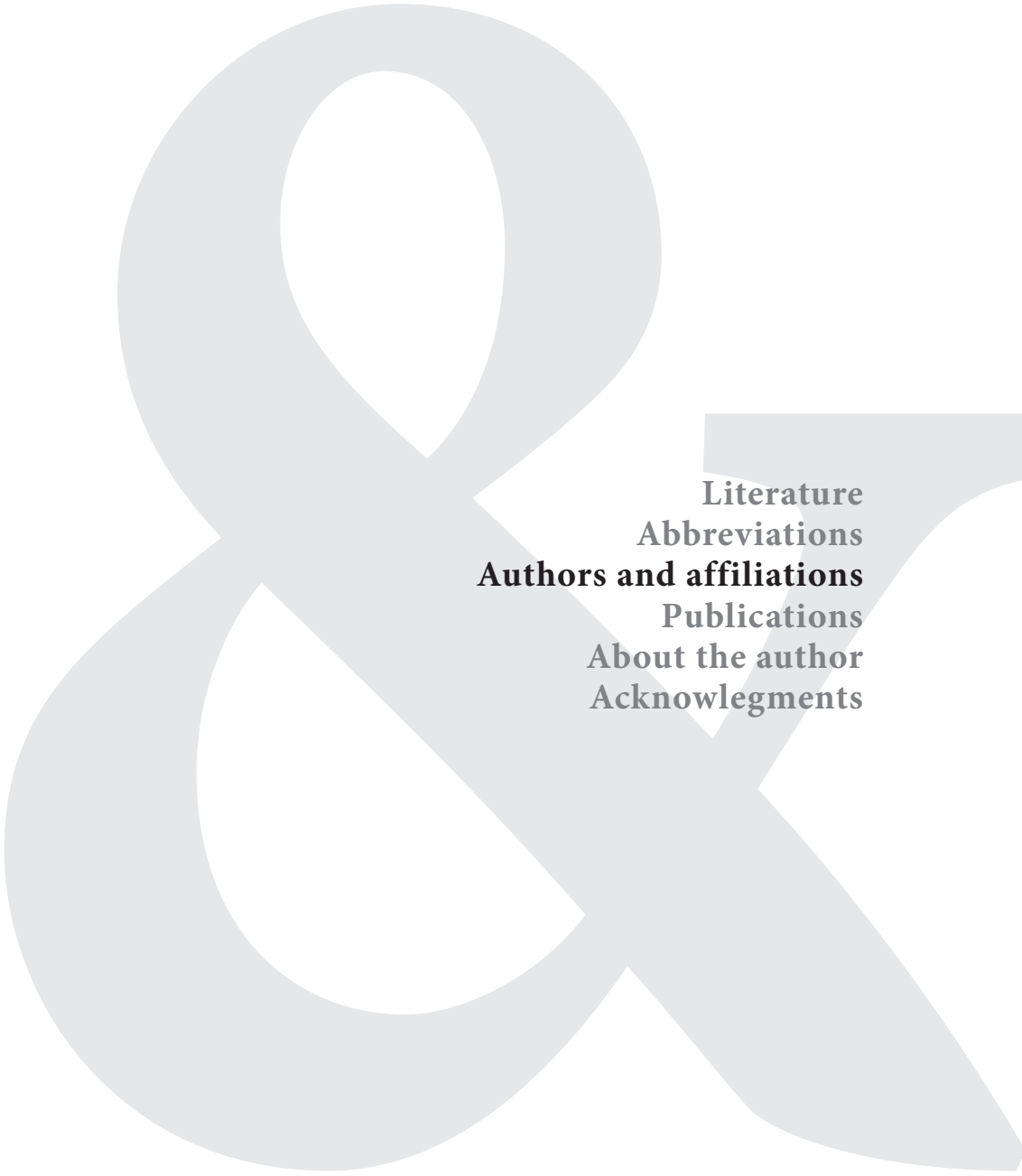


APC	antigen-presenting cell
ASCUS	atypical squamous cells of undetermined significance
CBA	cytometric bead array
CCL	chemokine ligands
CIN	cervical intraepithelial neoplasia
COPV	canine oral papilloma virus
CRPV	cotton tail rabbit papilloma virus
CTL	$CD8^+$ cytotoxic T lymphocyte
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FCS	fetal calf serum
FR	frequency of responders
HBV	hepatitis B virus
hc2	hybrid capture 2 assay
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
hrHPV	high-risk human papillomavirus
HSIL	high-grade squamous intraepithelial lesions
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Media
KC	keratinocytes
LA	linear array
LC	Langerhans cell
LEEP	loop electrosurgical excision procedure
LSIL	low-grade squamous intraepithelial lesions
