

# Elevated Systemic Levels of Inflammatory Cytokines in Older Women with Persistent Cervical Human Papillomavirus Infection

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## Abstract

**Background:** Defects in lymphoproliferative responses to mitogens/antigens in women >45 years old with a persistent type-specific human papillomavirus (HPV) infection have been reported.

**Methods:** To determine whether these defects were associated with altered cytokine profiles, plasma and peripheral blood mononuclear cell (PBMC) culture supernatants from 50 cases (oversampled for their reduced lymphoproliferative ability) and 50 uninfected controls (oversampled for their robust lymphoproliferative ability) were examined for 24 cytokines using multiplexed bead-based immunoassays and ELISA.

**Results:** The following plasma cytokines were significantly increased in cases relative to controls (cases versus controls; median pg/mL): interleukin (IL)-6, 393.1 versus 14.5; IL-8, 1,128.5 versus 43.9; tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), 164.1 versus 9.2; macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), 1,368.9 versus 25.5; granulocyte macrophage colony-stimulating factor (GM-CSF), 13.8 versus 7.3; IL-1 $\beta$ , 8.3 versus 1.6 (all  $P < 0.0001$ ); and IL-1 $\alpha$ , 218.2 versus 169.5 ( $P = 0.02$ ). We focused our analysis on the cytokines IL-6, IL-8, TNF- $\alpha$ , and MIP-1 $\alpha$  due to their high fold change (>10) and highly statistically significant difference between cases and controls. Length of persistence or type of infection (high risk and low risk) did not affect these differences. IL-6, TNF- $\alpha$ , and MIP-1 $\alpha$  levels were also increased in unstimulated PBMC culture supernatants from cases compared with controls ( $P < 0.05$ ), however, the cytokine levels from phytohemagglutinin-stimulated PBMC culture supernatants were significantly lower in the cases ( $P < 0.0001$ ).

**Conclusions:** Persistent HPV infection in older women with evidence of immune deficit is associated with an increase in systemic inflammatory cytokines.

**Impact:** Future studies are needed to determine whether the inflammatory profile is age dependent and to examine the role that inflammatory cytokines play in HPV-induced progression from infection to cervical cancer. *Cancer Epidemiol Biomarkers Prev*; 19(8); 1954–9. ©2010 AACR.

## Introduction

Cervical cancer and its precursor lesions have been extensively shown to be caused by human papillomaviruses (HPV; refs. 1, 2). The prevalence of HPV infections in women peaks shortly after sexual debut (20–25 years old) and declines thereafter. In many regions of the world, a second wave of increased HPV prevalence has been described among women older than 55 (3) and postulated to be the result of factors such as age-

induced immune suppression or cervical changes that occur with menopause.

We have previously observed a decrease in mitogen and recall antigen lymphoproliferation among women persistently infected with HPV (4), suggesting that HPV persistence in the cervix is associated with a decrease in peripheral immune fitness.

Several factors can lead to a decrease in peripheral immune responsiveness. Cytokines and chemokines are essential for a multitude of immune-related activities such

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as recruiting phagocytes to areas of insult, stimulating the expansion of T cells and B cells on antigen recognition, and regulating the activation state of the immune system (5-7). Previous studies have noted associations between cytokine alterations and various infection-related states.

To determine biomarkers of immune suppression in older women who are persistently infected with HPV, we evaluated cytokine profiles in a subpopulation of older women with evidence of persistent infection with HPV and similarly aged women without HPV infection.

## Materials and Methods

Participants in this study were selected from a population of 10,049 women who participated in a population-based natural history cohort study of HPV and cervical neoplasia in the province of Guanacaste, Costa Rica. Details on the design and methods of the main cohort and the nested case-control study evaluating lymphoproliferative responses among HPV-positive and HPV-negative older women have been published (4, 8, 9). Briefly, we previously conducted a nested case-control study to examine lymphoproliferative responses within the Costa Rican natural history cohort where a group of women ( $n = 284$ ) older than 45 who were infected with HPV were compared with a similarly sized control group of HPV-negative women ( $n = 291$ ) of the same age distribution. Testing was done on peripheral blood mononuclear cells (PBMC) collected at the final visit of natural history cohort study (7-9 years after enrollment).

