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1

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-speciic plasma IgG was measured by ELISA.	15/49 (GP5+/GP6+ PCR)	IgGresponses 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. HPV 16 E7-specific mCTLp activity was associated with previously published IL-2 release in response to HPV 16 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

this same population. This time, they used HPV16 L1-VLP or synthetic L1-derived 15-mer peptides P1 (amino acids 311-325) and P2 (amino acids 321-335) to stimulate the PBMCs and sorted them as before. Importantly, they found IgG responsiveness was significantly associated with HPV16 persistence alone, but that CD4 T helper IL-2 responsiveness was significantly associated with both HPV clearance and persistence. Further, they reported that neither cell-mediated nor humoral immune responses against HPV16 L1 seemed adequate for viral control. In another publication, this group took their study one step further and measured IL-2 levels in response to HPV E2 N-terminal and C-terminal protein fragments (Bontkes, de Gruijl et al. 1999). They reported that 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. The following year, Bontkes et al. (Bontkes, de Gruijl et al. 2000) evaluated HPV 16 E6- and E7-specific memory cytotoxic T-cell precursor (mCTLp) activity in the same cohort of patients with cervical dysplasia. They found that activity was significantly associated with persistent HPV16 infection but not observed in HPV negative women or women with viral clearance. Kadish et al. (Kadish, Ho et al. 1997) had previously observed a similar phenomenon. Subjects with positive lymphoproliferative responses to E6 and/or E7 peptides were more likely to be HPV negative at the same clinic visit than were nonresponders ($P = 0.039$). Subjects who were negative for HPV and those with a low viral load were also more likely to respond than were those with a high viral load (P for trend = 0.037). These data suggest that lymphoproliferative responses to specific HPV 16 E6 and E7 peptides appear to be associated with the clearance of HPV infection.

In 2007, three additional reports evaluating patient-derived PBMCs were published. Sharma et al. (Sharma, Rajappa et al. 2007) focused on IL-2, IFN-g, IL-4, and IL-10 levels in PBMCs stimulated with PHA. They observed that increasing levels of IL-4 and IL-10 levels were significantly associated with HPV infection and that decreasing levels of IL-2 and IFN-γ were associated with HPV status. Seresini et al. (Seresini, Origoni et al. 2007) measured lymphoproliferative responses and IL-2, IFN-g, IL-4, and IL-10 levels in PBMCs stimulated not with HPV16 peptides, but rather with HPV18-specific E6 peptides. Their analyses revealed that one or more HPV18 E6 peptides were able to induce a response in 40-50% of the women evaluated. Response percentages increased to 80-100% when HPV18-positive women alone were considered. Levels of IFN-γ released were also shown to predict HPV persistence and/or disease relapse after surgery. In addition, they showed that a higher number of infiltrating CD4(+) and T-bet(+) T cells in lesions correlated with favorable clinical outcomes. Finally, Molling et. al. (Molling, de Gruijl et al. 2007) evaluated cultured PBMCs again stimulated with 14 different 20mer HPV16 E7 peptides or with PHA and measured both IL-2 levels and CTL activity. Importantly, they also measured invariant natural killer T-cells (iNKT) and FoxP3+ regulatory T cells (Tregs) levels by flow cytometry (FACSCalibur). While iNKT cells did not appear to be associated with HPV persistence, Treg frequencies were significantly increased in women with persistent HPV16 infection; and the Tregs were significantly more common in women who had detectable HPV16 E7 specific IL-2 producing T-helper cells. These data suggest that HPV infection may affect Treg development – a finding that opens the door for a whole new avenue of research related to HPV-related immune research.

Immune Markers in Cervical Tissues PBMC responses and circulating or secreted cytokines can be useful indicators of immune response, but the best indications may come

from the actual site of interaction between HPV infection and the immune system: tissue. A number of studies have attempted to measure immune markers in HPV-positive compared to HPV-negative women in different ways. Among studies included in this review, these markers fall into three major categories: immune presentation molecules, cytokines or cytokine receptors, and immune cells.

