

Slumbering mucosal immune response in the cervix of human papillomavirus DNA-positive and -negative women

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Abstract. Persistent human papillomavirus (HPV) infection is a prerequisite for cervical cancer and results from bypassing the local immune response. Twenty-four volunteers underwent an ectocervical biopsy, Pap smear, tests for sexually transmitted infections including HIV and HPV genotyping. All answered a questionnaire regarding medical history. Repeat Pap smear and HPV genotyping was performed 9-26 months later. Quantitative reverse transcriptase (qRT)-PCR was used to assess expression of CD3, CD4, CD8, CD19, CD27, IL-2, IL-12, IL-4, IL-10, IL-17, HLA-DR α , TGF β , IFN γ , PD-1, PD-L1, CTLA-4, LAG3, IgA, IgG, CCR5, CCL5/RANTES and the IL-7 receptor in the biopsies. Eleven of 24 volunteers were HPV DNA-positive at baseline. Four of 10 were infected with a persistent HPV genotype at follow-up. All target molecules were successfully amplified and quantified except for IL-4. We found no difference in mRNA expression of these molecules when comparing HPV DNA-positive and -negative women, neither when comparing persistently infected individuals or those who cleared the infection. However, mRNA expression of the B cell phenotypic marker CD19 was higher in women using hormonal contraception than those not ($p < 0.05$). HPV infection does not evoke a local inflammatory immune response in the ectocervix measurable with qRT-PCR. Hormonal contraception may influence B cell activity in the cervix.

Introduction

Infection with oncogenic human papillomavirus (HPV) is a crucial factor in the development of cervical intraepithelial neoplasia (CIN) and cancer (1,2). HPV is a common sexually

transmitted virus with a cumulative life-time risk of any HPV-type of 80%, although only a small fraction of infected women progress to invasive cancer of the cervix (3-5). Failure of the host immune response to clear the HPV infection contributes to carcinogenesis, as suggested by the increased prevalence of HPV infection, CIN and cervical cancer in individuals with impaired cell-mediated immunity such as that in patients with HIV/AIDS, organ transplants and iatrogenic immunosuppression (6,7). The immunological barrier of the cervical mucosa is predominately regulated in the lamina propria, which contains plasma cells, antigen-presenting cells (dendritic cells), natural killer cells as well as helper and cytotoxic T lymphocytes. Successful regression of HPV infection occurs in the presence of local pro-inflammatory (Th1) cytokine expression (8-10) and CD4⁺ T cells as evident in immunohistological studies of HPV-induced warts (11), and a systemic lymphoproliferative response to HPV E7 protein (12). Therefore, cytokine-mediated immune responses may be a critical factor in HPV clearance.

Cytokines are usually expressed at low levels in a para- and autocrine fashion, but the small size of relevant tissue samples limits the opportunity to extract and analyze these proteins. Consequently, the detection of cytokine expression at the mRNA level has become an attractive approach, which we selected for this study in the form of semi-quantitative real-time reverse transcriptase PCR (RT-PCR) technology (13).

A dogmatic and simplified view of cytokine secretion divides the options into studies of pro-inflammatory cytokines that stimulate cell-mediated responses (Th1) or immune-inhibitory, tumor-permissive cytokines that mediate humoral immunity (Th2). The pro-inflammatory group includes interleukin-2 (IL-2) -12 and -17, interferon- γ (IFN γ), regulated upon activation normal T cell expressed and secreted (RANTES)/C-C chemokine ligand 5 (CCL5), as well as expression of C-C chemokine receptor 5 (CCR5) and HLA-DR. Those considered to be anti-inflammatory are IL-4, IL-10 and transforming growth factor- β (TGF β) (14,15). IL-17 also promotes mucosal expression of endogenous anti-microbial peptides, such as defensins, which act as natural antibiotics and exhibit chemotactic activity (16). Scott *et al* demonstrated a local Th1 response pattern (non-quantitative) in

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Table I. Entry characteristics.

Entry data	All	HPV status		p-value ^a
		HPV-positive	HPV-negative	
Number of women, N (%)	24	11 (46)	13 (54)	0.56
Age (median) [range], years	32 [24-50]	31 [24-46]	32 [26-50]	0.66
Current smoker, N (%)	5 (21)	2 (18)	3 (23)	0.58
Hormonal contraception, N (%)	11 (46)	5 (45)	6 (46)	0.98
Oral contraception, N (%)	7 (29)	5 (45)	2 (15)	0.18
Progesterone IUD, N (%)	4 (17)	0	4 (31)	0.10
Condom use $\geq 50\%$, N (%)	7 (29)	4 (36)	3 (23)	0.66
New sex partner previous 12 months, N (%)	6 (25)	3 (27)	3 (23)	1.00
Ever pregnant, N (%)	13 (57)	4 (40)	9 (69)	0.16
Additional cervical condition ^b , N (%)	5 (21)	2 (18)	3 (23)	1.00
Previous CIN1 ⁺ , N (%)	2 (8.7)	0	2 (15)	0.25
HSV-2 seropositive, N (%)	4 (17)	1 (9)	3 (23)	0.29
Preovulatory menstrual phase, N (%)	2 (8.7)	1 (10)	1 (7.7)	0.95
Postovulatory menstrual phase, N (%)	10 (43)	4 (40)	6 (46)	0.95

