High Number of Intraepithelial CD8+ Tumor-Infiltrating Lymphocytes Is Associated with the Absence of Lymph Node Metastases in Patients with Large Early-Stage Cervical Cancer

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Abstract

In a prospective study, we have examined the tumor-specific immune response in a group of 59 patients with human papillomavirus (HPV) 16-positive (HPV16+)–induced or HPV18+-induced cervical cancer. Local antitumor immunity was analyzed by the enumeration of tumor-infiltrating dendritic cells and CD4+, CD8+, and regulatory T cells as well as by calculation of the ratio of CD8+/CD4+ T cells and CD8+/regulatory T cells. Systemic tumor-specific immunity was assessed by determination of the HPV E6- and/or E7-specific T-cell response in the blood of these patients. Finally, these variables were evaluated with respect to known histopathologic prognostic variables, including the absence (LN−) or presence (LN+) of lymph node metastases. Stratification according to the lymph node status of patients revealed a significantly stronger CD8+ T-cell tumor infiltration, a higher CD8+/CD4+ T-cell ratio, and higher CD8+/regulatory T-cell ratio in the group of patients in which the tumor failed to metastasize to the tumor-draining lymph node. Subdivision according to the presence (IR+) or absence (IR−) of circulating HPV-specific T cells disclosed that the highest number of tumor-infiltrating CD8+ T cells was found in the group of LN− patients displaying a concomitant systemic tumor-specific immune response (LN−IR+). CD8+ T-cell infiltration in LN−IR− patients was comparable with that of LN+ patients. In cervical cancer, the absence of lymph node metastases is strongly associated with a better prognosis. Our data indicate that, especially in a subgroup of LN− patients, a strong and effective interaction between immune system and tumor exists. This subgroup of cervical cancer patients may have the best prognosis. [Cancer Res 2007;67(1):354–61]

Introduction

Cervical cancer is caused by high-risk types of human papillomavirus (HPV), particularly types 16 (HPV16) and 18 (HPV18), which account for approximately two third of all cervical carcinomas (1, 2). It is the second most common cancer in women worldwide (3, 4). Overall cure rates of early-staged tumors approach 85%. However, a spectrum of relapse risk exists depending on several prognostic factors. Clinical stage and lymph node status are the most powerful predictors of outcome in cervical cancer (5, 6), but other histopathologic factors, including size of primary tumor, infiltration depth, and vasoinvasion, have effect on the prognosis too (5–8).

Cervical cancer cells highly express two well-known tumor-specific oncoproteins that are encoded by HPV. These proteins, E6 and E7, are constitutively expressed because they are required to maintain the malignant phenotype (9). The tumor can be infiltrated by lymphocytes (10, 11), and both CD8+ and CD4+ T cells isolated from tumors are able to recognize the E6 and E7 tumor antigens (12–14)[5] as well as to kill tumor cells *in vitro* (15, 16). Tumor-specific T cells, directed against the two HPV-encoded oncoproteins, can be detected in the peripheral blood of ~50% of the patients albeit at low levels (17–23). Therapeutic vaccination strategies aiming at the activation of large numbers of E6- and/or E7-specific T cells in patients with precursor cancer lesions showed promising results (24–28), but no clear association between the presence of either a spontaneously induced or a vaccine-induced systemic tumor-specific immune response and clinical outcome was established.

Thus far, the only immunologic determinant associated with a better prognosis is a pronounced infiltration of cervical carcinoma by lymphocytes (10, 11). However, thorough evaluations on the prognostic significance of tumor-infiltrating lymphocytes (TIL) in other human cancers revealed that especially intraepithelial infiltrating CD3+CD8+ T cells contributed to a better prognosis (29–31). On the other hand, the infiltration by CD3+CD4+ T cells (32, 33) or a subpopulation of CD4+ T cells with immunosuppressive properties, so called regulatory T cells that were detected by staining for Foxp3 (34), was reported to counteract the beneficial effect of CD8+ T cells (33–35). High ratios between CD8+ T cells and the other cell types were associated with improved survival (32, 33). These types of analyses have not been done in patients with cervical carcinoma.

