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Alterations of T-cell surface markers in older women with persistent human papillomavirus infection

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Abstract

We previously reported decreased lymphocyte proliferative responses among older women with persistent human papillomavirus (HPV) infection. To characterize the phenotype of peripheral lymphocytes associated with persistent HPV infection, we evaluated the expression of different cell surface markers in peripheral blood mononuclear cells (PBMCs) from a case-control study within a 10,049-woman population-based cohort study in Guanacaste, Costa Rica. Women in the cohort aged 46 to 74 and with HPV results at their 5th year anniversary visit were considered, and all women (n=87) with persistent HPV infections, all women (n=196) with transient HPV infections and a random sample of HPV DNA-negative women (n=261) frequency-matched to cases on age were selected for this study. A median of 3 years after the case-control matching visit, cervical cells were collected for liquid-based cytology and repeat HPV DNA genotyping. Blood was obtained from which PBMCs were extracted and cryopreserved for immunological phenotyping via flow cytometry. Significant increases in risk of HPV persistence were observed for three marker subsets indicative of immune cell activation/differentiation. Relative risk estimates were 5.4 (95%CI=2.2–13.3) for $CD69^+CD4^+$, 2.6 (95%CI=1.2–5.9) for $HLADR^+CD3^+CD4^+$ and 2.3 (95%CI=1.1–4.7) for $CD45RO^+CD27^-CD8^+$. A significant decrease in HPV persistence was observed for a subset marker indicative of an immature, undifferentiated memory state $CD45RO^+CD27^+CD4^+$ (OR=0.36; 95%CI = 0.17–0.76). Adjustment for these markers only partially explained the previously reported association between decreased lymphoproliferative responses and persistent HPV infection. Whether phenotypic alterations observed predispose to HPV persistence or result from it should be the focus of future studies.

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Keywords

HPV persistent infection; T-cell distribution; T-cell activation and differentiation; older women

Introduction

Infection of the cervix with one or more of approximately 10–15 carcinogenic human papillomavirus (HPV) genotypes is very common and a necessary cause for cervical cancer [1–5]. Most cervical HPV infections clear (become undetectable by HPV DNA tests) without sequelae; cervical pre-cancer and cancer arise from the small subset of infections that persist, typically for more than 1–2 years [6–9].

The host immune response is believed to be an important determinant of the fate of incident HPV infections. Certain HLA alleles have been associated with cervical cancer [10]. Studies in HIV positive women have shown increased persistence and progression of HPV infection, with the most pronounced risk observed among women with reduced CD4 counts. This suggests that immune perturbations associated with HIV infection predispose to HPV persistence and its associated abnormalities [11]. Additional immune function alterations have been described in HPV infection, including impairment of $CD4^+$ T cell-mediated immunity and cytokine dysregulation in peripheral blood in women with cervical cancer or precancerous lesions [12–17]. At the tissue level, a lack of dendritic cells [18, 19], and decrease in Th1 immune infiltrates have been described in the presence of HPV associated lesions [20, 21].

Age-specific prevalence curves of HPV vary in different parts of the world, and in some countries a second peak of HPV infection has been described above age 50 [22–24]. The reasons for this second peak are not clear, but may include re-exposure through new partners of the woman or of her partner, reactivation of previous infections to detectable levels (re-emergence of latent HPV infections) due to previously-described senescence of the immune system or a cohort effect [11, 25–27].

We previously reported an association between HPV persistence and decreased responsiveness of peripheral blood mononuclear cells (PBMCs) to antigenic and mitogenic stimuli in women 46 – 74 years old who participated in a 10,049 women population-based cohort study in Guanacaste, Costa Rica [28]. Herein, to better characterize the peripheral immune phenotypes associated with persistent HPV infection in this same group of women, we have evaluated various T-cell subsets within $CD4^+$ and $CD8^+$ lymphocyte populations with a particular emphasis on markers of immune activation in T-cells and differentiation within naïve and memory T-cells.

Methods

Study design and population

A population-based cohort of women 18 years and older was established in Guanacaste, Costa Rica to study the natural history of human papillomavirus infection and cervical neoplasia. Between June 1993 and December 1994, 10049 women were recruited into the study. Participation rate was 93%. Sexually active women underwent a pelvic examination where exfoliated cells for conventional and liquid-based cytology and HPV DNA tests were collected. Women who had evidence of cervical abnormality were referred to colposcopy and treatment if needed. After exclusion of hysterectomized women and those with prevalent precancer (cervical intraepithelial neoplasia (CIN) grade 2 or worse) or cancer, remaining participants were eligible for the follow-up phase of the study (N=9100). During follow-up, women who presented evidence of precancer or cancer were colposcopically

evaluated, treated or referred for treatment as needed and censored. Details have been described elsewhere [29, 30].

Details of the methods employed for our case-control study among older women nested within the cohort described above have also been previously published [28]. In brief, individuals for the nested study were selected from the 7008 women enrolled in our prospective cohort and for whom PCR-based HPV DNA results from their 5^{th} – 6^{th} year of follow-up were available at the time this study was designed. All women who were between 45 and 75 years of age and positive for HPV DNA at the last visit during that period (N=324) were selected. A subset of women with negative HPV DNA result during this same period was also selected (N=310). The HPV-negative group was frequency matched to the HPV-positive group on age and time since enrollment. All women selected were invited to participate in an additional study visit that occurred on average 36.2 months (range: 12.0 – 53.4 months) after the time of the HPV DNA test that led to their selection into the nested study. At the time of the additional visit, a pelvic examination was performed, exfoliated cells were collected for liquid-based cytology and repeat PCR-based HPV DNA testing, and 40 ml of blood were collected in heparinized tubes from which PBMCs were isolated and cryopreserved.

The participation rate for the nested study was over 92% (591 of 634 women). Among participants, we excluded 7 women whose blood sample was not available for testing (5 cases and 2 controls), 3 women with missing HPV DNA results (1 case and 2 controls) and 9 women (all cases) whose cytology result at the time of the additional visit was high grade squamous intraepithelial lesion (HSIL). An additional 28 women initially in the HPV negative control group were excluded due to evidence of incident HPV infection at the time of the additional study visit. After these exclusions were applied, 283 HPV-positive cases (some persistent, some not as discussed below) and 261 HPV-negative controls remained.

The study protocol was reviewed by the National Cancer Institute and the Costa Rican INCIENSA Institutional Review Boards. All participants provided informed consent.