For the present evaluation, we were interested in screening for possible immune biomarkers associated with reduced lymphoproliferative ability among women with persistent HPV infection. We therefore selected a weighted subpopulation of women previously evaluated as part of the older women nested study described above based on the following criteria: cases ( $n = 50$ ), women with type-specific persistent HPV infection as denoted from PCR results obtained at enrollment, follow-up (5-6 years after enrollment), and final visit (~9th year after enrollment) and weak lymphoproliferative responses to phytohemagglutinin (PHA) or HPV16 L1 VLP; controls ( $n = 50$ ), women with no evidence of HPV infection at follow-up (5-6 years after enrollment) and final visit (7-9 years after enrollment) and strong lymphoproliferative responses to PHA and HPV16 L1 VLP. The characteristics of the selected cases and controls are described in Supplementary Table S1. All participants provided informed consent, and this study was approved by the U.S. National Cancer Institute and Costa Rica INCIENSA ethical committees.

HPV DNA testing was evaluated at enrollment, during follow-up (5-6 years after enrollment), and at the final study visit (~9th year after enrollment) as reported (4), PCR testing was done with the MY09/11 consensus primers and AmpliTaq Gold polymerase, and dot blot hybridization was used for genotyping

(10). The control group (HPV DNA negative women) is defined as not having a type-specific persistent infection at two essential time points within the natural history cohort study of HPV and cervical neoplasia [follow-up (5-6 years after enrollment) and final visit (7-9 years after enrollment)]. Although the controls were HPV DNA negative at these time points, some ( $n = 6$ ) had transitory HPV infections as detected during additional clinic visits between enrollment and final visit. The cases were divided into two groups: long-term persistors ( $n = 21$ ; median persistence, 108 months) and short-term persistors ( $n = 29$ ; median persistence, 19 months). The long-term persistors have a type-specific HPV DNA positive result at enrollment, follow-up (5-6 years after enrollment), and final visit (7-9 years after enrollment), and the short-term persistors have a type-specific HPV DNA positive result at follow-up (5-6 years after enrollment) and final visit (7-9 years after enrollment). Furthermore, we classified women as being infected with a high-risk HPV type if they were positive for HPV type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, or 68 and as being infected with a low-risk HPV type if they were positive for HPV type 6, 11, 26, 32, 34, 40, 42, 53, 54, 55, 57, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 85, or 89. Four women were excluded from the high-risk and low-risk analyses because they had high-risk and low-risk persistent infections.

All heparinized plasma specimens were collected at the final visit of the natural history cohort study of HPV and cervical neoplasia (~9 years following enrollment). The plasma was tested blindly for 22 cytokines and chemokines [interleukin (IL)-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-1 $\alpha$ , IFN- $\gamma$ , granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), IFN-inducible protein 10 (IP-10), RANTES, eotaxin, granulocyte colony-stimulating factor (G-CSF), IL-12, IL-15, IL-7, and IL-1 $\beta$ ] using the Linco-plex assay (Linco Research, Millipore). Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) was measured by ELISA (Biosource). IFN- $\alpha$  was measured as a single analyte in a bead array (Biosource). Cell supernatants from PBMCs incubated with AIM V medium or PHA for 48 hours were also assayed with the aforementioned cytokine assays. Specimens that were below the minimum detectable limit for any cytokine were assigned a value of 1/2 the lowest detectable limit.

The cytokine data were not normally distributed. The Wilcoxon rank-sum nonparametric test was used for all analyses comparing cases and controls.  $P < 0.05$  was considered significant.

## Results

The mean, median, and range of responses for each of the 24 different cytokines tested are shown in Supplementary Table S2. The following plasma cytokines were

**Table 1.** Elevated plasma cytokine levels from women with persistent HPV infection

	HPV-*	HPV+†
<b>IL-6</b>		
<i>n</i>	50	50
Responders (%)	50	96
Mean	206.1	715.5
Median	14.5	393.1
Range	8.0-3,202.5	8.0-3,338.0
<i>P</i> ‡		<0.0001
<b>IL-8</b>		
<i>n</i>	50	50
Responders (%)	96	100
Mean	392.5	1,545.7
Median	43.9	1,128.5
Range	1.6-3,615.8	17.8-7,535.2
<i>P</i>		<0.0001
<b>IL-1α</b>		
<i>n</i>	50	50
Responders (%)	100	100
Mean	244.8	328.5
Median	169.5	218.2
Range	52.4-2,714.8	57.6-2,113.9
<i>P</i>		0.02
<b>GM-CSF</b>		
<i>n</i>	50	50
Responders (%)	100	100
Mean	14.5	33.6
Median	7.3	13.8
Range	2.1-293.5	4.4-886.4
<i>P</i>		<0.0001
<b>TNF-α</b>		
<i>n</i>	50	50
Responders (%)	96	100
Mean	61.4	189.1
Median	9.2	164.1
Range	0.8-833.6	12.3-608.1
<i>P</i>		<0.0001
<b>MIP-1α</b>		
<i>n</i>	50	50
Responders (%)	54	100
Mean	576.0	2,164.9
Median	25.5	1,368.9
Range	8.0-10,097.2	17.9-10,097.2
<i>P</i>		<0.0001

