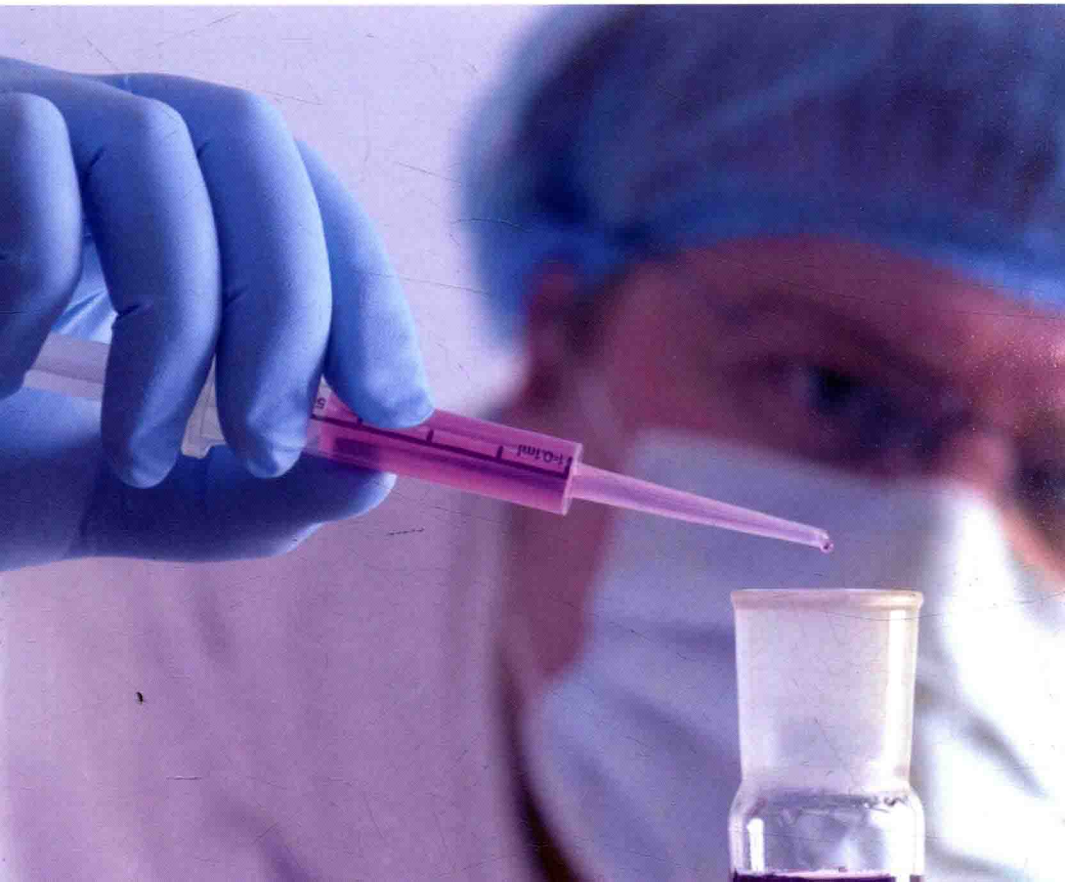
Essentials in Immunology

Infections, Cancer and Inflammations

Jim Wang



Essentials in Immunology: Infections, Cancer and Inflammations

Edited by **Jim Wang**

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Preface

The purpose of the book is to provide a glimpse into the dynamics and to present opinions and studies of some of the scientists engaged in the development of new ideas in the field from very different standpoints. This book will prove useful to students and researchers owing to its high content quality.

Immunology is a branch of biomedical sciences to study the immune system physiology in both diseased and healthy states. Some aspects of autoimmunity enable us to understand that it is not always related to pathology. For example, autoimmune reactions are effective in clearing off the unwanted, excess or aged tissues from the body. Also, autoimmunity occurs after the exposure of the non-self-antigen which is structurally similar to the self, assisted by the stimulatory molecules such as cytokines. Therefore, it can be said that there's a minor difference between immunity and auto-immunity. The question of how physiologic immunity changes to pathologic autoimmunity continue to interest researchers. Answer to such questions can be found by understanding physiology of the immune system. This book covers various topics about immunology, its related aspects and pathologies under two sections namely, Immunology of Viruses & Cancers and Basics of Autoimmunity & Multiple Sclerosis. The contributors of this book have carefully selected topics which would be of reader's interests.

At the end, I would like to appreciate all the efforts made by the authors in completing their chapters professionally. I express my deepest gratitude to all of them for contributing to this book by sharing their valuable works. A special thanks to my family and friends for their constant support in this journey.

