Human Papillomavirus Type 16-Positive Cervical Cancer Is Associated with Impaired CD4+ T-Cell Immunity against Early Antigens E2 and E6

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ABSTRACT

Cervical cancer is the possible outcome of genital infection with high-risk human papillomavirus (HPV) and is preceded by a phase of persistent HPV infection during which the host immune system fails to eliminate the virus. Fortunately, the majority of genital HPV infections are cleared before the development of (pre)malignant lesions. Analysis of CD4+ T-helper (Th) immunity against the E2, E6, and E7 antigens of HPV16 in healthy women revealed strong proliferative E2- and E6-specific responses associated with prominent IFN-γ and interleukin 5 secretion. This indicates that the naturally arising virus-induced immune response displays a mixed Th1/Th2 cytokine profile. Of all HPV16+ cervical cancer patients, approximately half failed to mount a detectable immune response against the HPV16-derived peptides. The other half of the patients showed impaired HPV16-specific proliferative responses, which generally lacked both IFN-γ and interleukin 5. This indicates that the HPV16-specific CD4+ T-cell response in cervical cancer patients is either absent or severely impaired, despite a relatively good immune status of the patients, as indicated by intact responses against recall antigens. It is highly conceivable that proper CD4+ T-cell help is important for launching an effective immune attack against HPV because infection of cervical epithelia by this virus is, at least initially, not accompanied by gross disturbance of this tissue and/or strong proinflammatory stimuli. Therefore, our observations concerning the lack of functional HPV16-specific CD4+ T-cell immunity in patients with cervical cancer offer a possible explanation for the development of this disease.

INTRODUCTION

Cervical encounter with human papillomavirus (HPV) generally results in a transient infection, with the majority of individuals showing clearance of the virus within 1 year of detection (1–4). Factors underlying the lag time between HPV infection and clearance are related to the minimal disturbance of the epithelial layer initially caused by the virus, lack of HPV-induced Langerhans cell activation, and the capacity of viral proteins to evade innate immune recognition by physically inhibiting specific components of the innate immune system, such as interference with the type I IFN pathway (5–10). Once the process of regression of HPV-induced dysplasia is initiated, it is characterized by an influx of macrophages and T lymphocytes, resembling a delayed-type hypersensitivity response (11). Indirect evidence that the adaptive cellular immune system plays a major role in the protection against HPV-induced lesions is given by the high incidence of persistent HPV infections and subsequent HPV-related dysplasia in both immunosuppressed transplant patients and HIV-infected individuals (12, 13). An underlying cause of the failing anti-HPV immune response in immunocompetent individuals with persistent infection was suggested to be a locally altered cytokine environment with an increase in interleukin (IL)-10 production and a decrease in proinflammatory cytokines (14–16). Others have shown that a so-called T-helper (Th) 2-type cytokine bias in the peripheral blood of cervical intraepithelial neoplasia (CIN) patients was associated with more extensive cervical disease (17), but the implication of such altered cytokine balance on HPV-specific immunity has not been determined.

Studies analyzing T-cell responses against viral antigens from the high-risk type HPV16 in the healthy population and several stages of disease do not present an unequivocal relationship between protection against HPV16-induced (pre)malignant lesions and virus-specific T-cell immunity (18–21). We have previously described the presence of IFN-γ-producing memory CD45RO+CD4+ Th cells specific for HPV16 E2 and E6 proteins in the peripheral blood of the majority of healthy individuals (22, 23), suggesting a role for these Th cells in protection against HPV16-induced progressive disease. To fully appreciate the importance of HPV16 E2- and E6-specific Th responses in managing infection and subsequent progressive (pre)malignant lesions, we have performed a detailed analysis with respect to the magnitude and cytokine polarization of this immunity and compared this between healthy subjects and HPV16+ patients.