LUMC	Leiden University Medical Center
MACS	magnetic cell sorting
MAdCAM	mucosal addressin cell adhesion molecule
MDA5	melanoma differentiation-associated gene 5
MRM	memory response mix
NF-kB	Nuclear Factor-KappaB
NTP	nucleotide triphosphate
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinine
PKR	protein kinase R
PRR	pattern recognition receptor
RIG-I	retinoic-acid-inducible gene I
RUL	relative light unit
SI	stimulation index
SIL	squamous intraepithelial lesion
SLP	synthetic long overlapping peptides
TCGF	T cell growth factor
Th cell	$CD4^+$ T helper cell
TLR	toll like receptor
TNF	tumor necrosis factor
Treg	$CD4^+$ regulartory T cell
VIA	visual inspection with acetic acid
VIN	vulvar intraepithelial neoplasia
VLP	virus-like particles



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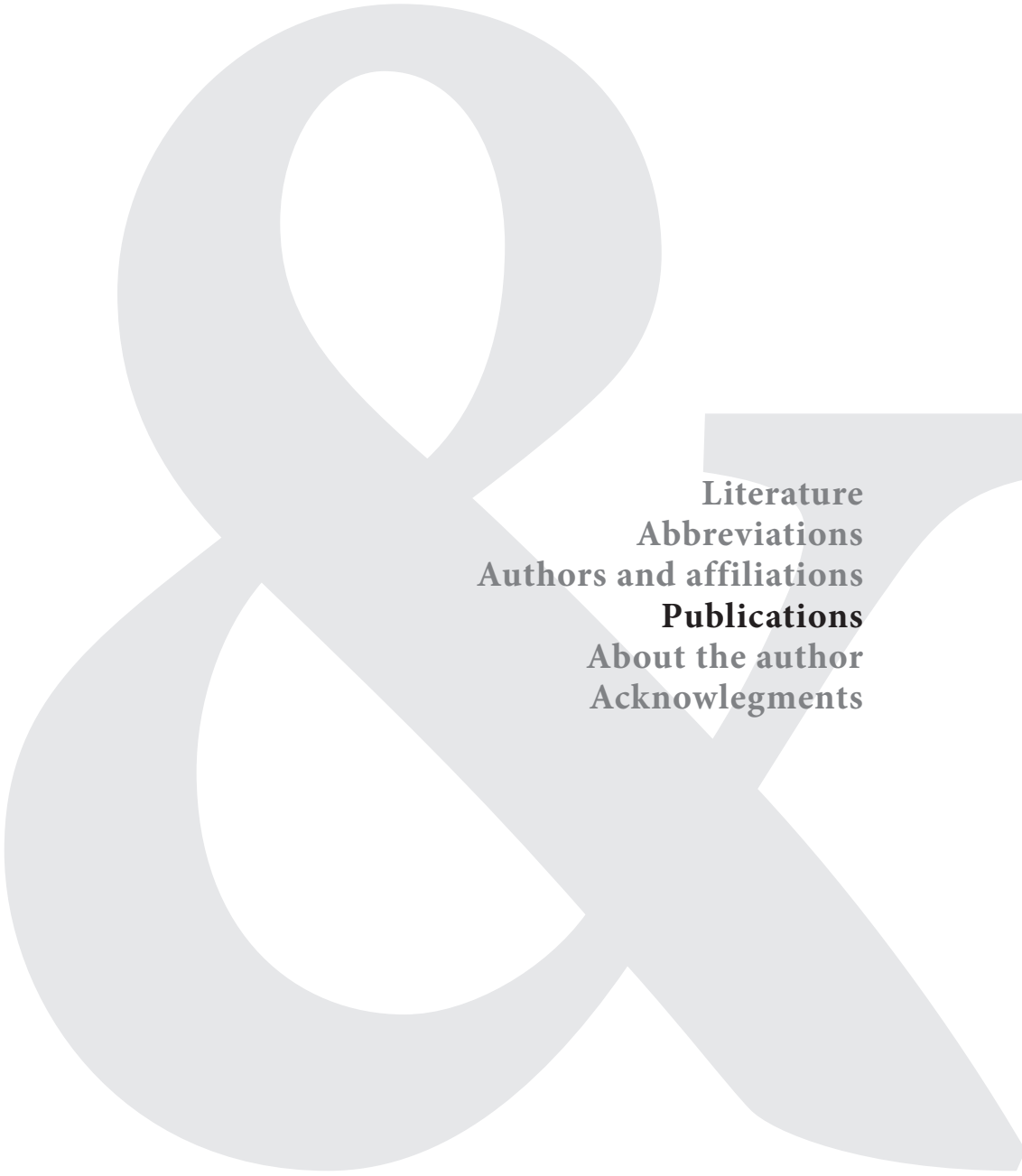