Editor

Contents

Section 1

Immunology of Viruses and Cancer

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Cytokines and Markers of Immune Response to HPV Infection

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1. Introduction

Cervical cancer is the third most commonly diagnosed cancer in women worldwide (Ferlay, Shin et al. 2010) and is a result of infection with cancer-causing types of human papillomavirus (HPV) (Bouvard, Baan et al. 2009). HPV is a very common infection, although in most circumstances, infection does not usually result in cervical disease (Trottier and Franco 2006). In fact, the natural history of HPV infection suggests that additional factors are required to drive progression from infection to the development of cancer. Most women are thought to clear their HPV infections within two years, but in approximately 10% of women, infection persists (Schiffman, Castle et al. 2007). Persistent HPV infection is, in effect, the strongest risk factor for progression to cervical precancer and cancer (Koshiol, Lindsay et al. 2008), and a dysfunctional immune response is likely to underlie the amplified risk that leads to HPV persistence and cervical cancer. Although efficacious prophylactic vaccines against the two types of HPV (16 and 18) that cause about 70% of cervical cancers (Munoz, Castellsague et al. 2006) are available, these vaccines are expensive, difficult to administer in poorer countries and will not protect women who have already been exposed to the virus (FUTURE II Study Group 2007; Hildesheim, Herrero et al. 2007) (Su, Wu et al. 2010). Thus, it is important to understand factors that predispose some women infected with a carcinogenic HPV infection to persist and progress.

HPV uses a variety of methods to avoid immune detection, such as maintaining an unobtrusive infectious cycle (e.g., non-viremic and non-cytolytic since replication occurs in cells already destined for natural cell death), suppressing interferon response, and down-regulating toll-like receptor (TLR)-9 (Stanley 2010). By employing such immune evasion tactics, HPV infection itself does not lead to a direct or obvious inflammatory response. Rather, inflammation due to other co-factors such as smoking, parity, oral contraceptive use, co-infection with other sexually transmitted diseases, multiple sexual partners etc. have long been hypothesized to lead to HPV incidence, persistence, and progression to cervical pre-cancer and cancer (Castle and Giuliano 2003). Studies that directly evaluate women's immune response to HPV infection may provide better insights into the role of inflammation and immunity in HPV persistence and cervical carcinogenesis.

Although humoral response to HPV infection has been well-characterized (Bhat, Mattarollo et al. 2011), cell-mediated response has not been well established. Numerous approaches have

been used to characterize cell-mediated immune responses to HPV. Such approaches include measurement of cytokines and other immune markers that commonly lead to infiltration of immune cells. Cytokines are pleiotropic glycoproteins that regulate cell survival, proliferation, differentiation and activation at both local and systemic levels. During inflammation, their excessive release may lead to both chronicity and pathogenicity. The purpose of this review is to describe the current state of knowledge regarding these important regulators or other important immune markers of cell-mediated immune response in HPV infection. To this end, we have evaluated studies in plasma or serum from peripheral blood, in cervical secretions, in unstimulated and stimulated PBMCs (and cellular subsets thereof), and in cervical tissues themselves. Importantly, this chapter will highlight not only the large amount of knowledge gained from these studies, but also the many scientific gaps in knowledge that remain.

2. Methods

Relevant studies were identified by searching MEDLINE (via PubMed) using broad search term categories for cervix and immunity (Appendix 1). The search included studies identified through 3 November 2011. Studies that evaluated cell-mediated immune response immune response by HPV status (positivity, persistence, or clearance) were included if there were at least 10 women in each comparison group (usually HPV-positive versus HPV-negative; sometime HPV persistence versus clearance or difference by HPV type). To focus on more functional aspects of immune response, only studies of immune-related proteins and mRNA (evidence of expression) and studies with HPV DNA detection were included. Studies were excluded if the HPV status and disease status of the referent group was unclear or if they focused on DNA polymorphisms alone. Given the focus on HPV infection, studies were also excluded if they include cervical cancer patients, but no other groups [i.e. normal women, women with low-grade squamous intraepithelial lesions (LSIL) or cervical intraepithelial neoplasia (CIN)]. Studies that included some cervical cancer patients along with CIN or normal patients were retained. Post-treatment studies or studies involving mice, cell lines, or HPV at extra-cervical anatomical sites were excluded as well.

Data were abstracted on the study characteristics, HPV measurement, immune marker measurement, and results pertinent to this review. Study characteristics included the country in which the study was conducted, the method of cervical secretion collection, and descriptions of comparison groups relevant for this review (e.g., women with incident HPV versus no HPV). The assay used to detect HPV was also noted. Immune marker-related data included the assay used to measure the immune marker and the specific markers measured, along with the results. Approximately 50% of studies were double abstracted.