Several studies used immunohistochemistry (IHC) to stain for major histocompatibility complex (MHC) proteins in cervical tissue (Table 4). MHC class I molecules present endogenous antigens (cytoplasmic proteins) to cytotoxic (CD8+) T cells and are typically present on all nucleated cells (Murphy, Travers et al. 2011). In contrast, MHC class II molecules present exogenous antigens from outside the cell to helper (CD4+) T cells and are typically present only on antigen presenting cells, such as dendritic cells and macrophages. Thus, normal cervical epithelial cells should be MHC class I positive and MHC class II negative. In humans, MHC class I consists of major human leukocyte antigens (HLA) A, B, and C and minor antigens E, F, and G, while MHC class II consists of HLA-DM, -DO, -DP, -DQ, and -DR.

Using a polyclonal stain specific for HLA-A, -B and -C heavy chains in formalin-fixed, paraffin-embedded (FFPE) tissue from biopsies and resection specimens from women with CIN1-3 or cancer, Cromme et al. (Cromme, Meijer et al. 1993) found that normal MHC class I expression, defined positive staining in ≥75% of cells, was reduced in women with HPV16, 18, or 31 infection versus HPV-negative women (p=0.04). MHC class II expression, as measured through a polyclonal HLA-DR antigen stain, was also altered, with normal staining (<25% positively stained cells) in 42% of women with HPV16, 18, or 31 infection versus 64% of HPV-negative women. This alteration was not statistically significant, however (p=0.14). Gonclaves el al (Goncalves, Le Discorde et al. 2008) also examined MHC class I expression in FFPE biopsy blocks, but in women with normal through cancerous histology. They found that HLA-A/B/C expression was not significantly elevated in HPV-positive compared to HPV-negative women (OR, 2.29; 95% CI, 0.77- 11.00; P = 0.14). Strangely, HPV16/18 infection was inversely associated with HLA-A/B/C expression (OR, 0.12; 95% CI, 0.02- 0.79; P = 0.04), but as reported, it was unclear whether this association was based on comparison to HPV-negative women, or a combination of both HPV-negative women and women with HPV infections other than HPV16 and 18. HLA-E expression tended to be increased in HPV-positive versus HPV-negative women (OR, 3.83; 95% CI, 0.49-30.10; P = 0.22), especially for HPV16/18 infections (OR, 11.25; 95% CI: 2.32-55.47; P = 0.003). Similarly, Dong et al. (Dong, Yang et al. 2010) stained for HLA-G in FFPE blocks from CIN1-3 patients and found higher HLA-G expression in HPV16/18-positive patients than HPV16/18-negative patients (P = 0.02).

In addition to interaction with an antigen MHC complex, T-cells require costimulation with an antigen nonspecific molecule to be full activated. T cells that encounter antigen MHC complex without costimulation may be come anergic and thus tolerant to the presence of HPV. To investigate this possibility, Ortiz-Sanchez et al. (Ortiz-Sanchez, Chavez-Olmos et al. 2007) evaluated expression of the CD80 and CD86 MHC class II costimulatory molecules through immunohistochemistry (IHC), quantitative reverse transcriptase PCR (qRT-PCR), and RNA in situ hybridization (ISH) in FFPE biopsies from histologically normal HPV-negative women and HPV16-positive women with LSIL. They found that CD86, but not CD80, was expressed in all HPV-negative normal cervical epithelial samples, while CD86