In the present study, we did an in-depth analysis of the tumor-specific immune response in patients with HPV-induced cervical cancer. Local antitumor immunity was analyzed by the enumeration of tumor-infiltrating dendritic cells and CD4+, CD8+, and regulatory T cells as well as by calculation of the ratio of CD8+/CD4+ T cells and that of CD8+/regulatory T cells. The presence of systemic tumor immunity was assessed by examination of HPV E6- and/or E7-specific T-cell immunity in the peripheral blood of

Note: S.J. Piersma, E.S. Jordanova, and M.I.E. van Poelgeest contributed equally to this work.

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doi:10.1158/0008-5472.CAN-06-3388

[5] S.J. Piersma, in preparation.

these patients. Finally, these variables were evaluated with respect to the lymph node status of patients because of its strong prognostic value in cervical cancer.

Materials and Methods

Subjects. Women presenting with histologically proven early-stage cervical carcinoma at the Department of Gynecology of the Leiden University Medical Center (Leiden, the Netherlands) and scheduled for radical hysterectomy were enrolled in the CIRCLE study that investigates cellular immunity against HPV16-positive cervical lesions after providing informed consent. The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center. Patient characteristics are given in Table 1. Blood was drawn at day of treatment before surgery. Peripheral blood mononuclear cells (PBMC) were obtained for the analysis of HPV-specific T-cell reactivity. The subjects were typed for HPV16 and HPV18 using HPV16- and HPV18-specific primers on DNA isolated from surgical resection specimens (36). $HPV16^+$ and $HPV18^+$ subjects were included in the immunologic and immunohistochemical analyzes. Due to the fact that the obtained materials of these patients are the subject of many different studies within the Leiden University Medical Center, a selection bias occurred for the inclusion of patients diagnosed with a bigger tumor volume. Because a larger tumor is associated with the presence of lymph node metastases (37), a larger fraction of the selected patients harbored metastatic disease in the pelvic lymph node than would be expected at these early disease stages (Table 1).

Histologic specimens of normal cervices from women ($n = 9$; median age, 46 years; range, 31–60) who underwent hysterectomies for benign uterine diseases with no cervical abnormalities served as control for the cervical carcinoma group. These specimens were obtained as paraffin-embedded blocks from the Department of Pathology of the Leiden University Medical Center.

Antigens and T-cell assay. A set of peptides spanning the whole HPV16 and HPV18 E6 and E7 proteins was used for the T-cell proliferation assays. For these assays, 32-mer peptides of the HPV16 E6 protein and the 35-mer peptides of the HPV16 E7 protein with an overlap of 14 amino acids were used in pools of two peptides per pool. The HPV18 peptides consisted of 15 E6 and 9 E7 22-mer peptides (overlap of 12 amino acids) and were used in pools of four peptides per pool. The peptides were synthesized and dissolved as described previously (38). 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., $E6_{1\text{-}50}$, residues 1–32 and 19–50). To examine the presence of circulating HPV-specific T cells, freshly isolated PBMCs were incubated with the indicated peptide pools in eight parallel microcultures as described previously (17). Medium alone was taken along as a negative control, and the memory response mix (dilution, 1:50) served as a positive control (17). Supernatant (50 μL) from the microcultures was taken at day 6 after incubation and stored at −20°C until cytokine analysis. Peptide-specific proliferation was measured at day 7 by [^{3}H]thymidine incorporation. Cultures 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 (39).