3. Literature review

In total, 35 studies met our inclusion criteria. These studies fell into four broad categories (Tables 1 to 4): circulating immune markers in plasma or serum (N = 7), those secreted locally in the cervix (N = 7), immune responses in patient-derived PBMCs (N = 10), and tissue-based immune markers (N = 12). One study contributed to both the circulating and PBMC-based immune marker categories.

Circulating Immune Markers in Plasma/Serum. Cytokines and soluble immune markers are increasingly being measured in readily accessible plasma and serum in the hope that they will provide useful diagnostic and prognostic information, as well as insight into the pathogenesis

of numerous diseases. Further, the availability of inexpensive enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and other bioassays to reliably measure cytokines in these samples make them enticing targets for discovery. Currently, seven studies that met our inclusion criteria have directly examined HPV-infection-related immune responses in either serum or plasma (Table 1). All of these studies have focused on associations with carcinogenic infection using a Hybrid Capture assay. Hildesheim et al. (Hildesheim, Schiffman et al. 1997) was among the first to use plasma to evaluate markers of immunity. However, their comparison of carcinogenic HPV positive women with low-grade lesions to carcinogenic negative women with low-grade lesions failed to find a statistical difference in the soluble IL-2 receptor (sIL-2R; p=0.63). Adam et al. (Adam, Horowitz et al. 1999) similarly compared 10 women with high risk HPV infection to 10 HPV negative women and reported that high risk HPV infection was indeed associated with higher mean serum CSF-1 levels. Abike et al. (Abike, Engin et al. 2011) measured neopterin, often considered a marker of immune activation, and found lower concentrations in HPV-positive versus HPV-negative women with normal through high-grade histology. Unlike the earlier studies, Bais et al. (Bais 2005) measured numerous cytokines simultaneously (IL-2, IL-4, IL-10, IL-12, IFN-γ, TNF-α), as well as soluble markers (sTNFRI and sTNFRII) in plasma. They discovered that higher mean IL-2 levels alone were associated with carcinogenic HPV positivity. Baker et al. (Baker, Dauner et al. 2011) evaluated eleven circulating markers (adiponectin, resistin, tPAI-1, HGF, TNF-α, leptin, IL-8, sVCAM-1, sICAM-1, sFas, MIF) and found elevated levels of resistin [odds ratio(OR) for 3rd versus 1st tertile, 103.3; 95 confidence interval (CI), 19.3–552.8; $P < 0.0001$], sFas (OR, 4.2; 95% CI, 1.5–11.7; $P = 0.003$), IL-8 (OR, 59.8; 95% CI, 11.4–312.5; $P < 0.0001$), and TNA-α (OR, 38.6; 95% CI, 9.1–164.3, $P < 0.0001$) were in women with persistent HPV infection compared to HPV-negative women. Kemp et al. (Kemp, Hildesheim et al. 2010) evaluated an even broader spectrum of cytokines in their comparison of 50 HPV-positive women older than 45 years and 50 HPV-negative similarly aged women from their population-based cohort study in Guanacaste, Costa Rica. Plasma levels of IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-1α, IFN-γ, GM-CSF, TNF-α, MCP-1, MIP-1α, IP-10, RANTES, eotaxin, G-CSF, IL-12, IL-15, IL-7, and IL-1β were measured by Lincoplex assay, IFN-α was measured by bead array, and TGF-β1 was measured by ELISA. Their analysis revealed statistically significant differences between cases and controls in levels of IL-6, IL-8, TNF-α, and MIP-1α, GM-CSF, IL-1β (all $P < 0.0001$) and IL-1α ($P = 0.02$). However, it should be noted that this study was intentionally designed to explore differences between the extremes of the immunological spectrum. Thus, differences between these groups are likely to be biased away from the null (upward) in comparison to the general population. All six of these studies failed to concurrently evaluate potential confounders, and with the possible exception of TNF-α, none of their findings have been confirmed by other studies.

Unlike the other studies, Hong et al. (Hong, Kim et al. 2010) evaluated several potential confounders (parity, menopausal status, smoking, oral contraceptive use, histological findings of colposcopic-directed biopsy) in their recently published report of HPV persistence and clearance among 160 carcinogenic HPV positive Korean women (normal women or women with histologically confirmed mild dysplasia). While their univariate analysis revealed that the number of women who were serum negative for TNF-α was significantly higher in the carcinogenic HPV clearance group (N=107) than their persistence group (N=53, $P = 0.0363$), their multivariate logistic regression analysis indicated that none of the four cytokines measured (IFN-γ, TNF-α, IL-6, and IL-10) had a significant association with clearance of the

carcinogenic HPV infection, pointing to the importance of these factors in future study design. In fact, they found that only age was significantly associated with clearance of carcinogenic HPV infections (OR, 0.95; 95% CI, 0.92- 0.98; P = 0.001).