Author & Year	Study source (Origin Country)	Immune Marker Measurement	HPV -/+ N (Measurement Method)	Major Conclusions
Cromme 1993	Oncological GYN oupatient department (Netherlands)	IHC for MHC-I & MHC-II expression in FFPE tissue from biopsies & resection specimens	14/107 (GP 5/6 PCR + TS PCR for HPV6, 11, 16, 18, 31, 33; RNA ISH for HPV16 E7)	Normal MHC-I expression reduced in women with HPV16, 18, or 31 vs. HPV-negative women (p=0.04). MHC-II expression was also altered with HPV16/18/31, but not significantly.
Fernandes 2005	Outpatient GYN Clinic (Brazil)	Double-sandwich ELISA for IFN-γ, TNF-α, IL-10 in snap frozen cervical biopsies	0/42 (GP5/6, MY09/11, HPV16E7.667/HPV16E7.774, HPV18E7.696/HPV18E7.799 PCRs)*	HPV16 associated with higher IL-10 and IFN-γ intralesional levels than other HPV types, but HPV18 was associated with reduced TNF-α and INF-γ levels. Thus, immune response may vary by HPV type.
Ortiz-Sanchez 2007	Women undergoing a routine hysterectomy due to uterine myomatosis and women with LSIL (Mexico)	IHC, qRT-PCR, ISH for CD80 and 86; IHC for IL10 in FFPE biopsies	30/30 (MY09/11 and p16-1 and p16-2R primer PCR. HPV typing by sequence comparison. Only the HPV-16 samples included in CD86 expression analysis.)	CD86 expression was decreased in patients with HPV16 positive LSIL versus normal women, independent of IL-10. Expression of CD86 on normal cervical keratinocytes could indicate the ability to activate cytotoxic T cells, while the shut-off of this molecule in HPV-16 positive lesions could be a mechanism for evading host immune surveillance, resulting in the persistent HPV infection and probable progression of cervical lesions.
Song 2007	OB/GYN clinic (Republic of Korea)	qRT-PCR for IL-6, IL-10, IFN-γ, TNF-α in frozen tissue biopsies	0/67 (Hybrid Capture 2 + HPV DNA Chip)*	IFN-γ was significantly associated with HPV-16 E6, E7, and high-risk HPV viral load among HPV-positive women.**
Bermudez-Morales 2008	Instituto Nacional de Cancerolog´ıa (National Cancerology Institute, Mexico)	RT-PCR for IL-10 in cervical scraping and biopsies (storage not specified)	28/47 (PCR-RFLPs)	Strong association between HPV positivity and IL10 mRNA levels
Butsch Kovacic 2008	The ASCUS/LSIL Triage Study for Cervical Cancer (ALTS) trial (US)	Visual counting of lymphocytes, neutrophils, macrophages, plasma cells, and eosinophils in 3 H&E sections per biopsy (stromal and epithelial sections of hematoxylin and eosin stain slides from FFPE biopsy tissue evaluated)	228/288 (Hybrid Capture 2 and PCR)	These data suggest that cervical inflammation varies with type of human papillomavirus infection, risk of persistence and progression and HPV cofactors.**

*Evaluated cytokine expression by HPV type in HPV-positive women. **Study adjusted for confounding factors in regression models. †Compared HPV persistence and clearance. Thus, all were HPV-positive at baseline. Abbreviations: HPV = human papillomavirus, CIN = cervical intraepithelial neoplasia, FFPE = formalin-fixed paraffin-embedded, GYN = gynecology, ISH = in situ hybridization, IHC = Immunohistochemistry, OB/GYN = obstetrics and gynecology, qRT-PCR = quantitative reverse transcriptase PCR, STI = sexually transmitted infection, TIL = tumor infiltrating lymphocytes, RFLPs = Restriction Fragment Length Polymorphisms, ASCUS = Atypical Squamous Cells of Undetermined Significance, LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, US = United States

Table 4. Part 1. Immune Markers in Cervical Tissues.

Author & Year	Study source (Origin Country)	Immune Marker Measurement	HPV -/+ N (Measurement Method)	Major Conclusions
Gonclaves 2008	GYN Reference Services (Brazil)	IHC for HLA-A/B/C and HLA-E in RNA from FFPE biopsy blocks	19/55 (GP5+/6+, MY09/11, HPV16E7.667/ HPV16E7.774, HPV18E7.696/ HPV18E7.799)	Some evidence that HPV infection was associated with increased HLA-E expression, especially HPV16/18 infection. Association with HLA-A/B/C was less clear.
Song 2008	OB/GYN clinic (Republic of Korea)	qRT-PCR for IL-6, IL-10, IFN-γ, TNF-α from frozen tissue biopsies	0/57 (Hybrid Capture 2)†	IFN-γ correlated with high-risk HPV clearance.**
Tirone 2009	Women with CIN or normal women with hysterectomies due to uterine myoma (Brazil)	RT-PCR for IFNAR 1, IFNAR 2, 2'5'OAS, IFN-α from cervical tissue biopsies	31/14 (Hybrid Capture 2)	Lower IFN-α receptor expression with HPV infection.
Brismar Wendel 2010	Healthy volunteers at Karolinska Division of Obstetrics and Gynecology (Sweden)	qRT-PCR for CD3, CD4, CD8, CD19, CD27, CCR5, CCL5/Rantes, IL-2, IL-4, IL-10, IL-12a, IL-17a, IL-7R, HLA-DRα, IFN-γ, TNF-β, PD-1, CTLA-4, LAG3, IgA, IgG from frozen biopsy of ectocervix outside the transformation zone	13/11 (Roche Linear Array)	HPV not associated with a local inflammatory immune response as measured by qRT-PCR.
Dong 2010	Department of Pathology (China)	IHC for HLA-G and visual counting of TILs in 5 high-power fields from FFPE blocks	22/33 (ISH for HPV16 & 18 in FFPE tissue section)	HLA-G elevated in HPV16/18+ lesions and associated with lower TIL counts, suggesting inhibition of immune response against HPV.
Øvestad 2011	Women refered to a hospital for abnormal Papanicolaou tests (Norway)	IHC for CD4, CD8, CD25, from RNA isolated from paraffin blocks of punch biopsies CD138, FOXp3	0/45 (AMPLICOR and Linear Array)*	HPV16 and related types were correlated with lower CD8-positive cell counts in the stroma compared to other HPV types.**