MATERIALS AND METHODS

Healthy Blood Donors and Patients. A selected group of 20 sexually active young females (age range, 19–31 years; median age, 23 years) participated in this study after providing informed consent. It was expected that a large fraction of these individuals had experienced previous transient HPV infection because most anogenital HPV infections are acquired soon after sexual debut and are also cleared at an early age. Older individuals were excluded because women who are HPV DNA positive at ages 35 years and above may represent failure of viral clearance (24). A Pap smear was performed for cytological examination, and an additional cervical swab specimen was obtained for HPV DNA analysis. Women presenting with histologically proven cervical carcinoma or CIN at the department of gynecology of the Leiden University Medical Center were enrolled in this study after providing informed consent. Blood was drawn at day of treatment before surgery. Carcinoma subjects enrolled were staged FIGO (International Federation of Gynecologists and Obstetricians) IB/IIA and treated by radical hysterectomy. The age of the cervical cancer patients (n = 17) ranged from 34 to 72 years (median age, 45 years), whereas the age of the CIN III patients (n = 13) ranged from 29 to 42 years (median age, 31 years). We preferred to use CIN III for analyses of cases in which the naturally occurring immune response has not been able to control the infection because there is a substantial heterogeneity in the microscopic diagnosis and biological meaning of CIN II lesions in particular. In general, the majority of CIN III lesions are incipient precancers that are destined to persist (2). Peripheral blood mononuclear cells (PBMCs) and serum were obtained for the analysis of HPV16-specific T-cell reactivity and virus-like particle (VLP) L1-specific antibodies.

The subjects were typed for HPV16 using HPV16-specific primers on DNA isolated from cervical swab specimens, paraffin-embedded sections of biopsies, or surgical resection specimens (25). The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center. Two of 20 healthy females were HPV+ (one for HPV62 and the other for HPV31),
but no HPV16 was detected. Of the CIN III and cervical carcinoma patients, only the HPV16+ subjects were included in the immunological analyses.

**Antigens.** A set of peptides spanning the whole HPV16 E2, E6, and E7 protein were used in pools of two peptides for the T-cell proliferation assays. The E2 peptides consisted of twenty two 30-mer peptides with a 15-amino acid overlap and the COOH-terminal peptide with a length of 35 amino acids. The length of the E6 and E7 peptides was 32 and 35 amino acids, respectively, with an overlap of 14 amino acids. The peptides were synthesized and dissolved as described previously (26). The peptide pools are indicated by the first and last amino acid of the region in the protein covered by the two peptides (e.g., E21–48, residues 1–30 + 16–45). Memory response mix (MRM), consisting of a mixture of tetanus toxoid (0.75 limus flocculentius/ml; National Institute of Public Health and Environmental, Bilthoven, the Netherlands), *Mycobacterium tuberculosis* sonicate (2.5 µg/ml; generously donated by Dr. P. Klatser; Royal Tropical Institute, Amsterdam, the Netherlands), and *Candida albicans* (0.005%; HAL Allergenen Lab, Haarlem, the Netherlands), was used to confirm the capacity of PBMCs to proliferate and produce cytokine in response to common recall antigens.

**HPV16 VLP ELISA.** For the detection of HPV16-specific antibodies in serum, we used an ELISA method described previously by Kurniawan et al. (27). Each serum was tested for reactivity against HPV16 VLPs (baculovirus-expressed capsids comprising the L1 protein) and against bovine papillomavirus capsids, the latter of which were disrupted by treatment with 0.1 M carbonate buffer to serve as a negative control. Both VLPs and bovine papillomavirus were kindly provided by Prof. J. Dillner (Malmö University Hospital, Malmö, Sweden). The patients (all proven HPV16+ by PCR) were tested for HPV16-specific IgG only, whereas the healthy controls were tested for both IgG and IgA because it has been described that transient infections more frequently lead to specific serum IgA antibodies than IgG. Overall, HPV16-specific antibodies can only be detected in a small fraction of individuals experiencing a transient HPV16 infection and can only be detected for a limited time period after infection (28). Therefore, only the presence, rather than the absence, of HPV16-specific antibodies allows conclusions as to whether or not an individual has experienced a transient infection in the past.