HPV determination by PCR

Type-specific HPV DNA testing was performed by L1 MY09/11 degenerate-primer PCR with AmpliTaq Gold polymerase, as detailed elsewhere [23, 31]. In brief, after amplification, PCR products were analyzed by electrophoresis and hybridized with radiolabeled generic probes for HPV. Samples positive for HPV DNA were typed by dot-blot hybridization with biotinylated type-specific oligonucleotide probes (2, 6, 11, 13, 16, 18, 26, 31–35, 39, 40, 42–45, 51–59, AE9 (54v), 61, 62, 64, 66–74, AE10 (74v), 81, AE2 (82v), w13b (82), 83–85, and 89).

Flow cytometry analysis

PBMCs isolated by Ficoll-Paque (Amershan Pharmacia Biotech, Piscataway, NJ) gradient centrifugation were used in this study. Detailed methods have been described elsewhere [28]. In brief, blood samples were transported to the cryopreservation laboratory in coolers at ~20°C. PBMCs were isolated and cryopreserved a median of 25.5 hours (interquartile range 1.45 hours) after specimen collection. PBMCs were cryopreserved in 20% fetal calf serum (FCS, BioWittaker) and 7.5% dimethylsulphoxide (DMSO, Sigma, St. Louis, MO)-supplemented RPMI 1640 media (BioWhittaker) using a controlled rate freezer (Kryosave, Rockville, MD). Cryopreserved PBMCs were transported and stored in the vapor phase of liquid nitrogen. Cells were thawed as previously described [28, 32] and stained for a variety of cell surface markers. Although there is still some controversy regarding models of differentiation and functional activities characteristic of each subset within $CD4^+$ and $CD8^+$

T-cells [33, 34], in general T-cells can be divided into early or late activated cells, memory or naïve cells, and as differentiated or undifferentiated cells. We defined our T-cell sub-populations based on the presence/absence of the different cell surface markers as follows: 1) $CD4^+CD69^+$ and $CD8^+CD69^+$ were defined as early activated T-cells; 2) $CD4^+CD25^+$, $CD8^+CD25^+$, $CD4^+HLA\text{-}DR^+$, $CD8^+HLA\text{-}DR^+$, $CD4^+CD38^+$ and $CD8^+CD38^+$ cells were defined as late activated T-cells; 3) $CD45RO^+CD45RA^-$ cells were defined as memory T-cells; 4) $CD45RO^-CD45RA^+$ cells were defined as naïve T-cells; 5) $CD27^+CD45RO^+$ and $CD27^+CD45RA^+$ cells were defined as resting memory cells and differentiated naïve cells, respectively; 6) $CD57^+CD45RO^+$ and $CD57^+CD45RA^+$ cells were defined as differentiated memory cells and undifferentiated naïve cells, respectively; and 7) $CCR7^+CD45RO^+$ and $CCR7^+CD45RA^+$ cells were defined as central-memory and -naïve cells, respectively; and 8) $CCR7^-CD45RO^+$ and $CCR7^-CD45RA^+$ cells were defined as effector-memory and -naïve cells, respectively.

PBMC (2×10^5 cells/ml) were stained per manufacturer's recommendations for 15 minutes at room temperature in the dark. 1 mL of lysing solution (154 mM Ammonium chloride; 10 mM potassium bicarbonate; 0.12 mM EDTA; pH 7.4) was then added. After 5 minutes, cells were washed two times in fluorescence-activated cell sorting (FACS) buffer (0.7% bovine serum albumin and 0.01% sodium azide in PBS) and analyzed within 6 hours in a five-color flow cytometry on a FC-500 flow cytometer (Beckman Coulter). CD69 and CD25 markers in $CD4^+$ and $CD8^+$ T-cells are reported as percentages of the total number of lymphocytes, with the lymphocyte population selected based on the forward scatter/side scatter pattern. The CD45RO, CD45RA, CD27, CCR7 and CD57 markers are reported as percentages of $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes. HLA-DR and CD38 marker populations were estimated as percentage of the $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes. The CD57 subpopulation was estimated as a percentage of $CD3^+CD4^+$ or $CD3^+CD8^+$ lymphocytes that were $CD27^+CD45RA^+$ or $CD27^+CD45RO^+$.

During flow cytometric analysis, we excluded results from consideration if the observed number of events (cell count) was below 5,000. Data were pre-checked before statistical analysis to assess tube-to-tube reproducibility. Repeated measurements among tubes for the same subject were compared, and these values needed to be within 5% of each other to be accepted. Additionally, the lymphosum was calculated by adding the percentage of T-cells, B-cells and NK-cells. This value needed to be 100±5%. If variation was higher for any of these checks, all data from this sample were excluded from analysis. Also, samples in a particular batch were excluded if an anomalous cell population was observed in some of the batch tubes. For most markers, observations from ≤ 25 women were excluded due to these technical reasons.

Lymphoproliferation assays

Lymphoproliferation responses were assessed as previously published [28]. Briefly, PBMCs were cultured in triplicate in the presence of AIM-V media as a negative control; HPV-16 L1 VLP (Novavax, Malvern, PA) or influenza A virus (Flu) (Infectious virus, H3N2, A/Hong Kong/8/68, 1:100, ATCC) as recall antigens; or phytohemagglutinin (PHA) (1:100, Sigma) as mitogen for a total of 5 and 3 days, respectively. 1 μCi of [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ) was added for 18 hours before harvesting and counted in an automated scintillation counter (Microbeta, Perkin-Elmer, Boston, MA).

Statistical analysis

For this analysis, the initial group of participants who were HPV DNA positive at the time of their follow-up visit was subdivided into two case groups. Those who had at least one type-specific HPV infection still present at the time of their additional visit for the present

study (HPV Persistence Group; n = 87, median persistence time = 42 months) and those who cleared all of their type-specific HPV infections by the time of their additional visit (HPV Clearance Group; n = 196).

These groups were compared against the HPV negative group (HPV Negative Group; n = 261) for the main analysis. The HPV negative group included those women with negative HPV results at selection and the additional visit. This group was selected as the control/comparison group since it is representative of women 46 – 74 years old from our population-based study. Given how common HPV infection is among sexually active individuals, we postulate that the majority of these women have been exposed to HPV and thus had the opportunity to develop persistent HPV infection (but did not). We also compared the HPV Persistence Group against the HPV Clearance Group as an ancillary analysis.

For some analyses, the HPV persistence group was further restricted to women with evidence of long term persistence. We considered women to be long-term HPV-DNA persistent if the same HPV type was detected since enrollment into the cohort in 1993–4 (n = 32; median persistence time of 107.4 months). Of note, only 13 women had more than one type-specific HPV-DNA persistent infection; of these 4 had long-term infections.