Cytokine analysis. The detection of cytokines in the supernatants of the short-term proliferation assays was done using the cytometric bead array (CBA; Becton Dickinson, Etten-Leur, the Netherlands). This technique allows the simultaneous detection of six different T-helper (Th) 1 and Th2 cytokines: IFN-γ, tumor necrosis factor-α, interleukin (IL)-2, IL-4, IL-5, and IL-10. The CBA was done according to the manufacturer's instructions. Cutoff values were based on the standard curves of the different cytokines (100 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 more than twice the concentration of the medium control (17, 40).

Quadruple immunostaining of tumor tissues and infiltrating lymphocytes. A newly developed technique for simultaneous immunohistochemical staining of four different epitopes was applied to 4-μm formalin-fixed, paraffin-embedded tissue sections (41). The AE1/AE3 antibody (IgG1; DAKO, Glostrup, Denmark) was added and slides were incubated overnight. Slides were washed and incubated first with cationic bovine serum albumin (Aurion, Wageningen, the Netherlands). After 2 h of incubation with ultrasmall gold-labeled goat anti-mouse antibody (Aurion), silver enhancement was done using the Aurion enhancement kit.

The silver-stained slides were incubated overnight with a mix of either ab828 (anti-CD3, rabbit polyclonal antibody; Abcam, Cambridge, United Kingdom), hNK-1 (anti-CD57, mouse IgM, culture supernatant of a hybridoma grown in our own laboratory), and 4B11 (anti-CD8, mouse IgG2b; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-CD4 (rabbit polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) and CD45RO (mouse monoclonal IgG2a; DAKO). Slides were washed and incubated with the appropriate combination of fluorescent antibody conjugates (goat anti-rabbit IgG-Alexa Fluor 546, goat anti-mouse IgM-Alexa Fluor 488, and goat anti-mouse IgG2b-Alexa Fluor 647 or goat anti-rabbit IgG-Alexa Fluor 546 and goat anti-mouse IgG2a-Alexa Fluor 647). Alexa Fluor conjugates were obtained from Molecular Probes (Leiden, the Netherlands).

Our pilot study revealed that, without counterstaining with anti-CD3, the enumeration of infiltrating $CD4^+$ T cells based only on their morphologic appearance was difficult. In a selected set of tumors, we compared the numbers of $CD4^+$ T cells by direct staining with anti-CD4 or by indirect identification using a double staining of anti-CD3 and anti-CD8 by counting the $CD3^+CD8^-$ cells. Because the indirect technique resulted in similar numbers of T cells, we used $CD3^+CD8^-$ cells as a marker for $CD4^+$ T cells in all other tumor sections.

The images were captured with a confocal laser scanning microscope (Zeiss LSM510, Zeiss, Jena, Germany). Fifteen images were scanned per slide. For each case, one successive negative control slide was included. These controls were silver stained but the primary lymphocyte-specific

Table 1. Patient characteristics

No. patients	59
Age (y)	
Median	44
Range	29–76
FIGO stage	
IA	0
IB	49
IIA	8
IIB	2
HPV type	
$HPV16^+$	45
$HPV18^+$	14
Lymph node metastases	
Positive	23
Negative	36
Tumor size (cm)	
<4	35
≥4	24
Unknown	0
Infiltration depth (mm)	
<15	34
≥15	21
Unknown	4
Vasoinvasion	
Yes	36
No	15
Unknown	8

Abbreviation: FIGO, Fesddration Internationale des Gynaecologistes et Obstetristes.

antibody step was omitted to ensure the specificity of the secondary antibody binding. Three images were taken at different positions on the control slide using exactly the same confocal laser scanning microscope multitrack settings as for the respective case. Intraepithelial and stromal infiltrating lymphocyte cell counts were done by two independent investigators (M.I.E.v.P and E.S.J.) and represented as the number of cells per mm^2.