**Short-Term T-Cell Proliferation Assay.** Freshly isolated PBMCs were incubated with 12 pools of HPV16 E2-derived 30-mer peptides, 4 pools of E6 32-mer peptides, and 2 pools of E7 35-mer peptides (each pool contained two overlapping peptides). PBMCs were seeded at a density of 1.5 × 10^5 cells/well in a 96-well U-bottomed plate (Costar, Cambridge, MA) in 150 µl of Iscove’s medium (BioWhittaker) supplemented with 10% autologous serum. HPV16 E2-, E6-, and E7-derived peptides were added at a concentration of 10 µg/ml/peptide.Medium alone was taken along as a negative control, and MRM (dilution, 1:50) served as a positive control. For each peptide pool, eight parallel microcultures were incubated. Fifty µl of supernatant from the microcultures was harvested 5 days after incubation, and stored at –70°C until cytokine analysis. Peptide-specific proliferation was measured at day 7 by [3H]thymidine incorporation. Cells were scored positive when the proliferation of ≥75% of the test wells exceeded the mean proliferation + 3 × SD of the control wells containing medium only, and the stimulation index, defined as the mean of all test wells divided by the mean of the control wells, was ≥3. Previously performed short-term proliferation assays with immunologically naïve cord blood cells (10 samples) revealed no specific proliferation against the E6 or E7 oncoprotein-derived peptides, indicating that the 7-day stimulation with peptides does not result in the *in vitro* induction of HPV16-specific proliferative responses.

**Carboxy-fluorescein Diacetate Succinimidyl Ester Labeling.** Freshly isolated PBMCs were washed in cold PBS and incubated with 0.5 µM carboxy-fluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, OR) in PBS at 37°C for 30 min. 5% autologous serum was added, and the PBMCs were washed twice in PBS. The carboxy-fluorescein diacetate succinimidyl ester-labeled PBMCs were then resuspended in Iscove’s modified Dulbecco’s medium supplemented with 10% autologous serum and incubated with the HPV16-derived peptides as described for the short-term proliferation assays.

**Analysis of Cytokines Associated with HPV16-Specific Proliferative Responses.** The detection of cytokines in the supernatants of the short-term proliferation assays was performed using the cytokometric bead array (Becton Dickinson). Analysis of the cytokine kinetics revealed that at day 5, antigen-specific production of all cytokines could be detected. IL-2 and IL-4 can both be consumed by proliferating cells; therefore, levels of these cytokines in this assay may be underestimated. Supernatants from the individual microcultures harvested at day 5 were pooled per peptide pool, and 50 µl of this supernatant were used for cytokine analysis. The cytokometric bead array was performed according to the manufacturer’s instructions. Cutoff values were based on the standard curves of the different cytokines (50 pg/ml for IFN-γ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and ≥2× the concentration of the medium control (29).

**Analysis of Phytomagglutinin (PHA)-Induced Proliferation and Cytokine Production.** Liquid nitrogen-stored PBMCs were used for these assays, enabling the analysis of all of the samples at the same time for optimal comparison. The cervical carcinoma and the healthy control group consisted of the same individuals as those analyzed in the short-term proliferation assays, minus those from whom an insufficient amount of PBMCs was available (healthy subjects, n = 17; cervical carcinoma patients, n = 13). The CIN group included additional samples, which had been collected previously and stored in liquid nitrogen (CIN patients, n = 13). PBMCs were thawed and resuspended at a concentration of 10^6/ml in serum-free x-vivo15 medium (BioWhittaker). One hundred µl of cell suspension were seeded per well of a 96-well round-bottomed plate (total, 24 wells/subject). Fifty µl of 1.5 µg/ml PHA (Murex Diagnostics, Dartford, United Kingdom) in x-vivo15 were added to 12 wells, whereas 50 µl of plain x-vivo15 medium served as a negative control in the remaining 12 wells. After 48 h, 50 µl of supernant per well were harvested, and the supernatants from the PHA-stimulated wells were pooled, as were the controls. Supernatants were stored at –20°C until cytokine analysis by ELISA. PHA-induced proliferation was measured by [3H]thymidine incorporation 48 h after stimulation. IFN-γ was measured by ELISA as described previously (26). IL-4 and IL-10 (CLB, Amsterdam, the Netherlands) and IL-5 (PharMingen) ELISAs were performed according to the instructions of the manufacturer.