^aMann-Witney U or χ^2 /Fischer's exact test; ^bYeast infection, bacterial vaginosis, or low-grade squamous intraepithelial lesion/atypical squamous cells of uncertain significance in the first Pap smear.

cervical brushings from women who cleared their HPV infection (8). A few studies have shown that mixed inflammatory states or dysplasia of the cervix involves increased mRNA expression of Th1 cytokines (17-19). In contrast, amounts of Th2 response cytokines including IL-4 and IL-10 are increased in HIV-positive HPV-co-infected persons compared to those infected with HPV alone or not infected, and such an increase may contribute to the inability to clear HPV infection (20-22).

Immunoregulatory receptors, such as the IL-7 receptor (IL-7R), the programmed cell death receptor 1 (PD-1), its ligand PD-L1, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and lymphocyte-activation gene 3 (LAG-3) are considered to be suppressors of T cell activation and/or participants in T cell memory (IL-7R) (23,24). Moreover, the impact of these immunoregulatory receptors and ligands on chronic infection caused by HIV, hepatitis B and hepatitis C has generated growing interest. PD-1 is up-regulated on activated T cells specific for chronic viral infections in humans but not T cells specific for non-persisting vaccinia or influenza viruses (reviewed in ref. 25). Blockade of PD-1 in a macaque model enhanced T cell immunity, as well as memory B cell proliferation, decreased viral load and prolonged survival (26). Exhausted T cells have been stained for PD-1, LAG-3 and CTLA-4 among several other inhibitory receptors in a complex pattern, yet their levels of expression seem to vary with the severity of viral infection (27). Expression of these regulatory receptors and ligands has not yet been studied in HPV.

Here we have focused on the mRNA expression of several cytokines as well as on phenotypic markers including CD3, CD4 and CD8 for T cells, CD19 for B cells, and CD27 as a memory cell marker at the local site (28-30). CD4 and CD8 have been quantified using RT-PCR in the cervical samples

of patients with HPV-genotyped CIN (31), but this is the first report of CD4 and CD8 mRNA expression in HPV DNA-positive women without CIN. Our results present a comprehensive analysis of the cytokines and phenotypic markers on cells involved in the local mucosal immunity in the genital tract of HPV DNA-positive and -negative women without CIN.

Materials and methods

Study subjects. A total of 24 subjectively healthy female volunteers were enrolled from March 2007 through September 2008 at the Division of Obstetrics and Gynecology, Karolinska University Hospital Huddinge, Sweden, after ethical approval and written informed consent were obtained. All subjects underwent a pelvic exam with collection of samples for cytological diagnosis, HPV DNA analysis, diagnosis of chlamydia trachomatis and neisseria gonorrhea. An ectocervical biopsy was also collected from each woman. Blood samples were drawn for HIV and herpes simplex virus (HSV) serological tests. All assays were performed at the accredited microbiological laboratory service at the hospital. Each woman also completed a questionnaire regarding previous medical conditions, reproductive history, menstrual cycle, contraception, smoking and sexual partnership status, as compiled in Table I. Nine to 26 months (median 23.8) later women were drafted for a second pelvic exam, cervical cytological sampling and HPV DNA testing. Nineteen (79%) of the 24 women completed the study, while five were lost to follow-up due to withdrawal (four) or pregnancy (one).

Cytology. Cells collected from the ectocervix with a plastic spatula and from the endocervix with a plastic brush were prepared as conventional Pap smears. The cytobrush was

Table II. Study subjects at inclusion and follow-up.

Id code	Age	Hormonal exposure ^a	1st Pap smear	1st HPV status	Time between visits (months)	2nd Pap smear	2nd HPV status
1	50	<i>Postovulatory</i>	Normal	neg	9.7	Normal	neg
2	41	Progesterone IUD	Normal	neg	9.6	Normal	neg
3	36	<i>Preovulatory</i>	Normal	neg	- ^c	-	-
4	31	Progesterone IUD	Normal, yeast	neg	9.4	Normal	neg
5	41	Progesterone IUD	Normal, yeast	neg	-	-	-
6	37	<i>Postovulatory</i>	Normal	neg	25.2	Normal	neg
7	37	Copper IUD, <i>postovulatory</i>	Normal	neg	-	-	-
8	32	<i>Postovulatory</i>	Normal	neg	25.1	Normal	neg
9	31	Progesterone IUD	Normal, BV	neg	-	-	-
10	31	Combined OC	Normal	neg	20.8	Normal	neg
11	30	<i>Postovulatory</i>	Normal	neg	25.2	Normal	HPV 81
12	26	<i>Postovulatory</i>	Normal	neg	24.3	Normal	HPV 53 , 61
13	26	Combined OC	Normal	neg	26.7	Normal	neg
14	41	Oral contraception ^b	Normal	HPV 79	9.1	Normal	HPV 70
15	36	<i>Preovulatory</i>	Normal	HPV 55	10.6	Normal	HPV 55
16	26	Combined OC	Normal	HPV 55	25.9	Normal	neg
17	46	<i>Postovulatory</i>	Normal	HPV 52	25.2	Normal	neg
18	41	<i>Postovulatory</i>	Normal	HPV 16	24.1	Normal	neg
19	37	<i>Postovulatory</i>	Normal	HPV 51	25.8	Normal	neg
20	31	Combined OC	Normal	HPV CP6108, 51 59, 53, 68, 73	10.3	Normal	HPV 51, 53, 73
21	30	<i>Postovulatory</i>	LSIL	HPV 33	23.8	Normal	neg
22	29	Combined OC	Normal	HPV 35, 39	-	-	-
23	25	Unknown	Normal	HPV 39	23.8	Normal	HPV 39
24	24	Combined OC	ASCUS	HPV 16, 31, 33, 70	10.6	Normal	HPV 18, 31, IS39