*Evaluated cytokine expression by HPV type in HPV-positive women. **Study adjusted for confounding factors in regression models. †Compared HPV persistence and clearance. Thus, all were HPV-positive at baseline. Abbreviations: HPV = human papillomavirus, CIN = cervical intraepithelial neoplasia, FFPE = formalin-fixed paraffin-embedded, GYN = gynecology, ISH = in situ hybridization, IHC = Immunohistochemistry, OB/GYN = obstetrics and gynecology, qRT-PCR = quantitative reverse transcriptase PCR, STI = sexually transmitted infection, TIL = tumor infiltrating lymphocytes, RFLPs = Restriction Fragment Length Polymorphisms, ASCUS = Atypical Squamous Cells of Undetermined Significance, LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, US = United States

Table 4. Part 2. Immune Markers in Cervical Tissues.

expression was lower (73% by IHC) in HPV16-positive LSIL samples. This decrease in CD86 expression in HPV-positive women could represent and immune evasion mechanisms through the down-regulation of costimulatory molecules.

The next major category of immune markers measured in cervical tissue includes cytokines and their receptors. In addition to testing for MHC costimulatory molecules, Ortiz-Sanchez et al. (Ortiz-Sanchez, Chavez-Olmos et al. 2007) used IHC to stain for IL-10, which inhibits CD86 expression. IL-10 detection was likewise poor in both HPV-negative normal tissue and HPV16-positive LSIL tissue, but detection was higher in a high-grade SIL (HSIL) control sample. Fernandez et al. 2005 tested for IFN-γ, TNF-α, and IL-10 protein from snap frozen cervical biopsies from HIV-positive or HIV-negative LSIL and HSIL patients infected with HPV using a double-sandwich ELISA approach. They reported that HPV16 was associated with higher IL-10 ($P = 0.03$) and IFN-γ ($P = 0.04$) intra-lesional levels than other HPV types, but HPV18 was associated with reduced TNF-α ($P = 0.009$) and INF-γ levels ($P = 0.01$) suggesting that immune responses may vary by the infecting HPV type.

The majority of studies measured cytokines with quantitative reverse transcriptase PCR (qRT-PCR). Bermudez-Morales et al. (Bermudez-Morales, Gutierrez et al. 2008) found a strong association between HPV positivity and IL10 mRNA levels, especially for HPV16. Song et al. evaluated IL-6, IL-10, IFN-γ, and TNF-α and both HPV16 positivity (Song, Lee et al. 2008.) and HPV clearance versus persistence (Song 2008) among women positive for carcinogenic HPV. They found that IFN-γ was associated with HPV-16 *E6* (OR, 28.20; 95% CI, 2.66-299.11) and *E7* (OR, 19.62; 95% CI, 2.14-180.25) expression (Song, Lee et al. 2007), as well as with clearance of carcinogenic HPV (OR, 8.26; 95% CI: 1.24-54.94) (Song, Lee et al. 2008.). Tirone et al. (Tirone, Peghini et al. 2009) found some evidence that the IFN-α receptor subunits IFNAR 1 and IFNAR 2 were under-expressed in HPV-positive women with CIN1-3 compared to HPV-negative women with normal through CIN3 histology. Brismar Wendel et al. (Brismar Wendel, Kaldensjo et al. 2010) measured a number of different cytokines and other immune markers and found no difference between HPV-positive and HPV-negative healthy volunteers (22/24 with normal cytology).