Author & Year	Study source (Origin Country)	Immune Marker	HPV -/+ N (Measurement Method)	Major Conclusions
Hildesheim 1997	Kaiser Permanente clinics (US)	CellFree IL-2R test kits for sIL-2R from plasma recovered by centrifugation of peripheral blood	45/60 (Hybrid Capture)	No statistically significant association between sIL-2R and high risk HPV positivity in plasma.
Adam 1999	Centers for Disease Control collection (United States and Panama)	ELISA for Macrophage colony-stimulating factor (CSF-1) in serum	10/10 (ViraPap + ViraType dot blot hybridization assay for screen positives)	High-risk HPV infection is associated with higher mean serum CSF-1 levels.
Bais 2005	Outpatient GYN clinic (The Netherlands)	ELISA for IL-2, IL-4, IL-10, IL-12, IFN-γ, TNF-α, sTNFRI, sTNFRII in plasma and leucoctye count for leucocytes, neutrophils, monocytes, and lymphocytes in peripheral venous blood	11/10 (GP5+/GP6+ PCR)	High-risk HPV infection is associated with higher mean plasma IL-2 levels.
Hong 2010	University hospital and women's health center (Korea)	ELISA for IFN-γ, IL-6, IL-10, TNF-α in serum	0/160* (Hybrid Capture 2)	Based on univariate analysis, the number of women that were serum negative for TNF-α was significantly higher in the high risk HPV clearance group than the persistence group (P = 0.0363). Based on multivariate logistic regression, none of the 4 cytokines had a significant association with clearance of the high risk HPV infection. Only age was significantly associated with clearance of the high risk HPV infection (OR, 0.950; 95% confidence interval, 0.92-0.98; P = 0.001).**
Kemp 2010	Population-based cohort (Costa Rica)	Linco-plex assay for IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-1α, IFN-γ, GM-CSF, TNF-α, MCP-1, MIP-1α, IP-10, RANTES, eotaxin, G-CSF, IL-12, IL-15, IL-7, and IL-1β; ELISA for TGF-β1; single analyte in a bead array for IFN-α.	50/50 (MY09/11 PCR, dot blot hybridization for genotyping)	Persistent HPV infection in older women with evidence of immune deficit is associated with an increase in systemic inflammatory cytokines and weak lymphoproliferative responses.
Abike 2011	GYN Department (Turkey)	ELISA for neopterin in serum	78/44 (Amplisense HPV multiplex PCR typing kit)	Neopterin levels were lower in women with HPV than women without HPV.
Baker 2011	Population-based cohort (Costa Rica)	Millipore Multiplex Bead Assay for adiponectin, resistin, tPAI-1, HGF, TNF-α, leptin, IL-8, sVCAM-1, sICAM-1, sFas, MIF in PBMCs from heparinized blood	50/50 (MY09/11 PCR, dot blot hybridization for genotyping)	Resistin, sFas, IL-8, and TNA-α were elevated in women with persistent HPV infection compared to HPV-negative women.

* Compared HPV persistence and clearance. Thus, all were HPV-positive at baseline. **Adjusted for age, parity, menopause, oral contraception, histological findings of colposcopic-directed biopsy, and cytokines. Abbreviations: US = United States, HPV = human papillomavirus, DNA = deoxyribonucleic acid, GYN = Gynecology, PCR = polymerase chain reaction, ELISA = enzyme-linked immunosorbent assay, PBMCs = peripheral blood mononuclear cells

Table 1. Studies of circulating immune markers in plasma and serum.

Local Immune Marker Secretions in the Cervix. It is believed that measurement of cytokines in cervical secretions may better reflect local cytokine production relevant to cervical carcinogenesis than circulating cytokines. Currently, seven studies that met our