Immunostaining of immature/activated dendritic cells and regulatory Foxp3$^+$ T cells. Paraffin sections (4 μm) were used for standard immunohistochemical staining of infiltrating antigen-presenting cells. The mouse monoclonal antibodies directed against CD1a (IgG1; NeoMarkers-Lab Vision, Fremont, CA) and DC-Lamp (IgG1; Immunotech, Marseille, France) were used for staining immature or activated dendritic cells, respectively. The mouse monoclonal Foxp3 antibody (clone 236A/E7; Abcam, Cambridge, MA) was used for detecting regulatory T cells. Briefly, slides were deparaffinized and antigen retrieval was done using EDTA for the DC-Lamp staining and trypsin pretreatment for the CD1a staining. After an overnight incubation, the biotin-labeled rabbit anti-mouse immunoglobulins and a biotinylated horseradish peroxidase-streptavidin complex (both were from DAKO) were applied. To visualize immune complexes, a 0.05% solution of diaminobenzidine (Sigma, St. Louis, MO) containing 0.0018% H_2O_2 in a 0.05 mol/L Tris-HCl buffer (pH 7.6) was used. Counterstaining of the slides was done with Mayer's hematoxylin. Cells were quantified by counting the tumor-infiltrating Langerhans cells and the stromal DC-Lamp$^+$ cells per 10 randomly selected high-power fields (×400 magnification). Foxp3$^+$ T cells were counted separately both in the tumor fields and in the stromal compartment and represented as the number of cells per mm^2.

Statistical analysis. A two-tailed t test with Welch correction or Mann-Whitney test (when applicable) was applied for the analysis of the numbers of infiltrating immune cells or ratios in the different patient groups divided into categories that were based on the following clinical prognostic factors: invasion depth of tumor in cervical tissue (<15 or ≥15 mm), tumor size (<4 or ≥4 cm), vasoinvasion, and lymph node metastases (5, 7, 8).

χ^2 analyses for trend were used to test the relationship between different categories of CD8$^+$ T-cell infiltration versus lymph node status. Categories were based on 25th percentile and 75th percentile. Spearman rank analysis was done to test the association between the different types of infiltrating immune cells. Fisher's exact test (two tailed) was used to analyze both the HPV-specific immunity and lymph node metastases and the relationship between different categories of infiltrating CD8$^+$ T cells, CD4$^+$ T cells, and regulatory T cells and combinations hereof versus lymph node status. Statistical analyses were done using GraphPad InStat software (version 3.0) and GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Results

The tumors of cervical carcinoma patients display a wide variety in the number of tumor-infiltrating T cells. A detailed examination of the antitumor response was done in a group of 59 patients with HPV16$^+$ or HPV18$^+$ cervical cancer (Table 1). The numbers of CD3$^+$ T cells and regulatory T cells (Foxp3$^+$) present in stroma or between epithelial tumor cells were counted. Because previous reports strongly indicated that, especially intraepithelial infiltration by T cells was correlated with prognosis in cervical cancer (10, 11), the numbers of CD3$^+$CD8$^+$ T cells, CD3$^+$CD8$^+$CD57$^+$ T cells, and CD3$^+$CD8$^-$ (CD4) T cells specifically infiltrating the tumor epithelium were examined. Additionally, tumor-infiltrating immature dendritic cells (CD1a$^+$) and activated dendritic cells (DC-Lamp$^+$) in the tumor were enumerated. To be able to assess to which degree immune infiltration was due to tumor-specific immunity, we also analyzed the infiltration of immune cells in sections of normal cervical epithelium that had been obtained from a group of nine patients with no evidence of cervical abnormalities. In Fig. 1*A*, an example of the analysis of CD3$^+$ (red) and CD3$^+$CD8$^+$ (purple) T-cell infiltration by quadruple-color confocal microscopy is shown. CD3$^+$ T cells were present both in normal cervical tissue and in tumors. More stroma resident CD3$^+$ T cells were detected in tumor tissue than in normal cervical tissue (patients: mean, 966 ± 635 cells per mm^2; controls: mean, 385 ± 96 cells per mm^2; $P < 0.0001$). The mean number of intraepithelial CD3$^+$CD8$^+$ or CD3$^+$CD8$^-$ (CD4) T cells that infiltrated tumors of all patients was about as twice as high when compared with those infiltrating normal cervices, but the number of intraepithelial tumor-infiltrating T cells varied enormously (range, 2.9–592 per mm^2) between patients (Fig. 2). The number of both intraepithelial (range, 0.6–56 mm^2) and stroma (range, 22–249 per mm^2) resident regulatory (Foxp3$^+$) T cells was significantly enhanced in cervical carcinoma when compared with control cervical tissue ($P < 0.001$; Figs. 1 and 2). Furthermore, small numbers of intraepithelial CD3$^+$CD8$^+$CD57$^+$ terminally activated effector T cells (42) and natural killer (NK)-like cells (CD3$^-$CD57$^+$) were observed in cervical tumors (Fig. 2).