Another major category of immune markers is the immune cells themselves. Two studies in this review evaluated infiltrating immune cells in cervical tissue by visually counting the cells. Butsch Kovacic et al. (Butsch Kovacic, Katki et al. 2008) counted lymphocytes, neutrophils, macrophages, plasma cells, and eosinophils among women with typical squamous cells of undetermined significance or LSIL and found that cervical inflammation varies with type of HPV infection, as well as risk of persistence and progression. Women with carcinogenic HPV infections also had more severe epithelial inflammation and less severe stromal inflammation than HPV-negative women. These associations were limited to carcinogenic and not the non-carcinogenic HPV types. Dong et al. (Dong, Yang et al. 2010) determined that among HPV16/18-positive CIN lesions, moderate to strong HLA-G expression was associated with weak immune response, as measured by few tumor infiltrating lymphocytes (TIL), whereas weak HLA-G expression was associated with strong immune response (high numbers of TIL). HLA-G expression was not associated with TIL in HPV-negative women, suggesting that the increased HLA-G expression in HPV-positive lesion may reflect an inhibition of immune response against HPV. Brismar Wendel et al. (Brismar Wendel, Kaldensjo et al. 2010) used qRT-PCR to measure CD3, CD4, CD8, CD19, and CD27 expression but found no difference by HPV status. Finally, Øvestad et al. (Ovestad, Vennestrom et al. 2011) used IHC to stain for cell surface marker in biopsies from CIN2-3 patients and found that HPV16 and related types were correlated with lower CD8-positive cell counts in the stroma compared to other HPV types ($P = 0.02$).

4. Conclusions and future perspectives

Taken together, these studies support the role of cell-mediated immune response in HPV-related carcinogenesis although their findings, particularly for those measuring cytokines, are largely inconsistent. There are many potential explanations. There has been a real lack of consistency in sample collection methods, cytokine measurement methods and even the outcome definitions used for analyses.

For example, some studies assessed HPV positivity, regardless of timing and/or disease state, while others evaluated incident HPV infection or HPV persistence or clearance. Further, these studies more than often focused on HPV 16, on carcinogenic HPV types, or any HPV type infection together. However, those few studies that did evaluate immune

markers by individual HPV type found evidence that immune responses vary by HPV type (Fernandes, Gonçalves et al. 2005; Butsch Kovacic, Katki et al. 2008; Ovestad, Vennestrom et al. 2011). Thus, HPV type is an important consideration. Moreover, while we chose to focus on immune markers' associations with HPV infection, most of the studies reviewed in this chapter predominantly assessed associations between immune markers and disease state (LSIL, HSIL, cancer or CIN1-3 and cancer). Ideally, future studies would evaluate differences by individual HPV type and better consider the timing of disease.

There are also other notable differences in the study populations considered by these studies (e.g., sample size, young versus old women, inclusion of HIV-positive women). Many studies used convenience samples of women. In fact, there is a general lack of consideration for factors that could confound or modify both cytokine production and the infectious outcomes. Only eight of the 35 studies made any attempt to account for co-factors that may influence cytokine level. The importance of adjusting for such potential confounders was recently highlighted at an international workshop that addressed best practices for sampling techniques and assessment of mucosal immune responses. The workshop identified a number of characteristics that should be considered when studying female genital tract immunity, including age, race, body mass index, sexually transmitted infections, other genital tract infections, vaginal flora, alcohol or substance use, recent immunization, pregnancy, phase of menstrual cycle, genital inflammation, recent douching, gynecologic procedures, recent intercourse/semen, and contraception (Anderson and Cu-Uvin 2011). The number of women included in each study is another important consideration in the evaluation of these studies. Twenty-two of the 32 (69%) studies meeting our criteria included less than 30 women in one or more groups (e.g., the HPV-positive or HPV-negative group). Fifteen (47%) included less than 20 in one or more groups. Small numbers of women in the comparison groups can lead to unstable results and may help explain why results for individual immune markers are so inconsistent.

Most studies have measured only a few cytokines, and few have evaluated infiltrating immune cells concurrently with cytokines, making it challenging to explore the activation pathways of cells involved in the immune response against HPV. One research group has made extensive use of their study population to characterize several aspects of immune response as measured in PBMCs (de Gruijl, Bontkes et al. 1998; Bontkes, de Gruijl et al. 1999; de Gruijl, Bontkes et al. 1999; Bontkes, de Gruijl et al. 2000; Molling, de Gruijl et al. 2007). However, few studies have been so thorough. In fact, more than half of the studies of PBMCs (five of nine studies), have come from this same research group with the same study population. Additional studies characterizing many aspects of immune response in different study populations would help clarify whether the results are broadly applicable.