inclusion criteria have measured immune responses in cervical secretions (Table 2). Unlike the studies of circulating cytokines above, most of these studies have tested for a broad range of HPV types, although one (Guha and Chatterjee, 2009) only tested for carcinogenic HPV types using the Hybrid Capture 2 assay, and another only analyzed results for women with carcinogenic HPV infection compared to women without carcinogenic HPV infection (Marks, Viscidi et al. 2011). Scott et al. (Scott, Stites et al. 1999) evaluated RNA expression of IL-4, IL-12, IFN-γ, and TNF and found that a T-helper type 1 (TH1) cytokine expression pattern (as defined by IFN-γ and TNF positivity and IL-4 negativity, with variable IL-12 expression) preceded HPV clearance. Crowley-Nowick et al. (Crowley-Nowick, Ellenberg et al. 2000) measured IL-2, IL-10, and IL-12 cytokine levels in HIV-positive and HIV-negative adolescents recruited from 16 clinical care settings in 13 US cities. Crowley-Nowick et al. found that HPV-positive girls had higher IL-12 concentrations compared to HPV-negative women ($P = 0.01$). Race, age, SIL status, smoking, other vaginal infections, and CD4 count were considered as potential confounders, but all were dropped out of the backwards regression model. Tjiong van der Vange et al. (Tjiong 2001) evaluated IL-12p40, IL-10, TGF-β1, TNF-α, and IL-1β levels by HPV status in CIN patients referred to an outpatient gynecology department. Similar to Crowley-Nowick et al., Tjiong van der Vange et al. found higher levels of IL-12 in HPV-positive compared to HPV-negative patients ($P=0.04$) (Tjiong 2001). However, no attempts were made to adjust for potential confounders. Unlike Crowley-Nowick et al. (Crowley-Nowick, Ellenberg et al. 2000) and Tjiong van der Vange et al. (Tjiong 2001), Gravitt et al. (Gravitt, Hildesheim et al. 2003) found no statistical differences in IL-10 and IL-12 concentrations by HPV-positivity versus HPV-negativity in women selected from a population-based cohort study in Guanacaste, Costa Rica, after adjusting for stage of menstrual cycle, recent oral contraceptive use secretion volume, and pH. Lieberman et al. (Lieberman, Moscicki et al. 2008) used a multiplex immunoassay kit to measure IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p40/p70), IL-13, IFN-γ in young women attending a family-planning clinic or university health center, or their friends. Although no significant differences were observed for women with incident or persistent HPV infections compared to women without HPV, there was some suggestion that IL-1β and IL-13 levels were reduced in women with incident or persistent HPV infections and that IL-6 and IL-2 levels were reduced in women with incident infections. Guha et al. (Guha and Chatterjee 2009) measured IL-1β, IL-6, IL-10, and IL-12 cytokine levels in commercial sex workers or spouses of HIV-positive men coming in for an HIV test. After taking HIV status into account, IL-1β, IL-10, and IL-12 seemed to be elevated in HPV-positive women compared to HPV-negative women. IL-6 was also higher in HPV-positive women compared to HPV-negative women ($P \leq 0.0004$). After stratifying by HIV status, however, IL-6 was only notably elevated in in women positive for both HPV and HIV, making the association with HPV less clear. This study also evaluated cytokine levels by abnormal versus normal cervical cytology and found that only IL-6 was related to abnormal cytology ($P = 0.03$). Finally, a recent study by Marks et al. (Marks, Viscidi et al. 2011) evaluated 27 different cytokines in a multiplex assay in cervical secretions from 35–60-year-old women attending outpatient obstetrics and gynecology clinics for routine examination. Similar to Gravitt et al. (Gravitt, Hildesheim et al. 2003) and Lieberman et al. (Lieberman, Moscicki et al. 2008), this study found no association between IL-12p70 and HPV status. However, IL-5 ($p = 0.03$), IL-9 ($p = 0.04$), IL-13 ($p = 0.01$), IL-17 ($p = 0.003$), EOTAXIN ($p = 0.04$), GM-CSF ($p = 0.01$), and MIP-1α ($p = 0.005$) levels were elevated in women with carcinogenic HPV infection compared to those without carcinogenic HPV. In addition, T-cell and pro-inflammatory cytokines tended to be correlated with EOTAXIN in women with carcinogenic HPV, while

they were correlated with IL-2 in women without carcinogenic HPV. The authors conclude that this shift from IL-2 to EOTAXIN may reflect a shift away from antigen-specific adaptive responses toward innate responses.