The degree of intraepithelial infiltration by CD8$^+$ T cells was paralleled by other types of immune cells infiltrating the tumor. Spearman rank analysis revealed significant correlations between the number of intraepithelial CD3$^+$CD8$^+$ T cells and the number of CD3$^+$CD8$^-$ T cells ($r = 0.64$; $P < 0.0001$), CD3$^+$CD8$^+$CD57$^+$ T cells ($r = 0.71$; $P < 0.0001$), CD3$^-$CD57$^+$ NK-like cells ($r = 0.43$; $P < 0.001$), and regulatory T cells ($r = 0.45$; $P < 0.001$) that infiltrated the tumor of each patient.

The number of tumor-infiltrating immature (CD1a$^+$) dendritic cells approximated two to three cells per 10 high-power fields both in the controls and in the patients. Small numbers of activated dendritic cells (DC-Lamp$^+$) were detected in all tumors but were virtually absent in the normal cervices ($P < 0.001$; Fig. 2). In general, the number of tumor-infiltrating immature and activated dendritic cells was correlated per tumor (Spearman rank analysis $r = 0.34$; $P = 0.008$). Together, these data indicate that there is great variation in the number of immune cells that can infiltrate HPV-induced cervical tumors. Furthermore, on the recruitment of higher numbers of CD8$^+$ T cells, there is also a more effective infiltration of the tumor by other immune cells, suggesting an intense interaction between tumor and immune system.

Strong tumor immunity is present in a subset of patients without lymph node metastases. Histopathologic factors known to affect on the prognosis of patients are vasoinvasion, the size of a tumor, and the depth of tumor invasion into the normal cervical stroma (5–8). Especially, the presence of lymph node metastases is a strong independent prognostic factor associated with a decrease in overall and disease-specific survival and an increase of recurrence in cervical cancer (5, 6). Stratification of the patient group based on clinical prognosis, as predicted by these histopathologic factors, revealed that the mean number of intraepithelial CD8$^+$ T cells was higher in the group of patients without lymph node metastases (LN$^-$) than in the group of patients with a poor prognosis (LN$^+$; 151 ± 126 versus 108 ± 147, respectively; $P = 0.02$). No differences were observed when other types of infiltrating immune cells, including CD3$^-$CD57$^+$ (NK) and CD8$^+$CD57$^+$ cells known to play a role in controlling tumor metastases in other cancers (43, 44), were analyzed. For the other histopathologic factors, we found a correlation neither between vasoinvasion ($P = 0.10$), infiltration depth of tumor ($P = 0.14$), or tumor size ($P = 0.31$) and CD8$^+$ T-cell infiltration nor for the infiltration by other immune cells.