significantly increased from cases relative to controls (median among cases, median among controls, *P* value for IL-6: 393.1, 14.5, *P* < 0.0001; for IL-8: 1,128.5, 43.9, *P* < 0.0001; for TNF-α: 164.1, 9.2, *P* < 0.0001; for MIP-1α: 1,368.9, 25.5, *P* < 0.0001; for GM-CSF: 13.8, 7.3, *P* < 0.0001; for IL-1β: 8.3, 1.6, *P* < 0.0001; and for IL-1α: 218.2, 169.5, *P* = 0.02; Table 1). Next, we focused our analysis on the cytokines IL-6, IL-8, TNF-α, and MIP-1α

**Table 1.** Elevated plasma cytokine levels from women with persistent HPV infection (Cont'd)

	HPV-*	HPV+†
<b>IL-1β</b>		
<i>n</i>	50	50
Responders (%)	48	84
Mean	6.6	23.6
Median	1.6	8.3
Range	1.6-73.9	1.6-536.5
<i>P</i>		<0.0001

\*HPV- women (controls) were negative for any HPV type at follow-up (5-6 y after enrollment) and final visit (9 y after enrollment).

†HPV+ women (cases) tested positive on at least the follow-up visit (5-6 y after enrollment) and final visit (9 y after enrollment) for a type-specific HPV infection.

‡The *P* value was calculated using the Wilcoxon rank-sum test to compare cytokine values between cases and controls.

due to their high fold change (>10) between controls and cases and having a highly statistically significant difference between controls and cases. The length of persistence or type of infection (high versus low risk; Fig. 1A-D) did not affect these differences. The predominance of inflammatory cytokines in the plasma led us to examine the transient secretion of cytokines from 48-hour cultured PBMCs to determine whether the levels of these cytokines produced by PBMCs paralleled those observed in plasma. The same inflammatory cytokines were also increased in unstimulated PBMC culture supernatant fluid from cases compared with controls (median among cases, median among controls, *P* value for IL-6: 471.4, 13.8, *P* < 0.0001; for TNF-α: 52.3, 11.8, *P* = 0.01; and for MIP-1α: 503.3, 8, *P* < 0.0001) except for IL-8 (6,360.9, 4,663.2, *P* = 0.09) as shown in Fig. 2A. Finally, we measured the concentration of cytokines in supernatants from PHA-stimulated PBMC cultures. In contrast to the results from the supernatants from the 48-hour culture of PBMCs with medium alone, the levels IL-6, IL-8, MIP-1α, and TNF-α measured in supernatants collected from PHA-stimulated PBMC cultures were significantly lower in cases (median among cases, median among controls, for IL-6: 1,287.2, 3,122.8; for IL-8: 14,059.8, 45,160.3; for TNF-α: 993.8, 3,312.1; for MIP-1α: 3,372.3, 9,449.3; *P* < 0.0001 for all analytes; Fig. 2B) compared with controls.

## Discussion

We have previously observed that older women who were persistently infected with HPV had a marked

decrease in lymphoproliferation in response to PHA, FLU, and HPV16 L1 VLP (4). To investigate possible underlying mechanisms for this phenomenon, we evaluated a weighted subset of participants from our previous study and explored whether alterations in their cytokine profile might account for the increased risk of HPV persistence observed among women with low proliferative response. Our results suggest that the cases have markedly increased levels of inflammatory cytokines as indicated by the significant increase in IL-6, IL-8, TNF- $\alpha$ , and MIP-1 $\alpha$  levels as compared with controls. These were the only peripheral markers we have noted to be markedly altered in this group of persistently infected women with evidence of immune dysfunction *in vitro*.