Author & Year	Study source (Origin Country)	Immune Marker Measurement	HPV -/+ N (Measurement Method)	Major Conclusions
Scott 1999	Family planning clinics (US)	RT-PCR of cDNA from total RNA for IL-4, IL-12, IFN-γ, TNF	13/22 (MY09/11 PCR)	HPV-positive subjects (especially those who cleared) tended to be IFN-γ positive, TFN positive, and IL-4 negative ("Th1 cytokine pattern").
Crowley-Nowick 2000	16 clinical care settings in 13 cities (United States)	ELISA for IL-2, IL-10, IL-12 in Weck-cel sponges	18/20 (PCR)	"Coinfection with HIV, human papillomavirus, and other STIs predicted the highest IL-12 concentrations."*
Tjiong 2001	GYN department (The Netherlands)	ELISA for IL-12p40, IFN-γ, IL-10, TGF-β1, TNF-α and IL-1β in cervical washes	13/50 (HPV-16-specific PCR; negative samples confirmed by CPI and CPIIG)	IL-12 was more often detected than in the HPV-DNA negative CIN patients (P=0.04, Chi Square test). No other significant associations between cytokine levels and the detection of HPV-DNA were found.
Gravitt 2003	Population-based cohort (Costa Rica)	ELISA for IL-10 & IL12 in Weck-cel sponges	194/51 (MY09/11 + reverse-blot hybridization	No significant association between HPV and IL-10 or IL-12.**
Lieberman 2007	Family-planning clinic or university health center or friends (US)	Protein Multiplex Immunoassay kits for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p40/p70), IL-13, IFN-γ in Merocel sponges	34/33 (PGMY09/11 PCR)	Although there were no significant differences between groups, IL-1β and IL-13 seemed to be depressed in women with incident or persistent HPV infections. IL-6 and IL-2 also seemed to be depressed in women with incident infections.
Guha 2009	Commercial sex workers or spouses of HIV+ men (India)	ELISA for IL-1β, IL-6, IL-10, IL-12 in lavage samples	28/17 (Hybrid Capture 2)	Taking HIV status into account, IL-1β, IL-10, and IL-12 seemed elevated in HPV+ vs. HPV- women. IL-6 seemed elevated when HIV was not taken into account (16.6 vs. 4.5 pg/ml, p≤0.0004), but otherwise was only notably elevated in women positive for both HPV and HIV.†
Marks 2011	Outpatient OB/GYN clinics (US)	Bio-Rad multiplex assay for BASICFGF, EOTAXIN, GCSF, GMCSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, VEGF in Merocel sponges	44/34 (Roche HPV Linear Array)	Carcinogenic HPV associated with elevated IL-5, IL-9, IL-13, IL-17, EOTAXIN, GM-CSF, and MIP-1α levels and a shift from IL-2 to EOTAXIN compared to no carcinogenic HPV, possibly reflecting a shift away from antigen-specific adaptive responses toward innate responses.

*Considered potential confounders, but all were dropped through backwards modeling. †Stratified by HIV status, but did not evaluate additional confounders. Abbreviations: US = United States, HPV = human papillomavirus, HIV = human immunodeficiency virus, CIN = cervical intraepithelial neoplasia, GYN = gynecology, OB/GYN = obstetrics and gynecology, PCR = polymerase chain reaction, qRT-PCR = quantitative reverse transcriptase PCR, STI = sexually transmitted infection

Table 2. Studies of immune markers in cervical secretions.

There is little consistency in the cytokines evaluated in these seven studies, but where there is overlap, the results tend to be contradictory. For example, one study found evidence that IL-6 levels were reduced in women with incident HPV infections (Lieberman, Moscicki et al. 2008), while another found that IL-6 levels tended to be elevated in HPV-positive women (Guha and Chatterjee 2009). Similarly, one study found no evidence that IL-12 levels varied by HPV status (Gravitt, Hildesheim et al. 2003), while two others (Crowley-Nowick, Ellenberg et al. 2000; Tjiong, van der Vange et al. 2001) observed higher levels of IL-12 in HPV-positive versus HPV-negative women. In addition, results from the study by Guha et al. (Guha and Chatterjee 2009) suggested a tendency toward increased levels of IL-1β in HPV-positive women versus HPV-negative women, while the results from Lieberman et al. (Lieberman, Moscicki et al. 2008) showed a trend toward decreased levels of IL-1β in women with incident or persistent HPV infection compared to HPV-negative women. These inconsistencies are not yet resolved.

Cytokine Responses in Patient-derived PBMCs. There is evidence that cell-mediated immune responses play an important role in the control of HPV infections. Cell-mediated immune responses are regulated by T lymphocytes [T-helper (Th) lymphocytes and cytotoxic lymphocytes (CTLs)] in cooperation with antigen-presenting cells such as monocytes and dendritic cells. These cells all are modulated by and release cytokines that can influence one another's synthesis. Characterization (including quality and quantity) of lymphocytes directed against HPV epitopes has been examined with the goal of providing insights into the clinical outcomes of HPV-positive patients. To this end, analyses of cytokines and concurrent lymphoproliferative and CTL responses in patient-derived peripheral blood mononuclear cells (PBMCs), T-cell fractions isolated from PBMCs or whole blood cultures after stimulation with several antigens and/or HPV peptides has been evaluated in 10 publications (Table 3).

