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# 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 3$^{rd}$ 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 (6$^{th}$) 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).

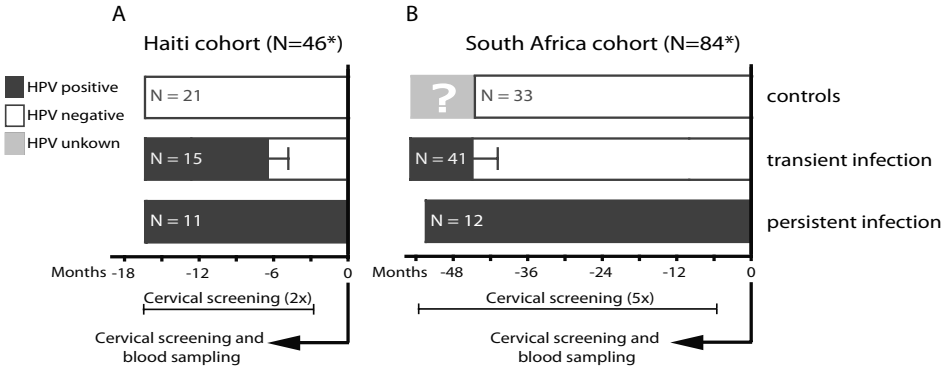


**Figure 1.** Study cohort: time frame of clinical examinations and collection of samples. Schematic overview of the Haitian (A) en South African (B) cohorts. Histograms depict the distribution of the subjects over the three subgroups: women consistently HPV negative at all visits (controls), women found 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.

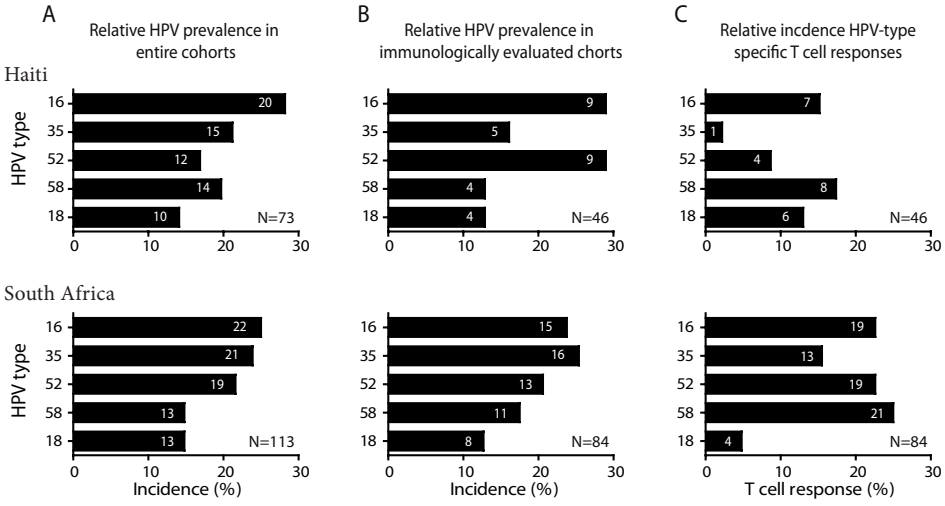


**Figure 2.** Relative frequency of infection and specific immune responses for selected HPV types in Haiti and South Africa cohorts. A. Relative prevalence of HPV types of interest (HPV 16, 35, 52, 58, 18) in the Haitian (upper, n = 73) and South African (lower; n = 113) study cohort. B. Relative prevalence 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.

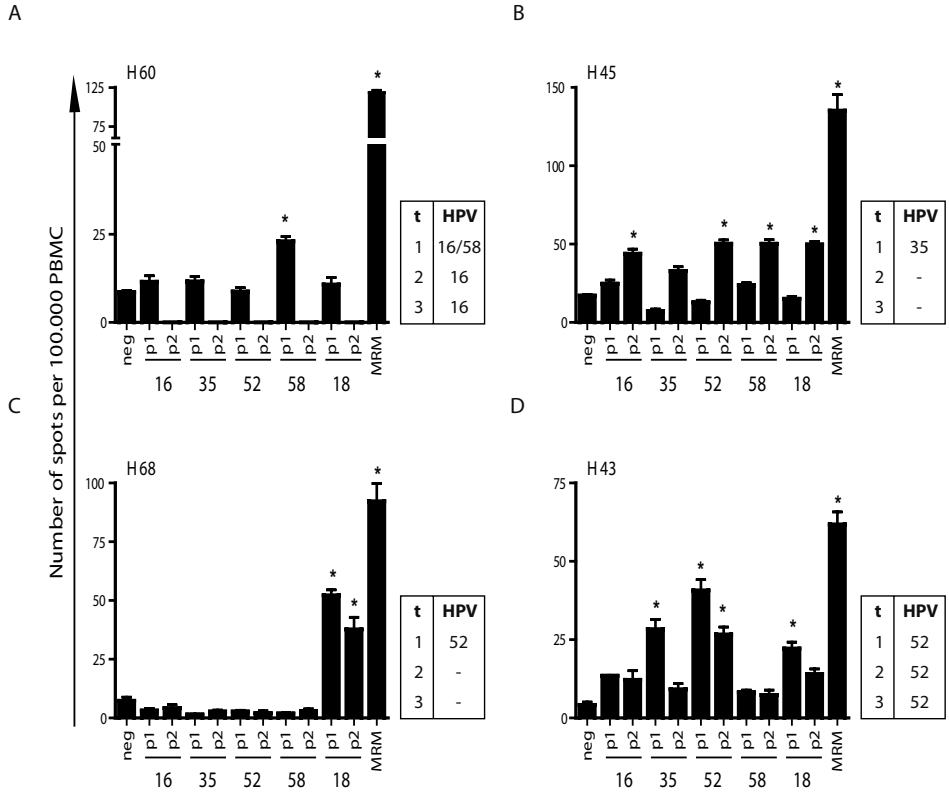


**Figure 3.** Examples of HPV E6-specific response patterns as measured by IFNγ-ELISPOT in HPV-positive subjects of the Haitian cohort. Responses as detected by IFNγ-ELISPOT analysis performed against specified peptide pools (p1, p2) per HPV type. MRM was used as a positive control. The mean number of 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.