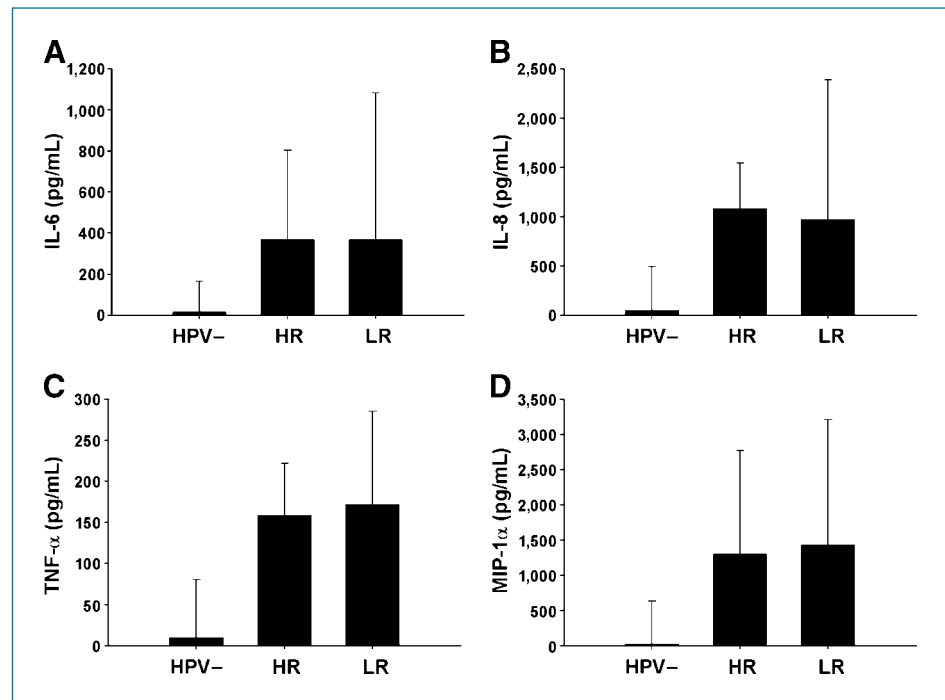
The finding of elevated systemic levels of inflammatory cytokines in women with persistent cervical HPV infections and marked decrease in immune function is novel and somewhat unexpected because there is little evidence for an HPV-induced systemic inflammatory reaction. Also, HPV infections described here are believed to remain localized to the cervix. Studies have shown that persistent HPV infection may lead to local immune tolerance (11), which suggests that local inflammation at the cervix would be minimal as well as the markers of peripheral inflammation. In addition, several studies have described HPV-induced anti-inflammatory mechanisms such as HPV16 E6 protein inhibiting the expression and signaling of IFNs (12) and IL-18 (13). Second, HPV does not have a lytic life cycle within differentiating keratinocytes that would induce an inflammatory response. Interestingly, the measured in-

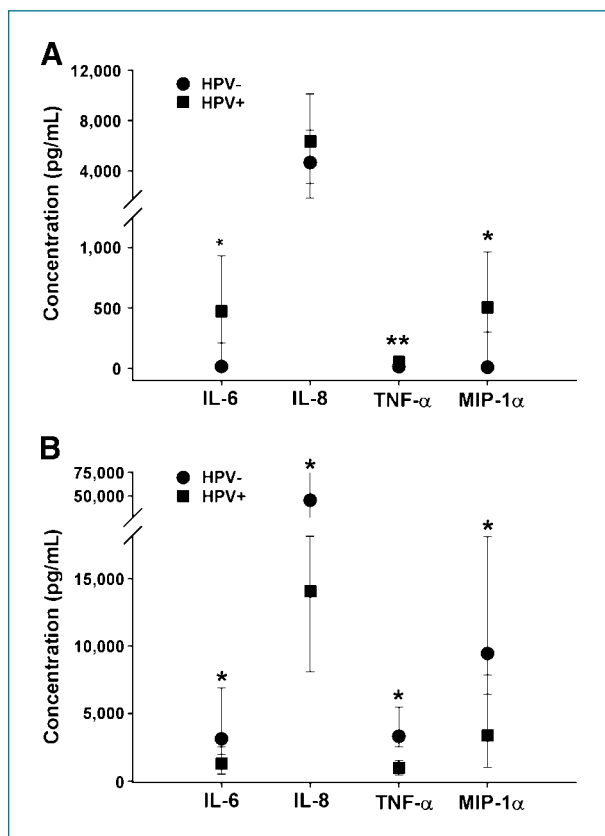
flammatory response in the periphery is not restricted to individuals infected with "high-risk" HPV types such as HPV16, HPV18, HPV31, and HPV45. We observed that women infected with "low-risk" HPV types also had a marked increase in IL-6, IL-8, TNF- $\alpha$ , and MIP-1 $\alpha$  when compared with controls. This finding suggests that the peripheral inflammatory response is not influenced by the oncogenicity of the HPV type involved. Although increased levels of inflammatory cytokines have been previously noted in a study of cervical cancer (14), women in our current study were healthy and had normal cervical cytology at each visit.