Tsukui et al. (Tsukui, Hildesheim et al. 1996) was one of the first to measure IL-2 levels in culture supernatants of PBMCs stimulated with predominantly 15mer overlapping peptides from HPV-16 E6 and E7 oncoproteins. The HPV early proteins E2, E6 and E7 are among the first of proteins that are expressed in HPV-infected epithelia. Stimulation with influenza served as a specificity control, and stimulation with phytohemagglutinin (PHA) served as a positive control since it is known to activate lymphocytes and induce rapid cell proliferation as well as lead to the release of inflammatory and immune cytokines. While the report itself focused on associations with IL-2 and disease progression, the study included both HPV typing data and IL-2 response data for each subject included in the study. Interestingly, by using the data presented in the paper for statistical calculation, we found that IL-2 levels were significantly increased in a group of 32 HPV positive healthy women and women with LSIL compared to a group of 51 HPV negative healthy women and women with LSIL (P=0.006). Among 18 women with HSIL with HPV typing and adequate IL-2 data, only 2 women had positive IL-2 levels (1 HPV positive, 1 HPV negative).

Several other studies also attempted to evaluate IL-2 levels in a similar manner. deGruijl et al. (de Gruijl, Bontkes et al. 1998) examined IL-2 reactivity in PBMCs stimulated with HPV16 E7 and sorted by anti-CD4 or anti-CD8 antibodies. They found that positive CD4+ T helper cell IL-2 reactivity was restricted to patients infected by HPV16 and related types and that reactivity was strongly associated with HPV persistence. Further, women with cervical carcinoma showed IL-2 responses at a significantly reduced rate [7 of 15 (47%); P = 0.014].

Author & Year	Study source (Origin Country)	Immune Marker Measurement	HPV -/+ N (Measurement Method)	Major Conclusions
Tsukui 1996	Kaiser Permanent or Simmons Cancer Center (US)	IL-2 was measured by radioimmunoassay in culture supernatants of PBMCs from whole blood that were stimulated with 15mer HPV16 peptides to E6 and E7, or stimulated with FLU or PHA	56/40 (ViraPap: Hybrid Capture with HPV-16-specific Hybrid Capture for + samples. Tumors: GP5+/GP6+ PCR)	IL-2 is signficantly increased in healthy HPV+ women and HPV+ women with LSIL. Few women with HSIL or cancer have detectable IL-2 levels.
Kadish 1997	Colposcopy clinic (US)	Measured lymphocyte proliferation in HPV16 E6 and E7 peptide stimulated cultures of PBMCs from heparinized blood	26/51 (PCR and Southern Blot assay; typing by dot blot for 39 types)	Lymphoproliferative responses to specific HPV16 E6 and E7 peptides are significantly associated with the clearance of HPV infection.
de Gruijl 1998	Non-intervention cohort follow-up study of patients with cervical dysplasia plus follow-up study of HPV-positive women with normal cervical cytology (Netherlands)	IL-2 was measured by IL-2 bioassay in culture supernatants of PBMCs from heparinized blood that were stimulated with 14 different 20mer HPV16 peptides to E7, or stimulated with PHA; T cell subsets were depleted by magnetic bead sorting and anti-CD4 and anti-CD8 antibodies	15/51 (GP5+/GP6+ PCR)	Positive CD4+ T helper cell IL-2 reactivity was restricted to patients infected by HPV-16 and related types and showed a strong association with viral persistence. Women with cervical carcinoma showed IL-2 responses at a significantly reduced rate [7 of 15 (47%); P = 0.014].
Bontkes 1999	Non-intervention cohort follow-up study of patients with cervical dysplasia (Netherlands)	IL-2 was measured by IL-2 bioassay in culture supernatants of PBMCs from heparinized blood that were stimulated with HPV16 N-terminal and C-terminal E2 protein fragments or with PHA.	22/52 (GP5+/GP6+ PCR)	HPV16 infection was not associated with IL-2 responsiveness against the N-terminal domain of E2, but HPV clearance was associated with IL-2 responsiveness against the C-terminal E2 domain
de Gruijl 1999	Non-intervention cohort follow-up study of patients with cervical dysplasia (Netherlands)	IL-2 was measured by IL-2 bioassay in culture supernatants of PBMCs from heparinized blood that were stimulated with HPV16 L1-VLP or synthetic L1-derived 15-mer peptides P1 (amino acids 311-325) and P2 (amino acids 321-335), or stimulated with PHA; T cell subsets were depleted by magnetic bead sorting and anti-CD4 or CD8 antibodies. HPV-16 L1-VLP-specific plasma IgG was measured by ELISA.	15/49 (GP5+/GP6+ PCR)	IgG responses were significantly associated with HPV16 persistence but CD4 T helper IL-2 responses were significantly associated with both HPV clearance and persistence. Neither cell-mediated nor humoral immune responses against HPV16 L1 seemed adequate for viral control.