^aWomen with intact ovulation have their menstrual cycle phase at the first visit recorded in the column hormonal exposure. ^b Uses high-dose progesterone only contraceptive pill (anovulation). ^cLost to follow-up. HPV, human papillomavirus (high-risk types in bold); OC, oral contraception; IUD, intrauterine device; BV, bacterial vaginosis; LSIL, low-grade squamous intraepithelial lesion; ASCUS, atypical squamous cells of uncertain significance; CIN, cervical intraepithelial neoplasia.

placed in 1 ml of sterile saline suspension for HPV genotyping. Blinded cytological evaluation was performed by a pathologist. According to Swedish recommendations, cases of koilocytosis without signs of dysplasia are reported as non-pathologic. The cytological diagnoses were based on the Bethesda nomenclature (32).

HPV DNA extraction and linear array. After the cytobrush saline suspension was centrifuged, the cell pellet was lysed

by using the Total Nucleic Acid Isolation kit (Roche, Basel, Switzerland). DNA was extracted with the MagNA Pure LC robot and analyzed with the Linear Array HPV genotyping test (both procedures by Roche). Details of this method have been published (33). The Linear Array test is a PCR- and probe hybridization-based genotyping assay covering 37 HPV types including 12 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), 6 probable high-risk HPV types (26, 53, 66, 68, 73, 82) and 19 low-risk or undeter-