For the specific T-cell subsets evaluated (Tables 1–2), the percentage of cells expressing each subset of markers was computed for each woman. Categorical quartile variables were derived based on the frequency distribution observed among the study population with valid results for the specific subset. The Kruskal-Wallis test, commonly used to determine whether the mean rank distribution within ordered groups differ between population samples, was used to test whether for each T-cell subset there were differences in the distribution of the categorical quartile variables between the study groups (e.g., HPV negative and persistent HPV positive groups). When correlation between markers was evaluated, the non-parametric Spearman's coefficient was used.

Logistic regression was used to estimate the odds ratios (OR) and 95% confidence intervals (95% CI) for the quartile variables while adjusting for age and other parameters as specified in the individual tables. To test for trend, categorical variables were considered as continuous in the models. In addition to the overall analyses presented in the tables, analyses were performed stratified by age at the time of the additional visit (<55, 55–64 and 65–74 years) and restricting the case group to women with carcinogenic HPV types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). When evaluating the impact of adjustment for immunophenotype subsets on the previously described association between proliferative potential and HPV persistence [28], we created a joint variable that combined across the four immunophenotype subsets found to be significantly associated with HPV persistence in our study. This joint variable was defined as the sum of the individual quartile levels for each of the four phenotypic markers of interest (e.g., women in the lowest quartile for all four markers had a combined score of four). Quartile order was reversed for $CD45RO^+CD27^+CD4^+$ to match risk for HPV persistence with the other markers. Based on the score, women were categorized into tertile groups (4 – 8, 9 – 10 and 11 – 16).

Results

As previously reported [28], the distribution of $CD3^+$ T-cells and $CD4^+$ and $CD8^+$ T-cell subsets within $CD3^+$ T-cells was similar across the three study groups: HPV negative, HPV persistence, and HPV clearance groups (Tables 1–2 and data not shown). When immunophenotypic subsets of $CD3^+CD4^+$ cells were evaluated and the frequency distributions compared between the HPV persistence group and the HPV negative controls (Table 1), statistically significant differences in the distributions of the respective marker

were observed with increases in the median levels among the persistence group for the following activation $CD3^+CD4^+$ T-cell subsets: $CD69^+CD4^+$, $HLADR^+CD3^+CD4^+$, $CD38^+HLADR^+CD3^+CD4^+$ and $CD38^-HLADR^+CD3^+CD4^+$. In addition, a statistically significant difference in the distribution was observed for the $CD45RO^+CD27^+CD4^+$ subset, a marker of undifferentiated memory cells with a decrease in the median level among the persistence group. Significant differences were also observed when the HPV persistence group was compared to the HPV clearance group for the same markers with median levels similarly increased or decreased as when compared to the HPV negative group (data not shown). No significant differences for any of the markers analyzed were observed when the HPV clearance group was compared to HPV negative controls (data not shown).

Next, we compared immunophenotypic subsets of $CD3^+CD8^+$ cells (Table 2). The only statistically significant difference noted between the HPV persistence and HPV negative group was for the mean rank distribution of the $CD45RO^+CD27^-CD8^+$ cell population, a marker of differentiated memory cells with an increase in the median level for the persistent HPV positive group. A higher median value of $CD45RO^+CD27^-CD8^+$ was also observed for the persistent HPV positive group when compared with the HPV clearance group with a significantly different quartile distribution (data not shown). No significant differences were observed when the HPV clearance group was compared to HPV negative controls (data not shown).

Associations between risk of HPV persistence and increasing percentages of specific subsets within $CD3^+CD4+$ and $CD3^+CD8+$ are presented in Tables 3 and 4, respectively. These analyses were restricted to the subsets whose population's mean rank distribution differed significantly between the HPV persistence and the HPV negative control groups. Although the distribution difference was significant for $CD38^+HLADR^+CD3^+CD4^+$ and $CD38^-HLADR^+CD3^+CD4^+$, these two subsets were not evaluated further since their observed effects were driven by the main effect seen for $HLADR^+CD3^+CD4^+$ (correlation between these two subsets and $HLADR^+CD3^+CD4^+$ was 0.53 and 0.96, respectively).

Consistent with findings based on the T-cell markers population distributions, a statistically significant trend in risk for HPV persistence was observed among women with increasing quartile categories when compared with women in the lowest quartile for the following markers of immune activation and/or differentiation after adjusting for age $CD4^+CD69^+$ (p-trend < 0.001; 4^{th} quartile OR = 5.4, 95% CI 2.2 – 13.3), $HLADR^+CD3^+CD4^+$ (p-trend = 0.04; 4^{th} quartile OR = 2.6, 95% CI = 1.2 – 5.9), and $CD45RO^+CD27^-CD8^+$ (p-trend = 0.01; 4^{th} quartile OR = 2.3, 95% CI 1.1 – 4.7;). Conversely, a decreased risk for HPV persistence was observed among women in the highest quartiles for the following marker of undifferentiated memory cells $CD45RO^+CD27^+CD4^+$ (p-trend = 0.006; 4^{th} quartile OR = 0.36, 95% CI 0.17 – 0.76). Effects of stronger magnitude were observed in analyses that restricted the case group to long term persistors (Tables 3 and 4). Similar effects were observed in analyses that evaluated persistence with carcinogenic HPV types (data not shown). We observed no evidence that the effects differed by age (45–54, 55–64, 65–74) (data not shown).

Next, we evaluated whether our previously reported association between proliferative responses and risk of HPV persistence could be explained by differences in the activation/differentiation state of $CD3^+CD4^+$ or $CD3^+CD8^+$ subsets (Table 5). Adjustment of the effects seen for proliferative responses against PHA and HPV-16 VLP for levels of the T-cell markers found to be associated with persistence ($CD69^+CD4^+$, $HLADR^+CD3^+CD4^+$, $CD45RO^+CD27^+CD4^+$, and $CD45RO^+CD27^-CD8^+$) only slightly attenuated effects (data not shown). A modest attenuation in the odds ratios was observed when we adjusted proliferative responses for the joint marker variable that combined across the four subsets

significantly associated with risk of persistence (Table 5. A). The age-adjusted OR comparing low to high responders to PHA was reduced from 2.7 (95% CI = 1.4 – 5.1) to 1.9 (95% CI = 0.94 – 3.7). Similarly, the age-adjusted OR comparing low to high responders to HPV-16 VLP was reduced from 2.7 (95% CI = 1.4 – 5.3) to 1.9 (95% CI = 0.90 – 3.8). Adjustment of the immunophenotype effects for proliferative responses to PHA or HPV-16 VLP had a minor effect on the magnitude of the immunophenotype associatiosn with HPV persistence (Table 5. B). Finally, although limited by study size, we observed no evidence that the proliferation effects differed by levels of specific immunophenotypic markers or vice versa (i.e., no evidence for effect modification) (data not shown).