**Statistical Analysis.** Statistical analysis of the HPV16-specific proliferative responses associated with cytokine production was performed using Fisher’s exact test. For statistical analysis of mitogen-induced proliferation and the cytokine levels in response to recall antigens, an unpaired two-tailed *t* test was applied, and an unpaired two-tailed *t* test with Welch correction was applied for the analysis of the mitogen-induced cytokine ratios.

**RESULTS**

**HPV16 E2- and E6-Specific CD4+ T-Cell Responses in Healthy Individuals Are Associated with Th1- and Th2-Type Cytokines.** Previously, we have demonstrated the presence of HPV16 E2- and E6-specific memory CD4+ Th cells in the CD45RO+ fraction of peripheral blood of the majority of healthy individuals (22, 23). These memory Th cells produced IFN-γ on stimulation, indicating that they are of a Th1 type. Thus far, it has not been determined whether the HPV16-specific responses in the healthy population consist solely of Th1 type cytokines or whether other cytokines are also involved. We therefore analyzed the cytokine profiles of the HPV16-specific T cells in a cohort of 20 young, sexually active, healthy women. All women had normal cytology and were proven HPV16 negative by PCR and VLP ELISA at the time of analysis; therefore, the presence of responses against the HPV16 antigens are expected to represent memory T cells induced on a transient HPV16 infection in the past. Short-term proliferation assays were performed against peptides derived from the HPV16 proteins E2, E6, and E7 as well as a mix of common recall antigens (MRM). In accordance with our previous data (22, 23), half of the individuals showed proliferative responses against E2 (10 of 20 subjects), and an even larger fraction shows responses against the E6-derived peptides (13 of 20 subjects). E7-specific responses were detected in only a minority of subjects (2 of 20 subjects; Fig. 1A). Carboxy-fluorescein diacetate succinimidyl ester labeling of PBMCs demonstrated that exclusively CD4+ Th cells contributed to the HPV16-specific proliferation because, on stimulation with E2 and E6 peptides, cell division could only be detected in the CD4+ fraction of PBMCs (Fig. 2). This is in line with our previous notion that
HPV16-specific IFN-γ secretion was solely derived from CD4+ T cells (Refs. 22 and 23; data not shown). Analysis of the supernatants of these T-cell cultures for the presence of IFN-γ, tumor necrosis factor α, IL-2, IL-4, IL-5, and IL-10 revealed the secretion of both Th1- and Th2-type cytokines in response to HPV16-derived peptides (Fig. 1B). The most predominantly secreted cytokines were IFN-γ and IL-5 (Figs. 1B and 3), often accompanied by IL-2 and, to a lesser extent, by tumor necrosis factor α and IL-10. Antigen-specific secretion of IL-4 was rarely observed. In response to recall antigens (MRM), the majority of Th1- and Th2-type cytokines for which we analyzed were detected. Taken together, the consistent HPV16-specific secretion of IFN-γ and IL-5 in healthy individuals suggests that both Th1- and Th2-type immunity play a role in the effective control of HPV16 infection.

Impaired HPV16-Specific Th Immunity in Patients with HPV16+ (Pre)Malignant Lesions. Having characterized the HPV16-specific Th responses in healthy individuals, a fraction of whom have apparently succeeded in clearing HPV16 infection, we questioned how these compare with patients who have evidently failed to establish an effective immune response against this virus. A group of HPV16+ patients, consisting of 8 subjects diagnosed with CIN III and 17 subjects diagnosed with cervical carcinoma (CxCa) patients (all human papillomavirus 16 positive) were tested in short-term proliferation assays as described in A. D). Supernatants of the positive proliferative responses of the patient group were subjected to cytokine analysis as described in B, n.t., not tested.