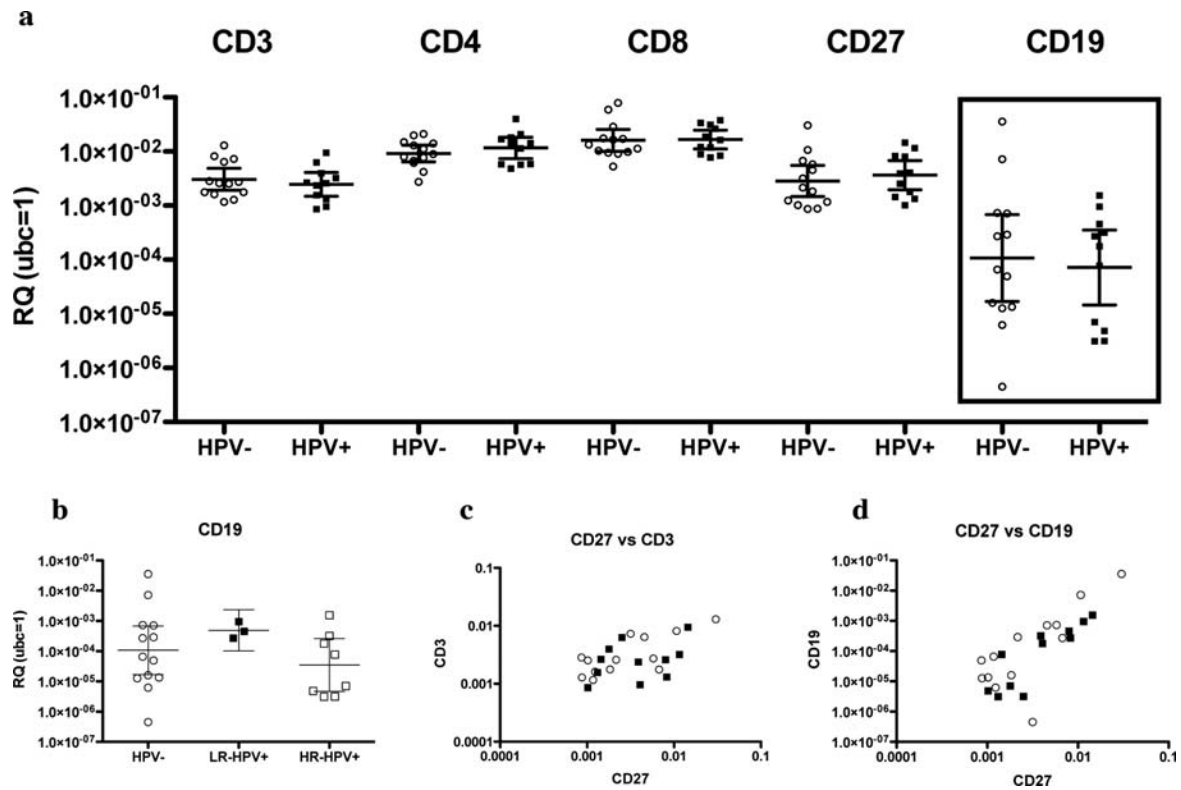


Figure 1. (a) Expression of phenotypic cell markers in HPV DNA-negative (HPV⁻) (circles) and HPV DNA-positive (HPV⁺) (boxes) individuals. Lines and bars depict geometric means with a 95% confidence interval. (b) Expression of CD19 in HPV⁻ (circles), low-risk HPV⁺ (LR-HPV⁺) (filled boxes) and high-risk HPV⁺ (HR-HPV⁺) (clear boxes) women. Lines and bars depict geometric means with 95% confidence interval, $p=0.25$. (c) Correlations between CD27 and T cell marker CD3 in HPV⁻ (circles) ($p<0.001$, $r^2=0.72$) and HPV⁺ (boxes) ($p=0.10$) individuals. (d) Correlations between CD27 and phenotypic B cell marker CD19 in HPV⁻ (circles) ($p<0.001$, $r^2=0.94$) and HPV⁺ (boxes) ($p<0.001$, $r^2=0.88$) individuals.

mined HPV types (6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, CP6108) (34). Linear Array uses biotinylated primers within the consensus L1 region of the HPV genome.

Detection and quantification of cytokine mRNA. Biopsies were taken by a single investigator (S.A.) from the superior ectocervix outside the transformation zone, immediately transferred to RNAlater buffer (Qiagen, Hilden, Germany), and stored at -70°C . The biopsies were thawed and disrupted in lysis buffer with a mechanical rotor, and RNA was extracted according to the manufacturer's protocol (the kit RNeasy, Qiagen). RNA was converted in equal dilutions to cDNA in a single reverse transcriptase reaction using superscript II reverse transcriptase (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and random hexanucleotide primers (Roche). The cDNA was stored at -20°C . Amplification of ubiquitin C (UBC), CD3, CD4, CD8, CD19, CD27, IL-2, IL-12a, IL-4, IL-10, IL-17a, HLA-DR α , TGF β , IFN γ , PD-1, PD-L1, CTLA-4, LAG3, IgA_{constant heavy}, IgG_{constant heavy}, CCR5, CCL5/RANTES and IL-7 receptor (IL-7R) cDNA was performed using the ABI PRISM 7500 sequence detection system and commercial FAMTM dye-labeled TaqMan MGB probes and primers (Applied Biosystems Inc, Foster City, CA, USA). UBC was chosen as endogenous control after a dilution series testing UBC, 18sRNA, and GAPDH revealed that UBC was the most constant highly expressed gene in our samples. Each sample and control was run in triplicates. The

relative quantity of target cDNA was computed using the comparative threshold (Ct) method (35). Ct values for target cDNA were normalized to UBC ($\text{Ct}_{\text{UBC}} - \text{Ct}_{\text{target}}$) and amounts of cDNA were computed through In-transformation ($2^{-\text{dCt}}$). This can be read out as number of target gene copies for each copy of UBC.

Statistical analysis. Data were analyzed with the software Statistica 8.0 (Statsoft Inc., Tulsa, OK, USA) and GraphPad Prism 4.00 (GraphPad Software Inc, La Jolla, CA, USA). Pearson's Chi-square and Fischer's exact test were used to compare proportions and Student's t-test to compare ages between groups. Data were not normally distributed even after logarithmic transformation therefore a non-parametric Mann-Witney U test was used to compare two groups and Kruskal-Wallis ANOVA to compare multiple groups, and Spearman rank-order correlation test to assess correlations. Multivariate logistic regression was performed to analyze effects of clinical parameters on CD19 expression, categorizing CD19 mRNA expression above or below median level. All tests were two-sided and differences considered significant at $p<0.05$.

Results

Distribution of HPV genotypes and cytological findings. At inclusion, a total of 22 of 24 (92%) women had cytological results within normal limits, one had atypical squamous cells

of uncertain significance (ASCUS) and one woman had a low-grade squamous intraepithelial lesion (LSIL). Eleven (46%) were positive for some HPV type, of whom 8 (33%) carried high-risk HPV types (Table II). Four women were HSV-2 seropositive and one of these had a reported history of genital herpes. None of the women was diagnosed with HIV, chlamydia or gonorrheal genital infection, although one woman's results from chlamydia PCR analysis and N. gonorrhea culture were missing. Two women had additional findings of yeast infection, and one had signs of bacterial vaginosis. These three women, including the two with minor cytological lesions, were designated as a group with an additional cervical condition. None of the HSV-2 seropositive women had ongoing genital lesions, and were not included in this group. No relevant differences in sexual or reproductive histories were noted between HPV-positive and -negative subjects (Table I). At follow-up, all women had normal findings in the cervical cytology sample. One woman had acquired genital herpes and seroconverted to HSV-2 (id code no. 12). Four (21%) women still carried at least one persistent HPV type, while three had acquired a new HPV type and five (26%) had succeeded in clearing the virus. Seven women were still HPV DNA-negative at follow-up. There were no significant differences found between these groups of HPV clearance status with regard to surveys of hormonal exposure, smoking, contraceptive methods, sexual and reproductive history or age (data not shown).