Discussion

Here we demonstrate that HPV persistence in older women is associated with peripheral phenotypic alterations of T-cells, in particular an increase in activated CD4 T-cells expressing CD69 or HLADR and an enrichment in differentiated memory $CD45RO^{+}CD27^{-}CD8^{+}$ T-cells. We observed differences in the distribution of peripheral T-cell subsets among older women with persistent HPV infections when compared to women of the same age who were not HPV infected. The three subsets found to be positively associated with persistence ($CD4^{+}CD69^{+}$, $HLADR^{+}CD3^{+}CD4^{+}$ and $CD45RO^{+}CD27^{-}CD8^{+}$) were indicators of cellular activation and/or differentiation, whereas the single subset found to be negatively associated with persistence ($CD45RO^{+}CD27^{+}CD4^{+}$) was an indicator of an undifferentiated state of memory CD4 T-cells. This suggests the possibility that chronic HPV infection is associated with an activation of lymphocyte subsets at the periphery that appears to be unsuccessful at eliminating the virus, as previously suggested by findings of increased IL-2 production among a small number of women with persistent HPV infection and normal cytology [35, 36]. Alterations in peripheral subsets of T-cell activation and/or memory differentiation have been reported in the context of other infections such as HIV, dengue and CMV [33, 34, 37–39] as well as other chronic inflammatory conditions [40, 41]. The magnitude of overall changes of the medians were modest and typically within the expected normal range, suggesting that subtle changes in the distribution of immune cells may have important effects on the ability of the host to handle HPV infections. The underlying mechanisms for activation are unknown and deserve further study.

In a previous report from this same population, we observed an association of decreased PBMCs responses to mitogenic and antigenic stimuli and HPV persistence [28]. Together with the present observation of an association between markers of lymphocyte activation/differentiation and HPV persistence, we hypothesize that chronic HPV infections, although local, are associated with activation and differentiation of peripheral subsets of T-cells, which is concomitant with an inability to respond *in vitro* to mitogenic or antigenic stimulation. Because the biological specimens used for immune assessment in the present study were collected at the end of the study (i.e., after persistence was established), it was not possible to determine whether immunological dysregulation leads to a predisposition to HPV persistence or whether HPV persistence induces the observed immunological changes. Future follow-up studies will be required to address whether HPV persistence is cause or a consequence of immune dysregulation.

Association between reduced proliferation and HPV persistence was only slightly attenuated after control for the distribution of T-cell immunophenotypes, suggesting that the T-cell phenotype changes cannot completely explain the previously observed proliferative effect. Similarly, the associations of phenotype changes with HPV persistence reported herein could not be completely explained by the changes in proliferative responses observed with persistent HPV infection. These data suggest the need for further studies to understand

underlying mechanisms of immune dysregulation associated with HPV persistence, such as role of immunoregulatory mechanisms involving regulatory T-cells [42] and of alterations at the level of antigen-presenting cells.

To the best of our knowledge this is the largest study to evaluate the distribution in peripheral blood of immunophenotypic lymphocyte subpopulations among women with different states of HPV infection without precancerous lesions and to show a dose response relationship between immunophenotypic T-cell distribution and risk of HPV persistence. Despite being the largest study of its kind to date, caution should be observed when interpreting our results given the multiple comparisons that were made and the possibility of false positive findings. There is a need for replication of our findings before they can be considered definitive. However, it is reassuring to note that all of the significant associations we observed pointed to a similar biological mechanism (an increase in the proportion of cells that were activated/differentiated), increasing the likelihood that the findings are real rather than false positive findings.

The small number of co-infections with other HPV types or other documented sexually transmitted diseases did not allow us to study co-infection as an effect modifier or confounder. As mentioned above, another limitation of the study is the collection of PBMCs after women had persisting HPV infections; therefore we are not able to disentangle the temporal relationship between immunological responses and HPV persistence. Finally, it should be noted that the present study could not evaluate the potential role of immune phenotype alterations on risk of progression to disease; additional studies that address this question would be of interest in the future.

In summary, markers of activation and/or differentiation of T-cells, mostly within the CD4 T-cells, are associated with HPV persistence. Our study suggests that a localized cervical infection is associated with peripheral T-cell phenotypic alterations. Future studies are needed to define the timing of these immune alterations and their role in disease progression.

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Abbreviations

HPV	Human papillomavirus
PBMCs	Peripheral blood mononuclear cells
CIN	Cervical intraepithelial neoplasia
HSIL	High grade squamous intraepithelial lesion