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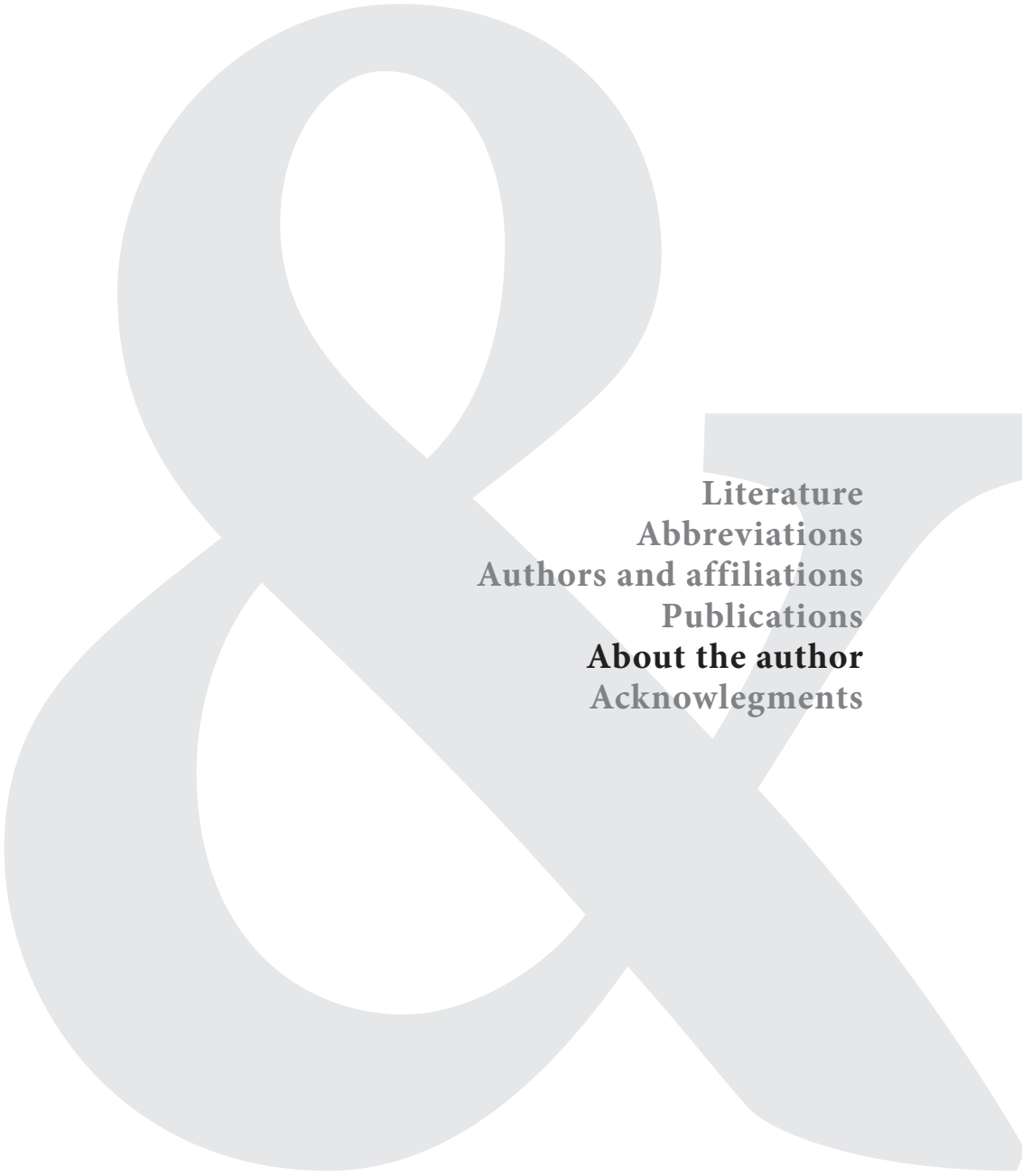

Muriel van den Hende was born on the 6th of July, 1976 in Leidschendam. She attended the gymnasium at the Erasmus College in Zoetermeer, which she finished in 1994.