Expression of phenotypic markers. Expression of the T phenotypic markers CD3, CD4 and CD8, and B phenotypic markers CD19 and CD27 was calculated in relative quantity compared to the endogenous control UBC (Fig. 1). None of these phenotypic markers of HPV-positive women differed significantly from those of the HPV-negative group ($p>0.05$). CD19 expression was widely dispersed in HPV DNA-negative women and women positive for high-risk and low-risk HPV DNA (Fig. 1), raising the question whether the outliers represent different compositions of B cells, with very high or low relative frequencies of CD19-negative cells such as memory B cells and plasma cells. Since no markers specific for human plasma cells are available, quantities of the memory B cell marker CD27 were evaluated and found to correlate linearly with CD19 (Fig. 1). Not forgetting that CD27 is also expressed by T cells, we tested further and found that the total CD27 expressed here correlated better with the amount of CD19 from B cells than with CD3 from T cells (Fig. 1). Interestingly, all four women with persistent HPV infection at follow-up displayed CD19 mRNA levels above the median ($p=0.06$). None of the other phenotypic markers differed significantly between groups of HPV status at follow-up (data not shown).

Expression of immunoglobulins. Expression of immunoglobulin mRNA was used as a marker for plasma cell presence. Immunoglobulin A (IgA) was detectable in all women, but immunoglobulin G (IgG) was absent in four of those who were HPV-negative (Fig. 2). There was no difference in expression of IgA or IgG in HPV DNA-negative women and women positive for high-risk and low-risk HPV DNA (Fig. 2). IgA expression correlated well with that of IgG in