Many studies focused on T-helper type 1 (TH1) versus T-helper type 2 (TH2) polarization, using a single cytokine (or small group of cytokines) to characterize the T-helper phenotype. However, advances in immunology have led to the shift of the TH1/TH2 paradigm to the TH1/TH2/TH17/T-reg hypothesis, a multi-lineage commitment from the same T-helper precursor cells. TH17 cells, in fact, have been shown to inhibit both TH1 and TH2 cells, and therefore are likely to play a critical role in HPV-related immune responses as well. Few studies have evaluated TH17 or Treg cells. The recent study by Molling et al. (Molling, de Gruijl et al. 2007) is among the few that have measured these cells. Using flow cytometry in HPV16 E7 stimulated PBMCs, they determined that Treg frequencies were significantly

greater in women with persistent HPV16 infection and in women with detectable HPV16 E7 specific IL-2 producing T-helper cells, suggesting that HPV infection may affect Treg development. These findings may also be supported by tissue-based studies of MHC class II expression. Although data are limited, one study found evidence of increased MHC class II expression in HPV-positive versus negative patients (Cromme, Meijer et al. 1993), while another found reduced expression of the CD86 MHC class II costimulatory molecule (Ortiz-Sanchez, Chavez-Olmos et al. 2007). It could be hypothesized that HPV upregulates MHC class II expression and down-regulates MHC class II costimulatory molecules in order to increase T-cell anergy through incomplete signaling. Additional studies are needed to better understand these relationships.

For studies of cervical secretions, the collection method can have a large impact on the results. Of the seven cervical secretion studies included in this review, two collected cervical secretions through cervicovaginal lavage (Tjiong, van der Vange et al. 2001; Guha and Chatterjee 2009), four used Weck-cel® (Crowley-Nowick, Ellenberg et al. 2000; Gravitt, Hildesheim et al. 2003) or Merocel® (Lieberman, Moscicki et al. 2008; Marks, Viscidi et al. 2011) ophthalmic sponges, and one used cytobrush sample suspensions (Scott, Stites et al. 1999). Cervicovaginal lavages may not be specific enough to the cervix and may overly dilute the specimen. Even studies that used ophthalmic sponges tended to use Weck-cel® sponges (Gravitt, Hildesheim et al. 2003; Moscicki, Ellenberg et al. 2004), which may not provide adequate cytokine recovery, especially compared to Merocel® sponges (Castle et al.(Castle, Rodriguez et al. 2004).

Studies evaluating tissue have seldom considered both stroma and epithelium. In this chapter, only two studies examined inflammation in both stroma and epithelium (Butsch Kovacic, Katki et al. 2008; Ovestad, Vennestrom et al. 2011). One study found opposite inflammatory patterns by HPV status ((Butsch Kovacic, Katki et al. 2008)). This study also found that neutrophils tended to be found only in the superficial epithelial layers, whereas mononuclear cells were found mainly near the basement membrane, suggesting that inflammatory patterns in the stroma and the epithelium may depend on the specific cell type. The second study only reported differences by CD8 in the stroma (Øvestad 2011).

Tissue-based studies of cytokines are also heterogeneous. Only two studies evaluated cytokine proteins in tissue (Fernandes, Gonçalves et al. 2005; Ortiz-Sanchez, Chavez-Olmos et al. 2007). It is not surprising that few studies have evaluated cytokine proteins in tissue since it can be challenging to find an appropriate antibody and optimize the assay. For example, antibodies that perform well in western blots may not work for staining since staining requires fixation, which can change the confirmation of the cytokine protein, thereby preventing antibody binding (Sachdeva and Asthana 2007). Most tissue-based studies of cytokines in this review measured RNA expression, but accurate measurement of RNA expression requires high quality tissue. If the tissue was not snap frozen immediately after surgery and well maintained, endogenous RNases may have degraded the RNA. RNA quality is rarely addressed. Although the presence of cytokine transcripts in tissue may be meaningful, the absence is not given the short-lived nature of RNA, even for high quality tissues (Sachdeva and Asthana 2007).