Fig. 1. A, freshly isolated peripheral blood mononuclear cells from 20 healthy female individuals (HD) were tested in short-term proliferation assays using a complete set of human papillomavirus 16 E2-, E6-, and E7-derived peptide pools (indicated by the first amino acid of the region in the protein covered by the peptides). Responses were scored positive when the proliferation (cpm) of ≥6 of 8 test wells exceeded the mean proliferation + 3 × SD of the control (medium only) wells, and the mean stimulation index of all test wells over control wells was ≥2. Memory response mix (MRM), consisting of a mixture of recall antigens, was used as a positive control. The stimulation indices of responses scored positive are indicated. B, supernatants of the positive proliferative responses indicated in A were analyzed for the presence of IFN-γ, tumor necrosis factor α, interleukin (IL)-2, IL-4, IL-5, and IL-10 by cytometric bead array. The indicated layout is used for the six measured cytokines; a filled square represents antigen-specific cytokine production. Cutoff values were based on the standard curves of the different cytokines (50 pg/ml for IFN-γ and 10 pg/ml for the remaining cytokines). Antigen-specific cytokine production was defined as a cytokine concentration above cutoff level and ≥2× the concentration of the medium control. C, 8 cervical intraepithelial neoplasia (CIN) III and 17 cervical cancer (CxCa) patients (all human papillomavirus 16 positive) were tested in short-term proliferation assays as described in A. D, supernatants of the positive proliferative responses of the patient group were subjected to cytokine analysis as described in B, n.t., not tested.
proliferation observed (Fig. 1C), which was associated with the production of both IFN-γ and IL-5 (Fig. 1D). The remaining seven patients with high-grade CIN did not show any proliferative reactivity against the E2, E6, or E7 antigens, whereas all eight patients did respond to the recall antigens. In contrast to the near absence of HPV16-specific responses in high-grade CIN patients, cervical carcinoma patients showed a response frequency that resembled that of the healthy subjects (Fig. 1C), with half of the patients showing proliferative responses against E2 (8 of 16 cervical carcinoma patients); cervical carcinoma 9 was not tested against E2), and less frequent responses against the E7 antigen (3 of 17 cervical carcinoma patients).

The number of cervical carcinoma patients showing a proliferative response against HPV16 E6 was lower (7 of 17 patients) than that of healthy individuals (13 of 20 patients), but this difference was not statistically significant.

Despite this resemblance of the healthy female group in terms of frequency of proliferative responses against HPV16 antigens, the corresponding cytokine production showed a completely different picture (Fig. 1D). In the group of cervical carcinoma patients, only a minor fraction of the proliferative responses was associated with cytokine production. In fact, in most cases, none of the cytokines were detected, despite the occasional broad proliferative response against all three HPV16 antigens (Figs. 1D and 4). In a large set of supernatants derived from microcultures lacking antigen-specific proliferation, we were not able to detect specific cytokine production, indicating that selection of supernatant samples based on proliferation does not result in an underestimation of the cytokine responses. Overall, compared with the healthy subjects, the responses against both E2 and E6 in cervical carcinoma patients are characterized by lack of IFN-γ and IL-5, as reflected by a significantly reduced fraction of total responses associated with either cytokine [IFN-γ, P < 0.05; IL-5, P ≤ 0.001 (Fisher’s exact test)]. The secretion of other cytokines besides IFN-γ and IL-5 is also reduced in cervical carcinoma patients, but the antigen-specific secretion of IL-10 appears to be less affected. As a consequence, HPV16 responses associated solely with the immunoregulatory cytokine IL-10 can be observed. HPV16-specific IL-10 production was accompanied by IFN-γ and/or IL-5 in 11 of 12 responses in healthy individuals but in only 2 of 6 responses in cervical carcinoma patients (P < 0.05, Fisher’s exact test). The low number of responses in CIN III patients and the low number of E7 responses in all groups precluded these parameters from statistical analysis. Importantly, no significant differences in the magnitude and cytokine profiles of the recall antigen (MRM) responses were observed between cervical carcinoma patients and healthy individuals. The mean IFN-γ and IL-5 levels in response to MRM were lower in cervical cancer patients than in healthy women; however, this difference was not significant [Fig. 1; IFN-γ, mean = 3,110 pg/ml and range = 307–10,500 pg/ml (healthy subjects) and mean = 2,229 pg/ml and range = 150–8,250 pg/ml (cervical carcinoma patients); IL-5, mean = 466 pg/ml and range = 11–1,091 pg/ml (healthy subjects) and mean = 280 pg/ml and range = 10–1,276 pg/ml (cervical carcinoma patients), two-tailed t test]. This indicates that the recall antigen-specific cytokine production was not significantly impaired in the patient population, which is in accordance with the fact that these patients had low-stage (mainly FIGO IB) disease.