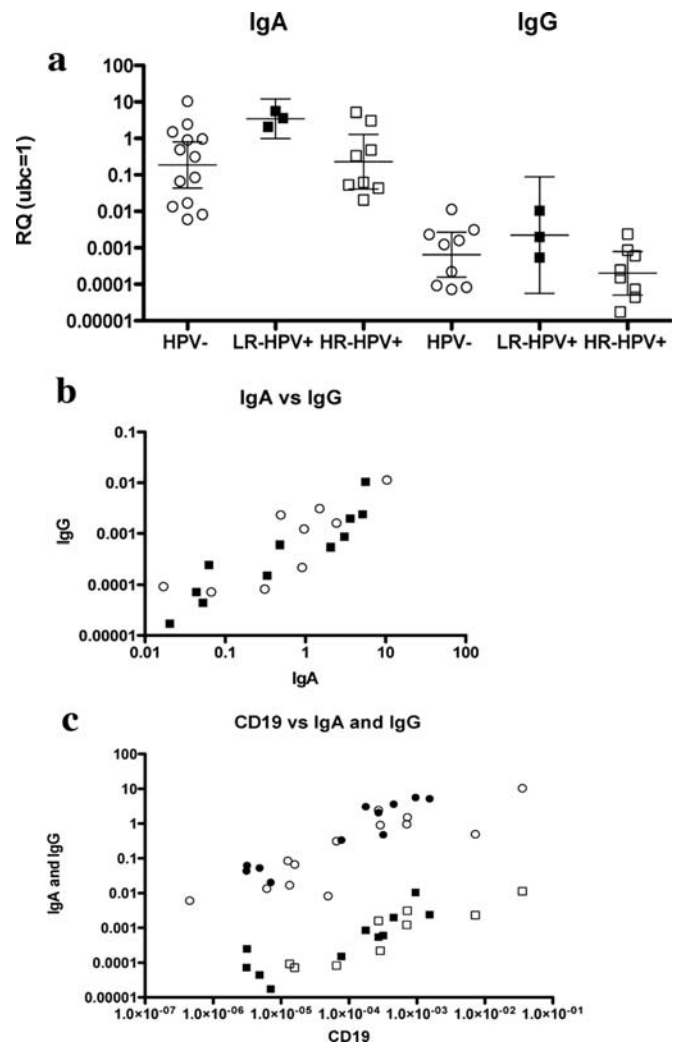


Figure 2. (a) Expression of IgA and IgG mRNA in HPV⁻ (circles), low-risk HPV⁺ (LR-HPV⁺) (filled boxes) and high-risk HPV⁺ (HR-HPV⁺) (clear boxes) women. Lines and bars depict geometric mean with 95% confidence interval, $p>0.05$ between groups for both IgA and IgG. (b) IgA and IgG expression strongly correlated in HPV⁻ (circles) ($p<0.001$, $r^2=0.93$) and HR-HPV⁺ (LR and HR combined; boxes) ($p<0.0001$, $r^2=0.97$) women. All values are in relation to UBC (=1). (c) IgA expression correlated with CD19 in HPV⁻ (clear circles) and HPV⁺ women (LR and HR combined; clear boxes) ($p<0.001$, $r^2=0.90$ and $p<0.001$, $r^2=0.75$, respectively) as did IgG expression with CD19 HPV⁻ (filled circles; $p<0.001$, $r^2=0.93$) and HPV⁺ (filled boxes; $p=0.046$, $r^2=0.37$) women. All values are in relation to UBC (=1).

all women (Fig. 2) and immunoglobulin expression correlated linearly with amounts of both CD19 (Fig. 2) and CD27 (data not shown), although neither CD19 nor CD27 should be expressed by plasma cells. No difference was found in expression of IgA or IgG mRNA between groups of HPV status at follow-up (data not shown).

Cytokine profiles, immunoregulatory receptors and ligands. As Fig. 3 depicts, expression of the pro-inflammatory cytokines IL-2, IL-12, IL-17 and IFN γ , RANTES/CCL5, its receptor CCR5, and HLA-DR was similar in the HPV DNA-negative and -positive women. Nor was any difference evident when sub-grouping HPV-positive women into low-risk or high-risk HPV carriers, nor between groups of HPV status at

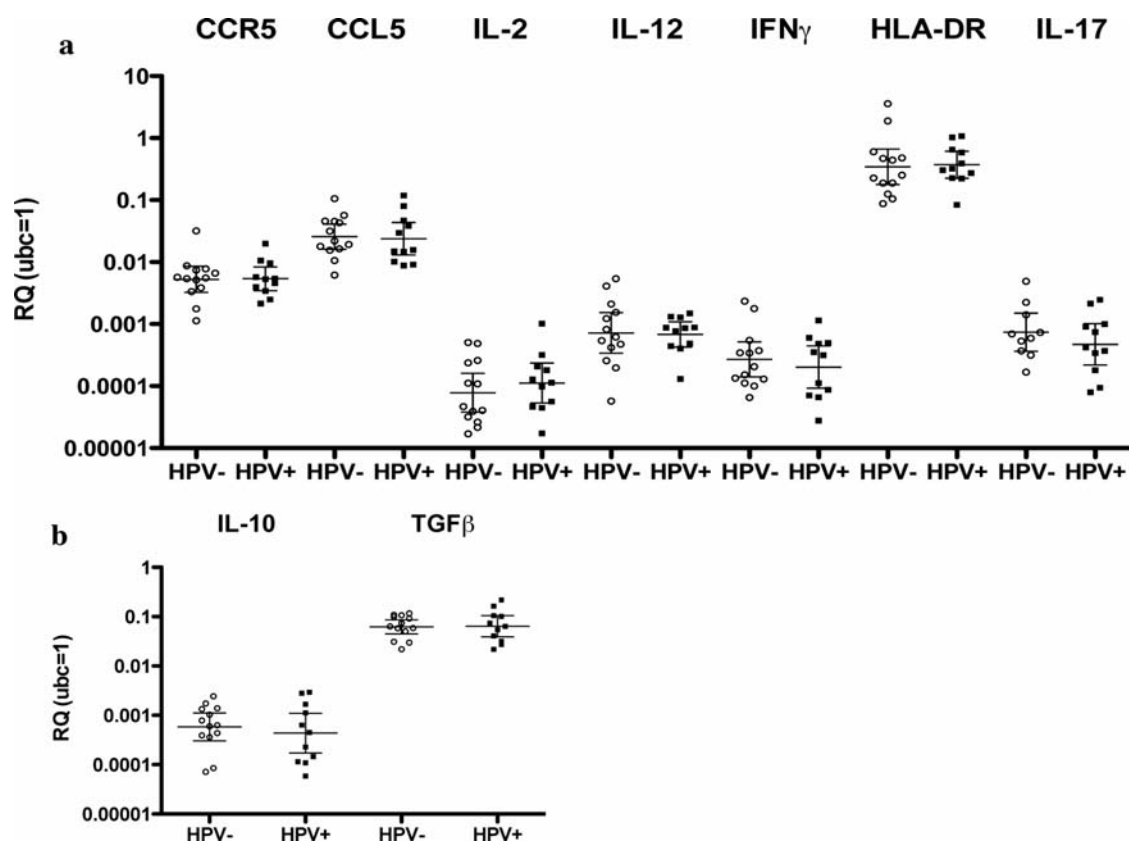


Figure 3. (a) No significant differences were found in the expression of pro-inflammatory cytokines in HPV⁻ (circles) and HPV⁺ (boxes) women. Lines and bars depict geometric means with 95% confidence interval. (b) Anti-inflammatory cytokines in HPV DNA-negative and -positive women. Lines and bars depict geometric means with a 95% confidence interval. No significant differences between HPV DNA-negative and -positive women were found.