Our study focused on comparing cytokine measures from age-matched cases and controls, suggesting that the presence of inflammatory cytokines in the periphery is associated with HPV persistence and weak lymphoproliferative responses, but not necessarily age. Because women ages 20 to 30 years old have the highest prevalence of HPV infection (15-18) and also are most likely to progress to CIN3 (19), it would be important to conduct a longitudinal study in younger women (20-30 years old) to define the associations between lymphoproliferative responses, inflammatory cytokine levels, and HPV persistence to track these events early on.

One of the limitations of our study is that the samples were all collected at the final visit. Future longitudinal studies will help define whether the increases in inflammatory cytokine levels observed are constitutive or induced following a persistent HPV infection. Furthermore, we do not know whether the inflammatory cytokines in the present study place women at risk

**Figure 1.** Comparisons of (A) IL-6, (B) IL-8, (C) TNF- $\alpha$ , and (D) MIP-1 $\alpha$  levels in plasma from women with high-risk (HR) or low-risk (LR) HPV type-specific persistent infection. HR and LR were each compared with the HPV- control group. There was not a significant difference between the HR and LR groups for the cytokines shown. All comparisons between HPV- and HR or LR were highly significant ( $P < 0.0001$ ) using the Wilcoxon rank-sum test. Vertical bars represent 75th percentile.





**Figure 2.** A, cytokine levels in supernatants collected from 48-h unstimulated PBMCs. Pooled supernatants from  $2 \times 10^5$  PBMCs cultured in AIM V medium for 48 h were collected and frozen at  $-80^\circ\text{C}$ . B, cytokine levels in supernatants collected from 48-h PHA-stimulated PBMCs. Pooled supernatants from  $2 \times 10^5$  PBMCs stimulated with PHA ( $2 \mu\text{g}/\text{mL}$ ) for 48 h were collected and frozen at  $-80^\circ\text{C}$ . Samples were tested with Linco-plex bead assay to examine the cytokine concentrations in the supernatants. Bars represent interquartile range. \*,  $P < 0.0001$ ; \*\*,  $P < 0.01$ , between HPV- (controls) and HPV+ (cases) groups for each cytokine (Wilcoxon rank-sum test).

for progression to CIN3 or if they are beneficial to these women.

There are several possible hypotheses about why cases have increased levels of systemic inflammatory cytokines, such as the presence of simultaneous infections with other microbial agents, which deserve attention in future studies. For example, chronic viral infections such as hepatitis B and C have been shown to induce inflammatory cytokines such as IL-8, TNF- $\alpha$ , and IL-6 in the

periphery (20, 21). Furthermore, high body mass index has been associated with an increase in peripheral inflammation in some studies (22, 23). Future studies are needed to define the potential role of concomitant infections and obesity in the inflammation and immune deficit seen in this subset of older women with persistent HPV infection.

Another hypothesis about why proliferation was significantly lower in the cases is that their PBMCs may be exhausted or more susceptible to cell death due to the peripheral cytokine milieu. Support for this hypothesis comes from our cytokine results that showed significantly lower concentrations for all of the cytokines tested in the supernatants from PHA-stimulated PBMCs. To find a dramatic decrease in nearly all of the cytokine values, we would have to suspect a global effect on the cells such as an increased susceptibility to cell death or anergy. Due to the limited number of cells isolated from our study participants, follow-up analysis of cell exhaustion or cell death mechanisms will require future studies to examine this hypothesis thoroughly.

In conclusion, we have described an increase in inflammatory cytokine levels among women persistently infected with HPV, with evidence of marked decrease in immune function. Future studies are needed to determine the role of this inflammatory cytokine profile in progression to precancer and cancer and whether this inflammatory profile is present in persistent infections in women from this cohort with preserved immune function and in younger women.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# BLOOD CANCER DISCOVERY

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