Even though the frequency at which HPV16 E2/E6-specific T-cell immunity was detected in patients and healthy females was roughly similar, it should be noted that all patients carried HPV16+ cervical lesions and therefore approximately half of the HPV16+ cervical carcinoma patients had failed to mount any detectable HPV16-specific Th response. Thus, failure of immune defense against cervical HPV16 infection can embody either HPV16 E2/E6-specific T-cell immunity with an impaired capacity to produce cytokines or a complete lack of such T-cell immunity.
Patients with HPV16+ Cervical Disease Display Altered Cytokine Profile of Peripheral T Cells. The local cytokine environment in HPV-induced lesions differs from that observed in healthy tissue (14, 16), and it has been suggested that this can influence the overall polarization of peripheral T cells toward a Th2-type cytokine profile in patients bearing these lesions (17, 30, 31). In our study, HPV16-specific CD4+ Th responses in patients with cervical cancer lack the clear Th1/Th2 cytokine profile that is exhibited by such responses in healthy subjects, whereas the cytokine profile of MRM-specific T-cell responses does not show significant differences between healthy and diseased individuals. We questioned whether the overall cytokine profiles of peripheral T cells, as determined by mitogenic stimulation of PBMCs, would reveal a difference between patients with HPV16-induced disease and healthy subjects. Supernatants from PHA-stimulated PBMCs were analyzed for the levels of IFN-γ, IL-4, IL-5, and IL-10. Per individual, the ratios of the different cytokines were calculated, which reflect the Th1/Th2 polarization of the T-cell repertoire. Cervical carcinoma patients revealed relatively higher levels of IL-10 and IL-4 as compared with IFN-γ, suggesting the loss of Th1 in favor of Th2 cytokines in the peripheral blood of the diseased population (Fig. 5, A and B; P < 0.01, unpaired two-tailed t test with Welch correction). However, the IFN-γ/IL-5 ratios did not differ between the groups (data not shown), indicating that the cytokine profiles in cervical carcinoma patients do not represent a general Th2 bias. In line with this, the ratios of the Th2 type cytokines IL-5/IL-4 are also significantly lower in cancer patients (Fig. 5C; P < 0.05). The high-grade CIN patients revealed cytokine profiles similar to that observed in the cervical carcinoma patients, although the difference with the healthy subjects was less pronounced and was only significant for the IFN-γ/IL-4 ratios (Fig. 5A; P < 0.05). Overall, the PHA-induced proliferation was slightly lower in both high-grade CIN and carcinoma patients compared with healthy individuals (Fig. 5D).

Taken together, the mitogen-induced cytokine profiles suggest the presence of an altered cytokine balance in HPV16-induced disease, which may have determined the lack in cytokine polarization of HPV16-specific Th responses found in patients and affected the proliferative capacity of these T cells.

DISCUSSION

Our analysis of CD4+ T-cell immunity against the HPV16 E2 and E6 antigens in healthy women and patients with HPV16+ cervical disease revealed that cervical cancer is associated with HPV16-specific immune failure. Of the HPV16+ carcinoma patients tested, approximately half lacked any detectable proliferative E2-, E6-, and/or E7-specific T-cell responses. The other half of the carcinoma patients did show systemic proliferative responses, but this immunity was, in general, not associated with a strong inflammatory cytokine profile. Because specific CD4+ T-cell help is of crucial importance for the development of humoral and cellular effector mechanisms against viral infections, these impaired responses against the viral antigens can be considered unfavorable in view of protection against...
progressive HPV-induced neoplasia. In contrast to cervical cancer patients, healthy women frequently displayed strong CD4+ T-cell responses against HPV16 E2 and E6, and these responses were generally associated with the secretion of inflammatory cytokines, most prominently IFN-γ and IL-5. Our observation that the HPV16-specific memory response in healthy subjects displays a mixed Th1/Th2 cytokine profile is in accordance with the notion that both humoral and cellular immunity are required to clear viral infection and provide subsequent protection against reinfection (Fig. 6, left panel). Others have previously observed a lack of E6- and E7-specific IL-2 secretion in PBMCs of cervical carcinoma patients and have, on the basis of this observation, suggested that this loss of IL-2, a cytokine indicative of a Th1-type response, might be accompanied by an increased Th2-type response (20). Our data indicate that the HPV-specific cytokine secretion in the diseased population indeed lacks IL-2 but is not skewed toward Th2 type. Instead, the HPV-specific T-cell response in patients features an overall defect in inflammatory cytokine production.