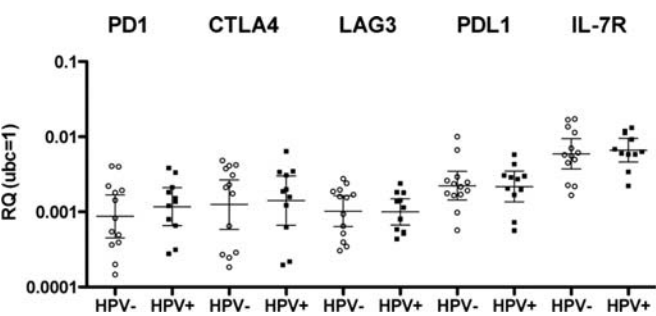


Figure 4. No significant differences were found in the expression of immuno-regulatory receptors and ligands in HPV⁻ (circles) and HPV⁺ (boxes) women. Lines and bars depict geometric means with 95% confidence interval.

follow-up (data not shown). The anti-inflammatory cytokines TGF β , IL-4 and IL-10 were expressed in a mutually similar fashion (Fig. 3). IL-4 mRNA expression was below the detection limit in all patients. Additionally, as outlined in Fig. 4, neither immunoregulatory receptors nor ligands differed to a significant extent between HPV DNA-positive and -negative women, nor between groups of HPV status at follow-up (data not shown).

Clinical correlates of cytokines and phenotypic markers. Surveys of hormonal exposure, smoking and treatment with inhaled or oral corticosteroids, sexual and reproductive history or age (data not shown) revealed only one distinctive

result, and that was mRNA expression of the phenotypic marker CD19. CD19 levels were significantly higher in women currently using oral contraception or progesterone intrauterine devices compared to women not using hormonal contraception (Fig. 5). Immunoglobulin expression was not associated with hormonal exposure. Among the other phenotypic markers, pro- or anti-inflammatory cytokines, immune regulatory receptors and corresponding ligands, there were no significant correlations with clinical parameters, including HSV seropositivity, in univariate analyses. Due to the dispersed expression of CD19 mRNA, values above or below median were analyzed in a multivariate logistic regression model combining possible confounders and effect modifiers (age, smoking, hormonal contraception, HSV status, additional inflammatory condition, new sexual encounter, HPV status at inclusion and HPV clearance). In such a model, no significant correlations were found. The two women with LSIL or ASCUS (id code nos. 21 and 24, Table II) had immune activity within the interquartile range regarding the expression of all markers. One woman (id code no. 2, Table II) had a very strong mRNA expression of CD3, CD8, CD19, CD27, IgA, IgG, PD-1, IL-7R, IFN γ and CCR5, despite lack of clinical or laboratory signs of inflammation or infection.

Discussion

Results from this study provide a unique baseline of the constituent mRNA expression for a panel of cytokines and

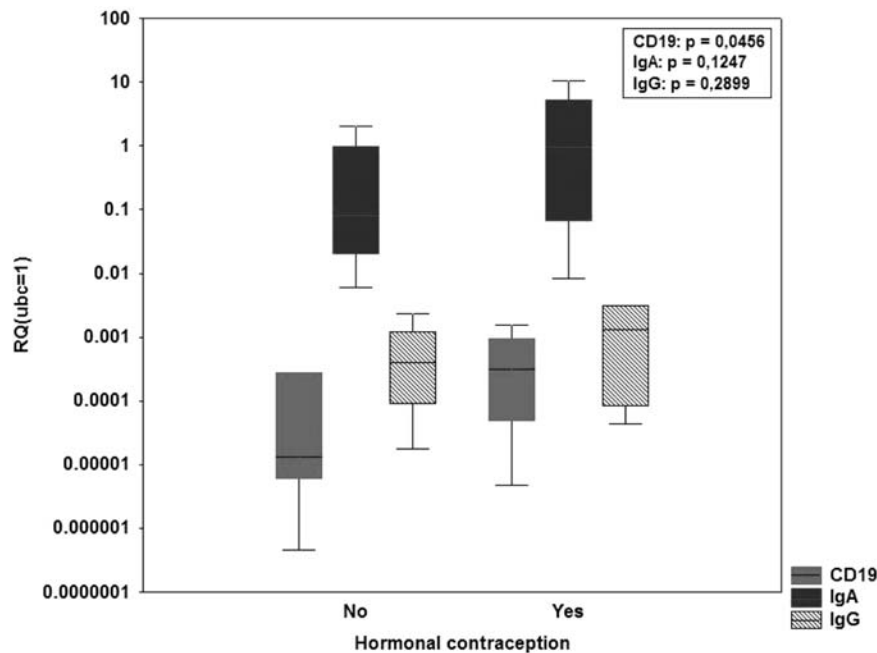


Figure 5. Expression of CD19 and immunoglobulins in women without and with current hormonal contraception (contraceptive pill or hormonal IUD). P-values were calculated with Mann-Witney U non-parametric test. Lines depict median values, boxes 25-75% range, and whiskers denote non-outlier range.

cellular markers in the ectocervices of subjectively healthy volunteers with asymptomatic HPV infection. No differences were found in this material in inflammatory or adaptive immune response regulation, suggesting that HPV DNA positivity with high- or low-risk types without dysplastic lesions does not affect the activity of the local mucosal immune response at the mRNA level. Yet, we did observe a significant association between exposure to hormonal contraception and higher CD19 mRNA expression.