To clarify the role of immune response in cervical carcinogenesis, future studies should be conducted in well-characterized epidemiologic studies that can address most or all of the

characteristics and considerations described above. Studies should include large numbers of women, evaluate a broader spectrum of cytokines/immune markers and measure and adjust for potential confounders concurrently. Possible usefulness of tissue microarrays and multiplex arrays with well-defined phenotypes should be considered as they are likely to make these studies more feasible. Emerging results must be repeated in different study populations and specimen types, but are encouraging. Accumulating evidence indicates that there is a cell-mediated immune response to HPV. As technologies improve, it should become possible to better characterize these responses to distinguish between women at risk of developing cervical cancer and women who can effectively resolve their HPV infections.

5. Appendix 1. Search strategy for immune function in cervical carcinogenesis

("humans"[MeSH Terms] AND "female"[MeSH Terms] AND English[lang]) AND ("cervix uteri"[MeSH Terms] OR "Uterine Cervical Neoplasms/immunology"[Mesh] OR "Uterine Cervical Neoplasms/pathology"[Mesh] OR "Uterine Cervical Neoplasms/blood" [Mesh] OR "Cervical Intraepithelial Neoplasia/metabolism"[Mesh] OR "Mucus/metabolism" [Mesh]) AND ("Cytokines/blood*"[Mesh] OR "Cytokines/metabolism"[Mesh] OR "Immunity, Innate"[Mesh] OR "Adaptive Immunity"[Mesh] OR "Immunity, Cellular"[Mesh] OR "Immunity, Humoral"[Mesh] OR "Immunity, Mucosal"[Mesh] OR "Immunity, Innate/immunology"[Mesh] OR "immune infiltrates" OR immunity OR "immune response" OR "immune cells" OR "immune cell" OR inflammation OR infiltration OR "Lymphocyte Subsets/immunology"[Mesh] OR "TH1 Cells/ immunology" [Mesh] OR "TH2 Cells/ immunology" [Mesh]) NOT (mice OR mouse OR "cell line" OR "cell lines" OR "mouth" OR "oropharynx" OR "Antiretroviral Therapy, Highly Active"[Mesh] OR "Models, Theoretical"[Mesh] OR "Papillomavirus Vaccines/administration & dosage"[Mesh] OR "Premature Birth/immunology"[Mesh] OR "HIV Infections/immunology"[Mesh] OR "Combined Modality Therapy"[Mesh] OR "Complementary Therapies"[Mesh] OR "Blood Vessels/chemistry"[Mesh] OR "Laser Therapy"[Mesh] OR "Labor Stage, First/physiology"[Mesh] OR "Foreign-Body Reaction/pathology"[Mesh] OR "Postoperative Complications/pathology"[Mesh] OR "Male"[Mesh] OR "Labor, Obstetric/metabolism"[Mesh])

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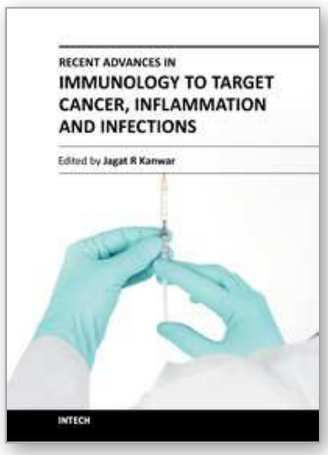

Recent Advances in Immunology to Target Cancer, Inflammation and Infections

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Immunology is the branch of biomedical sciences to study of the immune system physiology both in healthy and diseased states. Some aspects of autoimmunity draws our attention to the fact that it is not always associated with pathology. For instance, autoimmune reactions are highly useful in clearing off the excess, unwanted or aged tissues from the body. Also, generation of autoimmunity occurs after the exposure to the non-self antigen that is structurally similar to the self, aided by the stimulatory molecules like the cytokines. Thus, a narrow margin differentiates immunity from auto-immunity as already discussed. Hence, finding answers for how the physiologic immunity turns to pathologic autoimmunity always remains a question of intense interest. However, this margin could be cut down only if the physiology of the immune system is better understood. The individual chapters included in this book will cover all the possible aspects of immunology and pathologies associated with it. The authors have taken strenuous effort in elaborating the concepts that are lucid and will be of reader's interest.

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