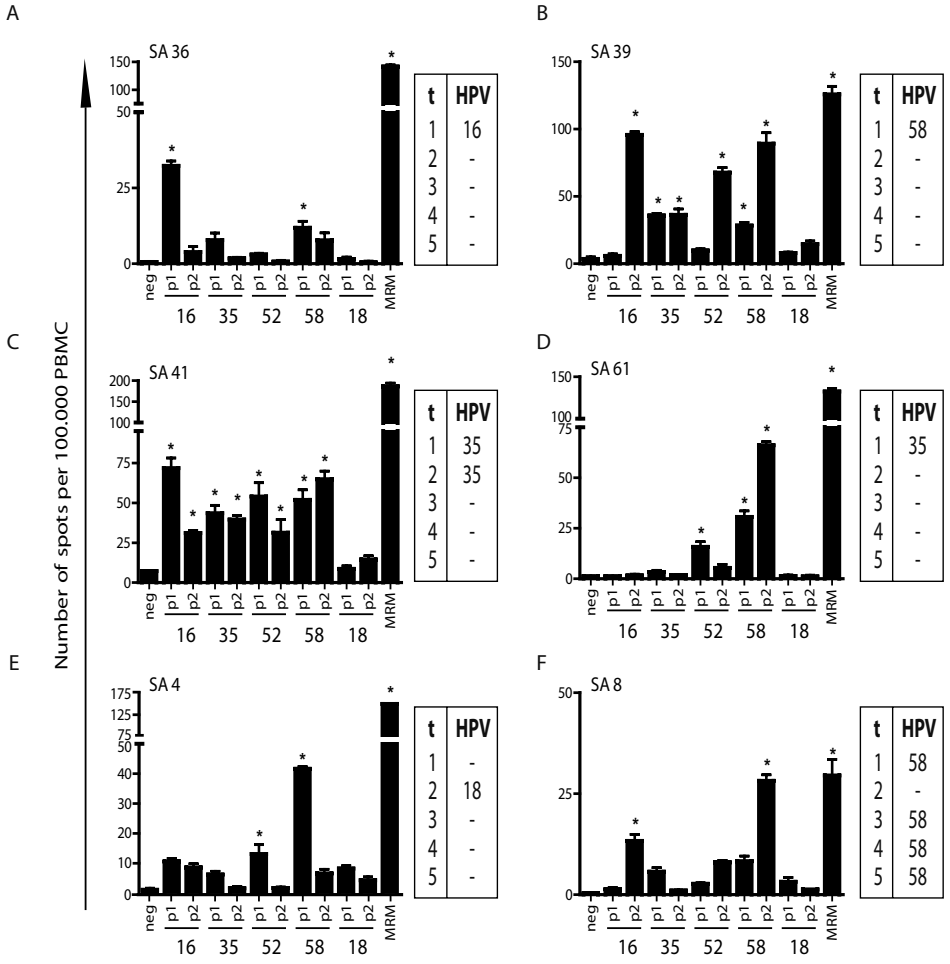


**Figure 4.** Examples of HPV E6-specific response patterns as measured by IFNγ-ELISPOT in subjects of the South African cohort. Responses as detected by IFNγ-ELISPOT analysis performed against specified peptide pools (p1, p2) per HPV type. MRM was used as a positive control. The mean numbers of spots 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

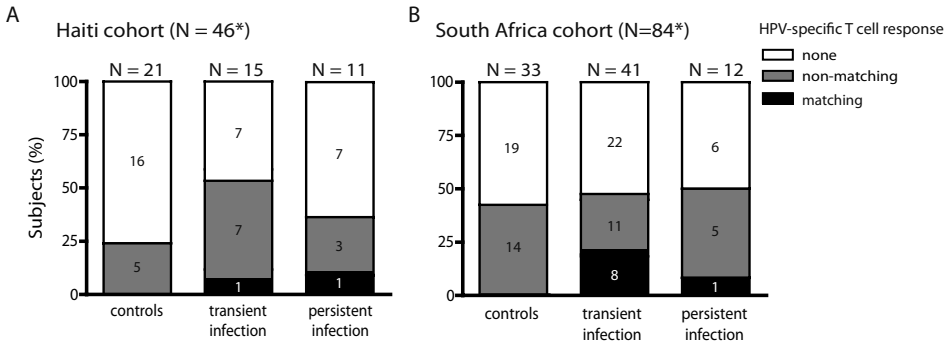


**Figure 5.** HPV E6-specific immunity in relation to virological status. Overview of the coincidence between HPV E6-specific immunity detected in PBMC samples collected at the final visit, and development of cervical HPV infections as observed through longitudinal cervical screening. For each of the 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.