From 1994 to 2000 she studied Medicine and Biomedical Sciences at the University of Leiden. A graduation project at the department of Ophthalmology resulted in her first immunologic research project at the Schepens Eye Research Institute, Harvard Medical School in Boston. Where she studied MHC class II expression in uveal melanoma under supervision of S.J. Ono, MD, PhD and Dr. M.J. Jager (dept. of Ophthalmology, LUMC).

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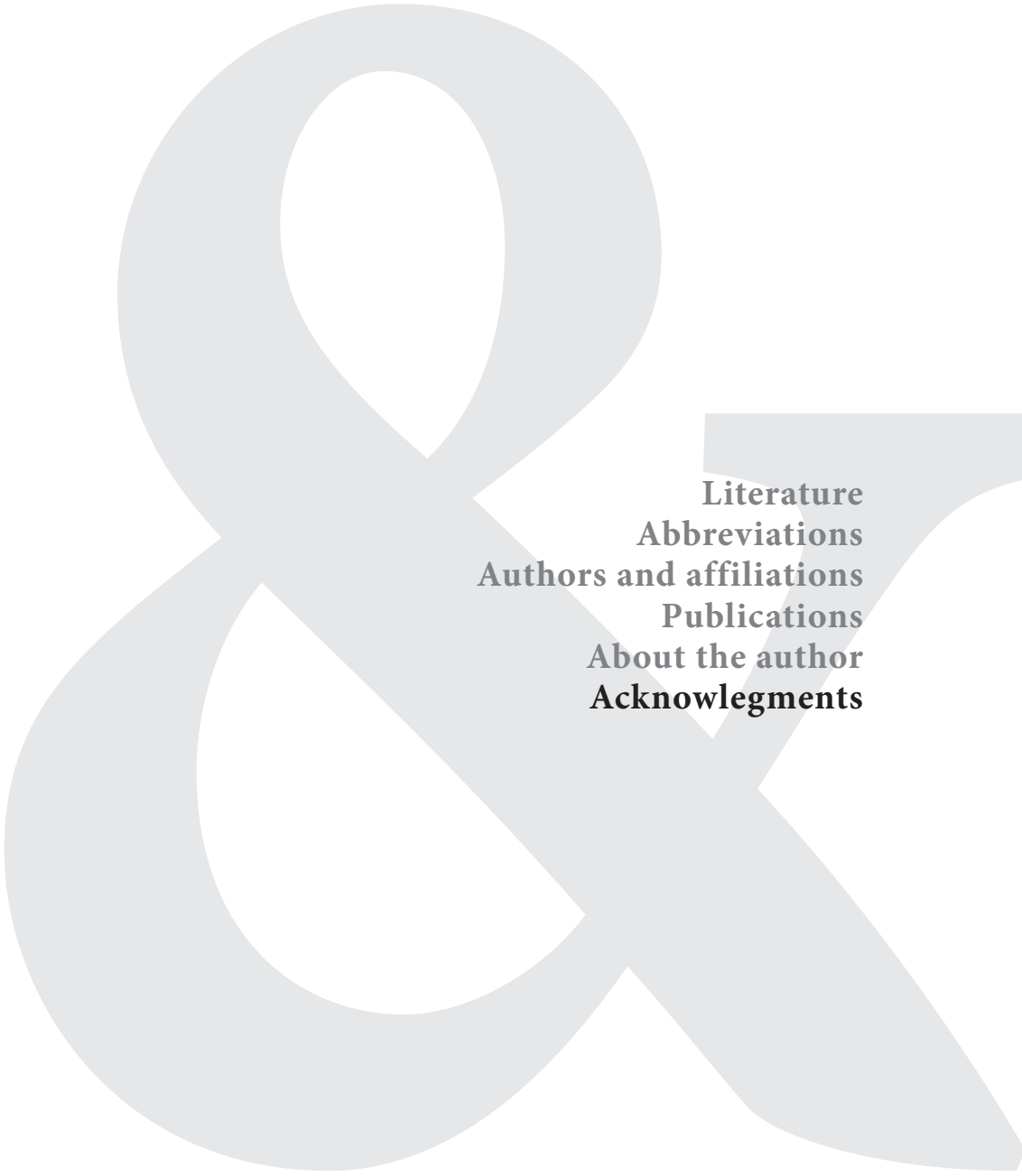

Promoveren doe je niet alleen!

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