Compared with the frequency at which HPV16-specific proliferative responses are detected in cervical carcinoma patients, the frequency of such immunity in patients with high-grade CIN lesions is surprisingly low, and this does not correspond with the idea of a gradual loss of HPV16-specific T-cell reactivity during disease progression. A similarly low frequency of HPV16-specific T-cell responses in CIN patients was also described by Nakagawa et al. (32). On the basis of these accumulated data, we hypothesize that failure of the HPV-specific CD4+ T-cell response allows persistent HPV infection and subsequent establishment of high-grade CIN (Fig. 6, right panel). Indirect evidence supporting this hypothesis is given by reports showing that low CD4+ T-cell counts in HIV-infected individuals are associated with multiple HPV infections, higher viral load, viral persistence, and cervical dysplasia (12). Without surgical intervention, the majority of established high-grade CIN lesions will evolve toward cervical carcinoma. Our data suggest that in approximately half of the cervical carcinoma patients, the presence of the tumor will eventually trigger the induction of a CD4+ T-cell response. However, it is conceivable that cervical cancers do not provide the appropriate proinflammatory environment for the induction of a potent and well-polarized T-cell response and that CD4+ T-cell priming at this stage of disease will most likely result in an ineffective HPV-specific antitumor immune response. The lack of responses in the remaining patients could reflect either a complete failure at the induction level or could be the result of silencing of preexisting impaired responses by tumor-induced or chronic infection-induced T-regulatory activity. We are currently performing longitudinal studies in cohorts of women to investigate the dynamics of the HPV16 E2/E6-specific CD4+ T-cell response in relation to clearance or persistence of HPV infection.

We frequently detected E2-specific CD4+ T-cell immunity in cervical carcinoma patients, despite the fact that integration of viral DNA into the cellular genome often results in the loss of functional E2 gene expression. This may be explained by the finding that in addition to integrated copies, episomal HPV16 DNA capable of encoding E2 can be found in cervical carcinoma (33, 34).

Comparison of the cytokine profiles of mitogen-induced T-cell responses in PBMCs from cervical cancer patients versus healthy subjects showed the loss of the Th1-type cytokine IFN-γ in favor of the Th2-type cytokines IL-4 and IL-10 in patients. Others have previously described a similar finding in patients with extensive CIN lesions compared with healthy controls (17). Although cross-sectional analysis does not allow definite conclusions with respect to the order of events, it is likely that this altered systemic cytokine balance is a consequence of HPV-induced disease because the local cytokine environment in HPV-induced lesions is directly and indirectly (via antigen-presenting cells) capable of modifying cytokine profiles of T cells (15, 16, 30, 31). Notably, we did not observe significant differences between patients and controls in the cytokine profile of recall (MRM)-specific T-cell responses. The discrepancy between the character of MRM-specific responses on one hand and mitogen-triggered and HPV-specific responses on the other can most readily be explained by the fact that MRM-specific T-cell memory was established and therefore properly polarized before the development of cervical neoplasia. These strongly polarized MRM-specific memory T cells are, in contrast to naïve T cells, relatively insensitive to modified antigen-presenting cell function (35, 36), such as that expected to be found in cervical cancer patients (37). Mitogen-induced cytokine secretion, on the other hand, reflects the cytokine production of all peripheral T cells, including that of naïve T cells and those that have been primed against other pathogens during this period of HPV-induced disease.

In conclusion, our data demonstrate an association between HPV16+ cervical disease and partial or complete failure of CD4+ T-cell function in E2- and E6-specific immunity. The sophistically designed infection cycle of HPV16, which does not involve destruction of virus-infected keratinocytes and avoids proinflammatory signals that could stimulate recruitment and activation of antigen-presenting cells, necessitates the role of CD4+ T-cell help in the process of anti-HPV immunity. In view of this consideration, our findings offer a possible explanation for the development of cervical disease.

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