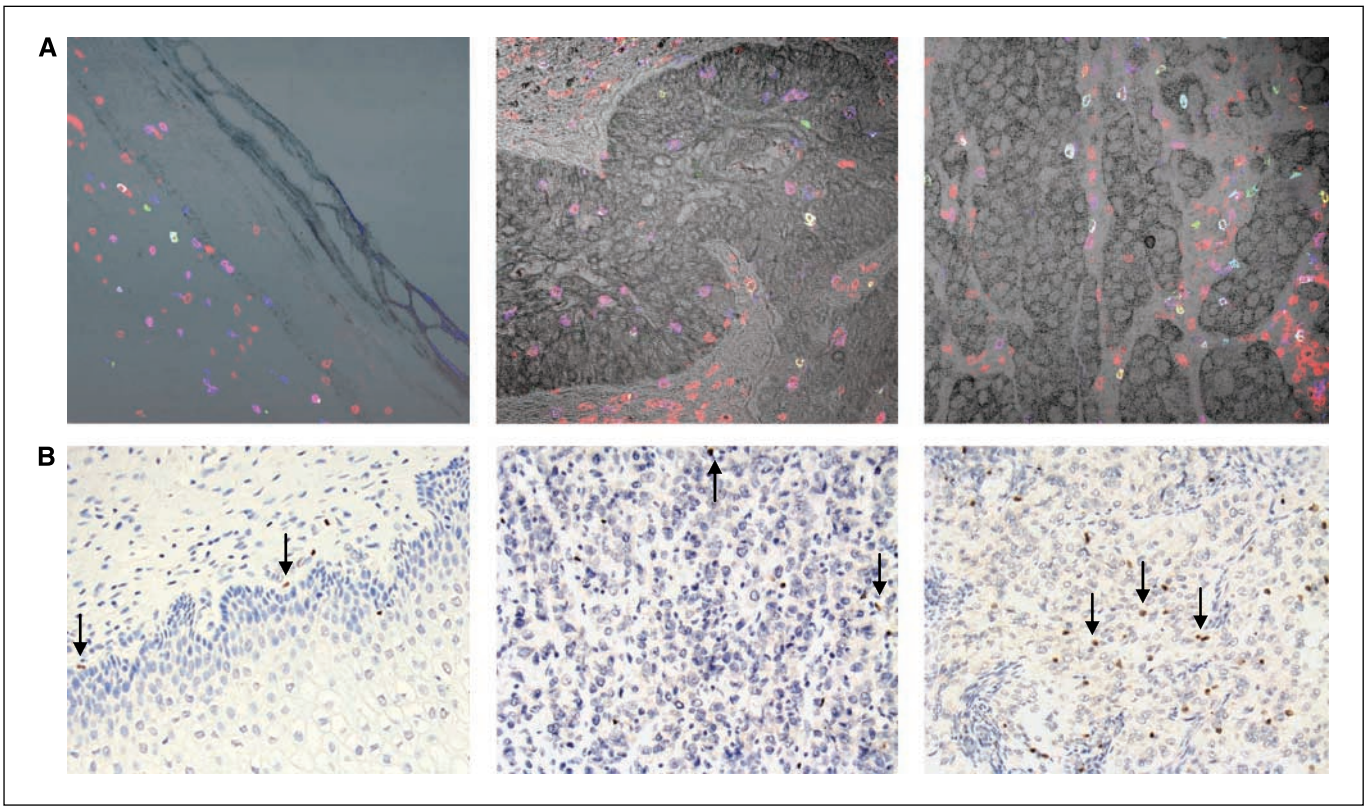

Figure 1. Intraepithelial infiltrating T cells in cervical cancer patients and in healthy controls. *A,* to detect tumor-infiltrating T cells, 4-μm tissue sections were stained with anti-CD3 (*red*), anti-CD8 (*blue*), and anti-CD57 (*green*) followed by the appropriate combination of fluorescent antibody conjugates. Images were captured with a confocal laser scanning microscope (Zeiss LSM510) and used to count all T cells that were located intraepithelial and in the stroma of 15 different (tumor) fields in each tissue section. The number of T cells per tumor area (T cells/mm^2 tumor epithelium) was calculated. Three representative examples are shown. *Left,* example of a normal cervix; *middle,* tumor tissue of a patient without a lymph node metastases displaying a systemic immune response to HPV (patient 161) that shows intraepithelial infiltration by CD3$^+$CD8$^-$ (CD4) T cells (*red*) and CD3$^+$CD8$^+$ T cells (*purple*); *right,* tumor tissue of a patient with a lymph node metastases and no systemic immune response (patient 148). *B,* to detect regulatory T cells, 4-μm paraffin sections were stained with the mouse monoclonal Foxp3 antibody (clone 236A/E7). The number of positive cells per 10 randomly selected high-power fields (×400 magnification) was counted separately both in the tumor fields and in the stromal compartment and represented as the number of cells per mm^2. Example of a normal cervix (*left*) and lymph node–negative patient 161 (*middle*) showing rare infiltration by regulatory T cells (*arrow*). *Right,* tumor tissue of a patient with lymph node metastases and strong infiltration by regulatory T cells (patient 148).