Abbreviations: US = United States, HPV = human papillomavirus, DNA = deoxyribonucleic acid, GYN = Gynecology, PCR = polymerase chain reaction, ELISA = enzyme-linked immunosorbent assay, PBMCs = peripheral blood mononuclear cells, FLU = influenze, PHA = phytohemagglutinin, LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, mCTLp = memory cytotoxic T-cell precursor

Table 3. Part 1. Cytokine Responses in Patient-derived PBMCs.

Author & Year	Study source (Origin Country)	Immune Marker Measurement	HPV -/+ N (Measurement Method)	Major Conclusions
Bontkes 2000	Non-intervention cohort follow-up study of patients with cervical dysplasia (Netherlands)	HPV16-specific mCTLp activity was measured in cultured PBMCs from heparinized blood stimulated with both HPV16 E6 and E7 peptides. IL-2 was measured by IL-2 bioassay in culture supernatants of PBMCs that were stimulated with 14 different 20mer HPV16 peptides to E7, or stimulated with PHA.	11/20 (GP5+/GP6+ PCR)	mCTLp activity was significantly associated with persistent HPV16 infection but not observed in HPV negative women or women with viral clearance. HPV16 E7-specific mCTLp activity was associated with previously published IL-2 release in response to HPV16 E7-derived peptides at the end of follow-up.
Molling 2007	Non-intervention cohort follow-up study of patients with cervical dysplasia (Netherlands)	Cultured PBMCs taken from heparinized blood were stimulated with 14 different 20mer HPV16 E7 peptides or with PHA. IL-2 levels were determined by bioassay. CTL activity determined by chromium release assay. iNKT and Treg counts were measured by FACS. FoxP3 staining was performed using an available kit. Lymphocytes were characterized by staining with monoclonal antibodies.	2458 (GP5+/GP6+ PCR and type specific PCR for 27 types)	Treg frequencies significantly increased in women with persistent HPV16 infection. Treg frequencies were increased in patients who had detectable HPV16 E7 specific IL-2 producing T-helper cells, suggesting HPV may affect Treg development. No evidence that iNKT cells affect persistence of HPV16 infection.
Seresini 2007	Healthy donors and women with cervical lesions (Italy)	CD4+ T cells were purified from cultured PBMCs from perifpheral blood stimulated with HPV18 E6 peptides or PHA and CTL activity was measured by chromium release assay as well as IL-4, IL-5, IL-10 and IFN-γ levels using cytometric bead array kits. The immune infiltrates in cervical lesions were also evaluated.	25/37 (Hybrid Capture 2 and typing by reverse hybridization assay)	One or more HPV18 E6 peptides were observed to be able to induce a response in 40-50% of the women evaluated. Response percentages increased to 80-100% when HPV18+ women alone were considered. Levels of IFN-γ released were shown to predict HPV persistence and/or disease relapse after surgery. A higher number of infiltrating CD4(+) and T-bet(+) T cells were observed in the lesions which correlated with favorable clinical outcomes.
Sharma 2007	Outpatient department or cancer clinic (India)	IL-2, IFN-γ, IL-4, and IL-10 was measured by ELISA in cultured PBMCs from heparinized blood stimulated with PHA	30/84 (HPV16 and HPV 18 PCR)	Increasing levels of IL-4 and IL-10 levels were significantly associated with HPV infection. Decreasing levels of IL-2 and IFN-γ were associated with HPV status.
Kemp 2010	Population-based cohort (Costa Rica)	Linco-plex assay for IL-6, IL-8, TNF-α, MIP-1α in unstimulated and PHA stimulated PBMCs	50/50 (MY09/11 PCR, dot blot hybridization for genotyping)	IL-6, TNF-α, MIP-1α levels were significantly higher in unstimulated PBMCs from HPV+ and HPV- women; IL-6, IL-8, TNF-α and MIP-1α levels were significantly lower in PHA stimulated PBMCs between HPV+ and HPV- women

Abbreviations: US = United States, HPV = human papillomavirus, DNA = deoxyribonucleic acid, GYN = Gynecology, PCR = polymerase chain reaction, ELISA = enzyme-linked immunosorbent assay, PBMCs = peripheral blood mononuclear cells, FLU = influenze, PHA = phytohemagglutinin, LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, mCTLp = memory cytotoxic T-cell precursor

Table 3. Part 2. Cytokine Responses in Patient-derived PBMCs.

These findings are consistent with Tsukui et al. (Tsukui, Hildesheim et al. 1996) and suggest that IL-2 responsiveness may differ by cytological and/or disease stage. In 1999, deGruijl et al. (de Gruijl, Bontkes et al. 1999) again evaluated IL-2 levels, as well as IgG responses, in