OR	Odds ratio
FACS	Fluorescence-activated cell sorting

References

1. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. Journal of clinical pathology. 2002; 55:244–65. [PubMed: 11919208]
2. Ho GY, Bierman R, Beardsley L, Chang CJ, Burk RD. Natural history of cervicovaginal papillomavirus infection in young women. The New England journal of medicine. 1998; 338:423–8. [PubMed: 9459645]
3. Richardson H, Kelsall G, Tellier P, Voyer H, Abrahamowicz M, Ferenczy A, Coutlee F, Franco EL. The natural history of type-specific human papillomavirus infections in female university students. Cancer Epidemiol Biomarkers Prev. 2003; 12:485–90. [PubMed: 12814991]
4. Kjaer SK, Chackerian B, van den Brule AJ, Svare EI, Paull G, Walbomers JM, Schiller JT, Bock JE, Sherman ME, Lowy DR, Meijer CL. High-risk human papillomavirus is sexually transmitted: evidence from a follow-up study of virgins starting sexual activity (intercourse). Cancer Epidemiol Biomarkers Prev. 2001; 10:101–6. [PubMed: 11219765]
5. Collins S, Mazloomzadeh S, Winter H, Blomfield P, Bailey A, Young LS, Woodman CB. High incidence of cervical human papillomavirus infection in women during their first sexual relationship. Bjog. 2002; 109:96–8. [PubMed: 11845815]
6. Plummer M, Schiffman M, Castle PE, Maucort-Boulch D, Wheeler CM. A 2-Year Prospective Study of Human Papillomavirus Persistence among Women with a Cytological Diagnosis of Atypical Squamous Cells of Undetermined Significance or Low-Grade Squamous Intraepithelial Lesion. The Journal of infectious diseases. 2007; 195:1582–9. [PubMed: 17471427]
7. Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee SK, Kuypers JM, Koutsky LA. Development and duration of human papillomavirus lesions, after initial infection. The Journal of infectious diseases. 2005; 191:731–8. [PubMed: 15688287]
8. Rodriguez AC, Burk R, Herrero R, Hildesheim A, Bratti C, Sherman ME, Solomon D, Guillen D, Alfaro M, Viscidi R, Morales J, Hutchinson M, et al. The Natural History of Human Papillomavirus Infection and Cervical Intraepithelial Neoplasia Among Young Women in the Guanacaste Cohort Shortly After Initiation of Sexual Life. Sexually transmitted diseases. 2007
9. Wright TC Jr, Schiffman M. Adding a test for human papillomavirus DNA to cervical-cancer screening. The New England journal of medicine. 2003; 348:489–90. [PubMed: 12571255]
10. Hildesheim A, Wang SS. Host and viral genetics and risk of cervical cancer: a review. Virus research. 2002; 89:229–40. [PubMed: 12445662]
11. Strickler HD, Burk RD, Fazzari M, Anastos K, Minkoff H, Massad LS, Hall C, Bacon M, Levine AM, Watts DH, Silverberg MJ, Xue X, et al. Natural history and possible reactivation of human papillomavirus in human immunodeficiency virus-positive women. Journal of the National Cancer Institute. 2005; 97:577–86. [PubMed: 15840880]
12. Hildesheim A, Schiffman MH, Tsukui T, Swanson CA, Lucci J 3rd, Scott DR, Glass AG, Rush BB, Lorincz AT, Corrigan A, Burk RD, Helgesen K, et al. Immune activation in cervical neoplasia: cross-sectional association between plasma soluble interleukin 2 receptor levels and disease. Cancer Epidemiol Biomarkers Prev. 1997; 6:807–13. [PubMed: 9332763]
13. Clerici M, Merola M, Ferrario E, Trabattoni D, Villa ML, Stefanon B, Venzon DJ, Shearer GM, De Palo G, Clerici E. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. Journal of the National Cancer Institute. 1997; 89:245–50. [PubMed: 9017005]
14. Tsukui T, Hildesheim A, Schiffman MH, Lucci J 3rd, Contois D, Lawler P, Rush BB, Lorincz AT, Corrigan A, Burk RD, Qu W, Marshall MA, et al. Interleukin 2 production in vitro by peripheral lymphocytes in response to human papillomavirus-derived peptides: correlation with cervical pathology. Cancer research. 1996; 56:3967–74. [PubMed: 8752165]

15. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, Kenter G, Offringa R, van der Burg SH. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer research. 2004; 64:5449–55. [PubMed: 15289354]

16. Kadish AS, Timmins P, Wang Y, Ho GY, Burk RD, Ketz J, He W, Romney SL, Johnson A, Angeletti R, Abadi M. Regression of cervical intraepithelial neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide. Cancer Epidemiol Biomarkers Prev. 2002; 11:483–8. [PubMed: 12010863]

17. Bais AG, Beckmann I, Lindemans J, Ewing PC, Meijer CJ, Snijders PJ, Helmerhorst TJ. A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions. Journal of clinical pathology. 2005; 58:1096–100. [PubMed: 16189158]

18. Giannini SL, Hubert P, Doyen J, Boniver J, Delvenne P. Influence of the mucosal epithelium microenvironment on Langerhans cells: implications for the development of squamous intraepithelial lesions of the cervix. International journal of cancer. 2002; 97:654–9.

19. Jimenez-Flores R, Mendez-Cruz R, Ojeda-Ortiz J, Munoz-Molina R, Balderas-Carrillo O, de la Luz Diaz-Soberanes M, Lebecque S, Saeland S, Daneri-Navarro A, Garcia-Carranca A, Ullrich SE, Flores-Romo L. High-risk human papilloma virus infection decreases the frequency of dendritic Langerhans' cells in the human female genital tract. Immunology. 2006; 117:220–8. [PubMed: 16423058]

20. al-Saleh W, Giannini SL, Jacobs N, Moutschen M, Doyen J, Boniver J, Delvenne P. Correlation of T-helper secretory differentiation and types of antigen-presenting cells in squamous intraepithelial lesions of the uterine cervix. The Journal of pathology. 1998; 184:283–90. [PubMed: 9614381]

21. de Gruijl TD, Bontkes HJ, van den Muysenberg AJ, van Oostveen JW, Stukart MJ, Verheijen RH, van der Vange N, Snijders PJ, Meijer CJ, Walboomers JM, Scheper RJ. Differences in cytokine mRNA profiles between premalignant and malignant lesions of the uterine cervix. Eur J Cancer. 1999; 35:490–7. [PubMed: 10448305]

22. Franceschi S, Herrero R, Clifford GM, Snijders PJ, Arslan A, Anh PT, Bosch FX, Ferreccio C, Hieu NT, Lazcano-Ponce E, Matos E, Molano M, et al. Variations in the age-specific curves of human papillomavirus prevalence in women worldwide. International journal of cancer. 2006; 119:2677–84.

23. Herrero R, Castle PE, Schiffman M, Bratti MC, Hildesheim A, Morales J, Alfaro M, Sherman ME, Wacholder S, Chen S, Rodriguez AC, Burk RD. Epidemiologic profile of type-specific human papillomavirus infection and cervical neoplasia in Guanacaste, Costa Rica. The Journal of infectious diseases. 2005; 191:1796–807. [PubMed: 15871111]

24. de Sanjose S, Diaz M, Castellsague X, Clifford G, Bruni L, Munoz N, Bosch FX. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis. 2007; 7:453–9. [PubMed: 17597569]

25. Castle PE, Schiffman M, Herrero R, Hildesheim A, Rodriguez AC, Bratti MC, Sherman ME, Wacholder S, Tarone R, Burk RD. A prospective study of age trends in cervical human papillomavirus acquisition and persistence in Guanacaste, Costa Rica. The Journal of infectious diseases. 2005; 191:1808–16. [PubMed: 15871112]

26. Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi JY, Craft J. Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. J Immunol. 2004; 173:673–81. [PubMed: 15210831]

27. Hong MS, Dan JM, Choi JY, Kang I. Age-associated changes in the frequency of naive, memory and effector CD8+ T cells. Mechanisms of ageing and development. 2004; 125:615–8. [PubMed: 15491679]

28. Garcia-Pineres AJ, Hildesheim A, Herrero R, Trivett M, Williams M, Atmetlla I, Ramirez M, Villegas M, Schiffman M, Rodriguez AC, Burk RD, Hildesheim M, et al. Persistent human papillomavirus infection is associated with a generalized decrease in immune responsiveness in older women. Cancer research. 2006; 66:11070–6. [PubMed: 17108147]