To confirm the relationship between intratumoral CD8$^+$ T-cell infiltration and lymph node status, we reanalyzed our patient group and stratified patients according to weak infiltration (25 percentile: ≤49.9 intraepithelial CD8$^+$ T cells per mm^2; n = 5 LN$^-$; n = 10 LN$^+$), intermediate infiltration (25–75 percentile: n = 19 LN$^-$; n = 10 LN$^+$), or strong infiltration (75 percentile: ≥144 intraepithelial CD8$^+$ T cells per mm^2; n = 12 LN$^-$; n = 3 LN$^+$) with intraepithelial CD8$^+$ T cells. Based on the fraction of LN$^-$ patients within these groups, there was a significant association between the number of infiltrating CD8$^+$ T cells and the absence of lymph node metastases (χ^2 for trend = 6.9; P < 0.01; Fig. 3).

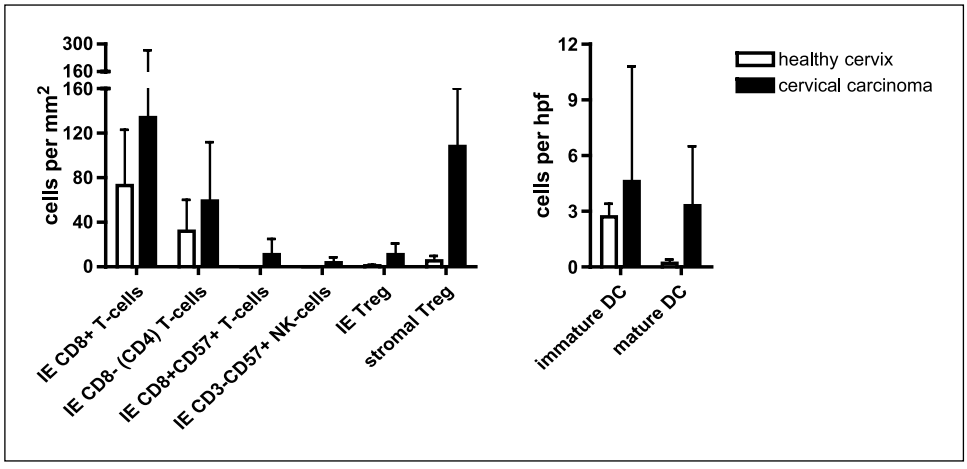

Figure 2. Comparison of immune cells infiltrating normal cervical tissue or cervical carcinoma. *Left,* cell densities of only the intraepithelial tumor-infiltrating T cells (expressed as cell number/mm^2 ±SD) were enumerated because of previous reports stating that the infiltration of cervical cancer cell nests displays the strongest correlation with prognosis (10, 11). The number of both intraepithelial (*IE*) and stromal tumor-infiltrating regulatory T (*Treg*) cells, identified through Foxp3$^+$ staining, was quantified because there was no data to support that either location is more important. *Right, columns,* cell densities of intraepithelial and stromal tumor-infiltrating CD1a and DC-Lamp$^+$ dendritic cells (*DC*) depicted per 10 high-power fields; *bars,* SD.