Few cytokines have been studied in cervical samples from women without CIN and no correlation with current HPV infection has been found (18,19), which supports our results. In women with HPV16-positive CIN, El-Sherif *et al* observed decreased levels of IFN γ and TGF β mRNA and increases of IL-10 compared to healthy HPV-negative controls (36,37). Others recently found that elevations in IFN γ and IL-10 were associated with decreased odds of having CIN 2 or 3 (38,39). Using immunohistochemistry, Behbahani *et al* located more IL-2 and IL-4 expressing cells as signs of immune activation in HPV-positive women compared to negative controls (22). Immunohistochemistry enables identification of cells expressing a certain target molecule, whereas quantitative RT-PCR will detect the amount of mRNA expressed, which may or may not be related to the number of cells. In the context of HIV susceptibility, the amount of target molecules such as CD4 and CCR5 may be of greater importance than the number of cells. The essence is that asymptomatic HPV infection alone does not seem to evoke a measurable inflammatory response or attract possible HIV target cells, instead oncogenic transformation or additional immunogenic conditions may be needed.

We assessed other covariates of cytokine expression, but could not repeat previous observations of increased IL-10, IL-12 or IFN γ mRNA expression in women with current or recent use of oral contraceptives (18,19). We did however

observe higher levels of CD19 mRNA in women engaged in hormonal contraception. CD19 is a phenotypic B cell marker lost in the late stages of B lymphocyte differentiation. CD19 $^{+}$ lymphocytes have previously been located in follicle-like structures in the subepithelial layers of the ectocervical mucosa with immunohistochemical technology (40), supporting our finding of CD19 mRNA in the ectocervix. Endometrial aggregates of CD19 $^{+}$ lymphocytes increase in size during mid-cycle and the secretory phase of the menstrual cycle (41), supporting the finding of hormonal influence on CD19 $^{+}$ cells. We did not observe any correlation between hormonal contraception and immunoglobulin mRNA expression. One may speculate that high levels of CD19 and no change in IgG or IgA expression represent hormonally blocked B lymphocyte maturation and, eventually, an impaired adaptive immunological response. In agreement with this, we noted that CD19 mRNA levels above the median correlated with chronic HPV infection ($p=0.06$). Combined oral contraception has long been regarded as an independent risk factor for HPV infection, CIN and cervical cancer (reviewed in ref. 42). However, that conclusion has been questioned, partly because no underlying mechanisms is evident and partly due to confounding effects by higher prevalence of HPV infection in oral contraception users (43). Here, we provide one clue to understanding how hormonal contraception may influence host immune defence, i.e., the influence of CD19 amplification.

Most cytokines, phenotypic cell markers, immunoregulatory receptors and ligands analyzed here have never before been characterized in the cervical mucosa. To determine whether the immune milieu was associated with subsequent clearance or persistence of HPV, the women were assessed for HPV diagnosis and clinical follow-up at 23.8 months following the baseline sample. Seven women were thus identified who either failed to clear their HPV infection or acquired a new HPV type, and 5 women who succeeded in

HPV clearance. Immunological host factors significantly associated with HPV persistence or clearance could not be identified. There was however a trend of CD19 mRNA levels above median being associated with type-specific HPV persistence which is subject to further investigations. We considered the possibility of false HPV DNA negativity in the liquid-based cytology sample of one case (id code no. 2) with several signs of inflammatory activation in the cervical biopsy. However, the test was negative for HPV DNA at inclusion as well as at follow-up, and the sensitivity of Linear Array is very high (44,45). None of the women had a clinically overt lesion; thus, we may not have taken the biopsy at the site of infection/inflammation. In addition, epithelial mRNA expression may dilute out differences in the immunologic microenvironment. Thus, future studies should consider including biopsies from several sites of the ectocervix and perform mRNA quantification on microdissections of such biopsies. The limited sample size of 24 women may also have hampered our ability to detect a small difference between groups. Nevertheless, major differences may be visible even between small groups of women. Most importantly the present study will serve as a pilot study.

In the present study, we have measured the mRNA expression of cytokines, phenotypic immune cell markers, immunoregulatory receptors and ligands, and immunoglobulins involved in mucosal immunity within the ectocervix of HPV-positive and -negative women. The fact that no significant differences were found in the two groups of women suggests that the immune response may not be activated in the cervixes of HPV-positive women without CIN. The level of mRNA expression of these substances was neither predictive of HPV clearance, although the association between B cell maturation and persistent HPV infection is worth further exploration.

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