29. Herrero R, Schiffman MH, Bratti C, Hildesheim A, Balmaceda I, Sherman ME, Greenberg M, Cardenas F, Gomez V, Helgesen K, Morales J, Hutchinson M, et al. Design and methods of a population-based natural history study of cervical neoplasia in a rural province of Costa Rica: the

Guanacaste Project. Revista panamericana de salud publica = Pan American journal of public health. 1997; 1:362–75. [PubMed: 9180057]

30. Bratti MC, Rodriguez AC, Schiffman M, Hildesheim A, Morales J, Alfaro M, Guillen D, Hutchinson M, Sherman ME, Eklund C, Schussler J, Buckland J, et al. Description of a seven-year prospective study of human papillomavirus infection and cervical neoplasia among 10000 women in Guanacaste, Costa Rica. Revista panamericana de salud publica = Pan American journal of public health. 2004; 15:75–89. [PubMed: 15030652]
31. Castle PE, Schiffman M, Gravitt PE, Kendall H, Fishman S, Dong H, Hildesheim A, Herrero R, Bratti MC, Sherman ME, Lorincz A, Schussler JE, et al. Comparisons of HPV DNA detection by MY09/11 PCR methods. Journal of medical virology. 2002; 68:417–23. [PubMed: 12226831]
32. Garcia-Pineres AJ, Hildesheim A, Williams M, Trivett M, Strobl S, Pinto LA. DNAse treatment following thawing of cryopreserved PBMC is a procedure suitable for lymphocyte functional studies. Journal of immunological methods. 2006; 313:209–13. [PubMed: 16737707]
33. Fletcher JM, Vukmanovic-Stejic M, Dunne PJ, Birch KE, Cook JE, Jackson SE, Salmon M, Rustin MH, Akbar AN. Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion. J Immunol. 2005; 175:8218–25. [PubMed: 16339561]
34. Green S, Pichyangkul S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Nisalak A, Kurane I, Rothman AL, Ennis FA. Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. The Journal of infectious diseases. 1999; 180:1429–35. [PubMed: 10515800]
35. de Gruijl TD, Bontkes HJ, Walboomers JM, Stukart MJ, Doekhie FS, Remmink AJ, Helmerhorst TJ, Verheijen RH, Duggan-Keen MF, Stern PL, Meijer CJ, Scheper RJ. Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study. Cancer research. 1998; 58:1700–6. [PubMed: 9563486]
36. de Gruijl TD, Bontkes HJ, Walboomers JM, Coursaget P, Stukart MJ, Dupuy C, Kueter E, Verheijen RH, Helmerhorst TJ, Duggan-Keen MF, Stern PL, Meijer CJ, et al. Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia. I. Differential T-helper and IgG responses in relation to HPV infection and disease outcome. The Journal of general virology. 1999; 80 (Pt 2):399–408. [PubMed: 10073700]
37. Miles DJ, van der Sande M, Jeffries D, Kaye S, Ismaili J, Ojuola O, Sanneh M, Touray ES, Waight P, Rowland-Jones S, Whittle H, Marchant A. Cytomegalovirus infection in Gambian infants leads to profound CD8 T-cell differentiation. Journal of virology. 2007; 81:5766–76. [PubMed: 17376923]
38. Pourgheysari B, Khan N, Best D, Bruton R, Nayak L, Moss PA. The cytomegalovirus-specific CD4+ T-cell response expands with age and markedly alters the CD4+ T-cell repertoire. Journal of virology. 2007; 81:7759–65. [PubMed: 17409149]
39. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nature medicine. 2002; 8:379–85.
40. Fritsch RD, Shen X, Illei GG, Yarboro CH, Prussin C, Hathcock KS, Hodes RJ, Lipsky PE. Abnormal differentiation of memory T cells in systemic lupus erythematosus. Arthritis and rheumatism. 2006; 54:2184–97. [PubMed: 16802356]
41. Petersen LD, Duinkerken G, Bruining GJ, van Lier RA, de Vries RR, Roep BO. Increased numbers of in vivo activated T cells in patients with recent onset insulin-dependent diabetes mellitus. Journal of autoimmunity. 1996; 9:731–7. [PubMed: 9115575]
42. Visser J, Nijman HW, Hoogenboom BN, Jager P, van Baarle D, Schuuring E, Abdulahad W, Miedema F, van der Zee AG, Daemen T. Frequencies and role of regulatory T cells in patients with (pre)malignant cervical neoplasia. Clinical and experimental immunology. 2007; 150:199–209. [PubMed: 17937675]

Table 1

CD4 Tcell immunophenotypic subgroups distribution by study group

Marker	HPV negative group‡		Persistent HPV positive group‡		
	N	Median %	N	Median %	p*
$CD3^+CD4^{+\dagger}$	250	39.7	81	37.5	=0.32
$CD69^+CD4^{+\dagger}$	254	0.09	80	0.22	**<0.001**
$CD25^+CD4^{+\dagger}$	251	3.4	81	3.6	=0.74
$HLADR^+CD3^+CD4^{+\dagger\dagger}$	250	11.2	81	13.3	**<0.01**
$CD38^+CD3^+CD4+^{\dagger\dagger}$	250	19.4	81	17.1	=0.06
$CD38^+HLADR^+CD3^+CD4^{+\dagger\dagger}$	250	1.4	81	1.6	**<0.01**
$CD38^-HLADR^+CD3^+CD4^{+\dagger\dagger}$	250	9.7	81	11.3	**<0.01**
$CD45RO^+CD4^+$	249	87.6	80	87.7	=0.96
$CD45RA^+CD4^+$	250	36.7	81	37.4	=0.89
$CD27^+CD4^+$	250	86.7	81	84.9	=0.17
$CD45RO^+CD27^+CD4^+$	251	72.0	81	68.6	**=0.01**
$CD45RO^+CD27^-CD4^+$	251	13.2	81	15.1	=0.18
$CD45RO^+CD57^+CD4^+$	251	3.1	81	2.8	=0.96
$CD45RO^+CD57^-CD4^+$	251	82.7	81	82.0	=0.39
$CD45RO^+CD57^-CD27^+CD4^{+\dagger\dagger\dagger}$	251	98.6	81	98.6	=0.55
$CD45RO^+CD57^+CD27^+CD4^{+\dagger\dagger\dagger}$	251	1.4	81	1.4	=0.63
$CD45RA^+CD27^+CD4^+$	251	34.7	81	33.5	=0.54
$CD45RA^+CD27^-CD4^+$	251	1.3	81	1.5	=0.36
$CD45RA^+CD57^+CD4^+$	251	0.67	81	0.67	=0.96
$CD45RA^+CD57^-CD4^+$	251	35.1	81	36.3	=0.82
$CD45RA^+CD57^-CD27^+CD4^{+\dagger\dagger\dagger\dagger}$	251	99.5	81	99.4	=0.34
$CD45RA^+CD57^+CD27^+CD4^{+\dagger\dagger\dagger\dagger}$	251	0.54	81	0.62	=0.35

†Markers expressed as percentage of total lymphocytes.

†† Markers expressed as percentage of total CD3 positive CD4 positive lymphocytes.

††† Markers expressed as percentage of total CD27 positive, CD45RO positive within CD4 positive lymphocytes.

†††† Markers expressed as percentage of total CD27 positive, CD45RA positive within CD4 positive lymphocytes. The rest of the markers are expressed as percentage of the CD4 positive lymphocytes

‡ HPV group as defined in the methods section

* Kruskal-Wallis test for equality of populations

Table 2

CD8 Tcell immunophenotypic subgroups distribution by study group

Marker	HPV negative group‡		Persistent HPV positive group‡		
	N	Median %	N	Median %	p*
$CD3^+CD8^{+\dagger}$	250	16.2	81	16	=0.98
$CD69^+CD8^{+\dagger}$	254	0.28	80	0.34	=0.15
$CD25^+CD8^{+\dagger}$	251	0.19	81	0.21	=0.92
$HLADR^+CD3^+CD8^{+\dagger\dagger}$	250	37.6	81	42.1	=0.12
$CD38^+CD3^+CD8^{+\dagger\dagger}$	250	6.6	81	6.3	=0.55
$CD38^+HLADR^+CD3^+CD8^{+\dagger\dagger}$	250	3.3	81	3.5	=0.71
$CD38^-HLADR^+CD3^+CD8^{+\dagger\dagger}$	250	32.9	81	37.4	=0.25
$CD45RO^+CD8^+$	252	58.6	80	62.9	=0.29
$CD45RA^+CD8^+$	251	77.0	80	76.0	=0.72
$CD27^+CD8^+$	251	51.3	81	47.5	=0.05
$CD45RO^+CD27^+CD8^+$	252	33.1	81	31.2	=0.35
$CD45RO^+CD27^-CD8^+$	252	23.2	81	27.1	**<0.01**
$CD45RO^+CCR7^+CD8^+$	252	0.10	81	0.11	=0.33
$CD45RO^+CCR7^-CD8^+$	252	58.4	81	62.2	=0.33
$CD45RO^+CD57^+CD8^+$	251	19.1	81	22.8	=0.11
$CD45RO^+CD57^-CD8^+$	251	36.1	81	33.9	=0.41
$CD45RO^+CD57^-CD27^+CD8^{+\dagger\dagger\dagger}$	251	88.6	81	85.6	=0.08
$CD45RO^+CD57^+CD27^+CD8^{+\dagger\dagger\dagger}$	251	11.4	81	14.4	=0.09
$CD45RA^+CD27^+CD8^+$	252	30.3	81	29.4	=0.47
$CD45RA^+CD27^-CD8^+$	252	41.1	81	41.9	=0.35
$CD45RA^+CCR7^-CD8^+$	252	76.7	81	75.7	=0.64
$CD45RA^+CD57^+CD8^+$	251	29.4	81	30.7	=0.33
$CD45RA^+CD57^-CD8^+$	251	41.6	81	39.8	=0.33

Marker	HPV negative group‡		Persistent HPV positive group‡		
	N	Median %	N	Median %	p*
$CD45RA^{+}CD57^{+}CD27^{+}CD8^{+}$††††	251	8.1	81	9.4	=0.06
$CD45RA^{+}CD57^{-}CD27^{+}CD8^{+}$††††	251	91.9	81	90.6	=0.07

† Markers expressed as percentage of total lymphocytes.

†† Markers expressed as percentage of total CD3 positive CD8 positive lymphocytes.

††† Markers expressed as percentage of total CD27 positive, CD45RO positive within CD8 positive lymphocytes.

†††† Markers expressed as percentage of total CD27 positive, CD45RA positive within CD8 positive lymphocytes. The rest of the markers are expressed as percentage of the CD8 positive lymphocytes

‡ HPV group as defined in the methods section

* Kruskal-Wallis test for equality of populations

Table 3

Odds ratios for HPV persistence by proportion of $CD4^+$ lymphocyte immunophenotypic subsets

Marker	Median quartile values*	HPV negative group‡	Persistent HPV positive group‡			Long term persistent HPV positive group‡		
		N	N	OR†	95% CI	N	OR†	95% CI
$CD69^+CD4^+$								
1st quartile	0.02%	80	8	1		1	1	
2nd quartile	0.08%	67	24	3.8	1.6 – 9.2	8	11.1	1.3 – 94.1
3rd quartile	0.31%	56	25	5.4	2.2 – 13.1	8	16.2	1.9 – 140
4th quartile	1.47%	51	23	5.4	2.2 – 13.3	10	24.2	2.9 – 204
p-trend				**<0.001**			**<0.001**	
$HLADR^+CD3^+CD4^+$								
1st quartile	6.97%	72	11	1		1	1	
2nd quartile	10.0%	61	22	2.3	1.02 – 5.1	4	3.9	0.42 – 36.8
3rd quartile	13.6%	63	21	1.9	0.83 – 4.3	14	10.8	1.3 – 86.8
4th quartile	19.7%	54	27	2.6	1.2 – 5.9	9	6.9	0.82 – 58.0
p-trend				**=0.041**			**=0.037**	
$CD45RO^+$ $CD27^+CD4^+$								
1st quartile	57.1%	58	29	1		11	1	
2nd quartile	67.4%	61	20	0.63	0.32 – 1.3	7	0.59	0.20 – 1.8
3rd quartile	74.8%	60	18	0.54	0.27 – 1.1	7	0.46	0.16 – 1.4
4th quartile	80.3%	72	14	0.36	0.17 – 0.76	3	0.17	0.04 – 0.70
p-trend				**=0.006**			**=0.011**	
$CD45RO^+CD27^-CD4^+$								
1st quartile	6.8%	71	20	1		6	1	
2nd quartile	11.4%	59	14	0.81	0.37–1.8	2	0.36	0.07–1.93
3rd quartile	17.0%	60	22	1.3	0.65–2.7	10	2.0	0.65–6.1
4th quartile	27.3%	61	25	1.1	0.70–2.8	10	1.9	0.61–5.8

Marker	Median quartile values*	HPV negative group‡	Persistent HPV positive group‡			Long term persistent HPV positive group‡		
		N	N	OR†	95% CI	N	OR†	95% CI
p-trend				=0.209			=0.086	

*For HPV Negative and HPV positive groups

‡HPV group as defined in the methods section

†Odds ratios for the HPV positive and long term HPV positive groups compared to the HPV negative group, adjusted for age.

Table 4

Odds ratios for HPV persistence by proportion of $CD8^+$ lymphocyte immunophenotypic subsets

Marker	Median quartile values*	HPV negative group‡	Persistent HPV positive group‡			Long term persistent HPV positive group‡		
		N	N	OR†	95% CI	N	OR†	95% CI
$CD69^+CD8^+$								
1st quartile	0.10%	78	16	1		4	1	
2nd quartile	0.23%	60	24	1.7	0.84–3.6	5	0.97	0.23–4.0
3rd quartile	0.46%	66	20	1.4	0.66–2.9	9	2.1	0.59–7.5
4th quartile	1.2%	50	20	1.8	0.86–3.9	9	2.7	0.76–10.0
p-trend				=0.195			=0.053	
$HLADR^+CD3^+CD8^+$								
1st quartile	20.6%	68	20	1		5	1	
2nd quartile	34.1%	64	12	0.60	0.27–1.3	1	0.17	0.02–1.6
3rd quartile	44.2%	56	23	1.2	0.57–2.4	10	1.5	0.46–5.0
4th quartile	57.3%	62	26	1.10	0.54–2.2	12	1.4	0.44–4.6
p-trend				=0.482			=0.190	
$CD45RO^+CD27^+CD8^+$								
1st quartile	21.3%	62	25	1		8	1	
2nd quartile	30.0%	65	21	0.77	0.39–1.5	10	1.1	0.37–3.0
3rd quartile	37.3%	65	16	0.67	0.32–1.4	5	0.79	0.23–2.7
4th quartile	45.4%	60	19	0.81	0.40–1.6	5	0.80	0.24–2.7
p-trend				=0.485			=0.619	
$CD45RO^+CD27^-CD8^+$								
1st quartile	11.5%	68	14	1		1	1	
2nd quartile	20.9%	68	16	1.1	0.49 – 2.4	8	7.5	0.90 – 63.3
3rd quartile	27.7%	57	21	1.9	0.87 – 4.1	9	11.5	1.4 – 96.2
4th quartile	41.5%	59	30	2.3	1.1 – 4.7	10	8.8	1.1 – 72.3

Marker	Median quartile values*	HPV negative group‡	Persistent HPV positive group‡			Long term persistent HPV positive group‡		
		N	N	OR†	95% CI	N	OR†	95% CI
p-trend				**=0.010**			**=0.041**	

*For HPV Negative and HPV positive groups

‡HPV group as defined in the methods section

†Odds ratios for the HPV positive and long term HPV positive groups compared to the HPV negative group, adjusted for age.

Table 5

Odds ratios and confidence intervals for persistent HPV positive group by PHA and VLP proliferation assays

A. Proliferation

Marker	Median ccpm*	Cases/Controls	OR†	95% CI	OR††	95% CI
PHA						
Highest tertile	186902	19/98	1		1	
Medium tertile	98055	28/89	1.6	0.85 –3.2	1.4	0.71–2.8
Low tertile	27117	40/74	2.7	1.4 – 5.1	1.9	0.94–3.7
p-trend			=0.002		=0.075	
VLP						
Highest tertile	7470	21/104	1		1	
Medium tertile	1168	27/86	1.6	0.81 – 3.0	1.2	0.61–2.4
Low tertile	71	29/57	2.7	1.4 – 5.3	1.9	0.90–3.8
p-trend			=0.003		=0.101	

B. T-Cell Immunophenotypes

Marker	Median quartile values*	Cases/Controls	OR†	95% CI	OR‡	95% CI	OR‡‡	95% CI
$CD69^+CD4^+$								
1st quartile	0.02%	8/80	1		1		1	
2nd quartile	0.08%	24/67	3.8	1.6–9.2	3.5	1.4–8.4	3.0	1.2–7.5
3rd quartile	0.31%	25/56	5.4	2.2–13.1	4.7	1.9–11.5	4.5	1.8–11.3
4th quartile	1.47%	23/51	5.4	2.2–13.3	5.4	2.2–13.5	4.5	1.8–11.4
p-trend			<0.001		<0.001		=0.001	
$HLADR^+CD3^+CD4^+$								
1st quartile	6.97%	11/72	1		1		1	
2nd quartile	10.03%	22/61	2.3	1.0–5.1	2.1	0.94–4.8	1.9	0.84–4.5
3rd quartile	13.58%	21/63	1.9	0.83–4.3	1.7	0.73–3.8	1.7	0.72–3.9
4th quartile	19.68%	27/54	2.6	1.2–5.9	2.2	0.94–4.9	1.9	0.83–4.6
p-trend			=0.041		=0.148		=0.203	

B. T-Cell Immunophenotypes

Marker	Median quartile values*	Cases/Controls	OR†	95% CI	OR‡	95% CI	OR‡‡	95% CI
$CD45RO^+CD27^+CD4^+$								
4st quartile	80.3%	14/72	1		1		1	
3rd quartile	74.8%	18/60	1.5	0.68–3.3	1.4	0.65–3.2	1.2	0.50–2.7
2nd quartile	67.4%	20/61	1.7	0.81–3.8	1.5	0.70–3.4	1.3	0.58–3.1
1st quartile	57.1%	29/58	2.8	1.3–5.8	2.4	1.2–5.2	2.7	1.2–5.8
p-trend			=0.006		=0.021		=0.011	
$CD45RO^+CD27^-CD8^+$								
1st quartile	11.5%	14/68	1		1		1	
2nd quartile	20.9%	16/68	1.1	0.49–2.4	0.96	0.43–2.2	0.93	0.40–2.2
3rd quartile	27.7%	21/57	1.9	0.87–4.1	1.6	0.75–3.6	1.4	0.60–3.2
4th quartile	41.5%	30/59	2.3	1.1–4.7	1.9	0.87–4.0	1.8	0.82–3.9
p-trend			=0.010		=0.042		=0.076	

*For HPV Negative and HPV positive groups

†Odds ratios adjusted for age

††Odds ratios adjusted for age and the combined T-cell subset marker variable defined in Methods.

‡Odds ratios adjusted for age and the proliferative response to PHA

‡‡Odds ratios adjusted for age and the proliferative response to HPV-16 VLP