In other cancers, a high ratio between the $CD8^+$ T cells and $CD4^+$ T cells or regulatory T cells is associated with favorable prognosis (32, 33), indicating that especially the proportion between the different subtypes of immune cells within the tumor of each individual patient is important. In view of the wide ranges observed for the numbers of infiltrating $CD3^+CD8^+$, $CD3^+CD8^-$, and regulatory T cells cells, the mean number of each type of infiltrating immune cells may not differ considerably between subgroups of patients, whereas the ratio between these subtypes can. Therefore, the ratios between $CD3^+CD8^+$, $CD3^+CD8^-$, and regulatory T cells were calculated for each patient. The mean $CD8^+/CD8^-$ (CD4) T-cell ratio did not differ between normal tissue and that of the group of all patients (3.1 ± 2.4 versus 2.8 ± 2.8, respectively). However, the CD8/CD4 T-cell ratio was considerably lower in the LN^+ patient group (1.9 ± 1.7) than in the LN^- patient group ($P = 0.01$), who displayed a $CD8^+/CD8^-$ (CD4) T-cell ratio (3.3 ± 3.2) similar to what is found in normal cervices. The ratio between intraepithelial $CD8^+$ T cells and either stromal (1.4 ± 1.4) or intraepithelial (27 ± 56) regulatory T cells was much lower in cancer patients than in normal cervical tissue (22 ± 18 and 118 ± 100, respectively; $P < 0.001$). Importantly, a higher $CD8^+$/stromal regulatory T-cell ratio was found in the LN^- patient group than for the LN^+ patient group (1.6 ± 1.5 versus 0.9 ± 1.0; $P < 0.05$). Thus, evaluation of cervical cancer patients with respect to their lymph node status revealed that the LN^- patient group, which in general has a good prognosis, displays significantly higher number of intraepithelial $CD8^+$ T cells, a higher $CD8^+/CD4^+$ T-cell ratio, and a higher $CD8^+$/regulatory T-cell ratio than the group of patients with lymph node metastases.

To assess the influence of $CD4^+$ T cells or regulatory T cells on the association of infiltrating $CD8^+$ T cells with lymph node status, two groups of patients that represented the two most extreme situations of tumor-infiltrating T cells were analyzed. In these two groups, patients who displayed either a favorable constitution of tumor infiltration (higher number of intraepithelial $CD8^+$ T cells, a higher $CD8^+/CD4^+$ T-cell ratio, and a higher $CD8^+$/regulatory T-cell ratio) or a nonfavorable constitution of infiltration (lower $CD8^+$ T-cell count and a low $CD8^+/CD4^+$ and $CD8^+$/regulatory T-cell ratio) were included. Although the fraction of LN^- patients was higher among the patients with a higher $CD8^+$ T-cell infiltration ($\geq$95.2 cells per mm^2; 50 percentile), this was not significant ($P = 0.06$). Selection based on $CD8^+$ T cells and both the $CD8^+/CD4^+$ T-cell ratio (50 percentile ratio, 2.235) and the $CD8^+$/stromal regulatory T-cell ratio (50 percentile ratio, 0.747) resulted in significantly increased fraction of LN^- patients within the group of patients with a favorable tumor infiltration when compared with the group of patients with a less favorable infiltration ($P = 0.01$), but this increase was not significant when compared with the fraction of LN^- patients within the group of patients selected only based on a higher $CD8^+$ T-cell infiltration. These data suggest that coinfiltrating $CD4^+$ T cells and regulatory T cells has a subtle influence on the lymph node status of patients but only in cases that they match the number of infiltrating $CD8^+$ T cells. However, the absence of lymph node metastases is most prominently associated with a robust $CD8^+$ tumor infiltration.

Strong $CD8^+$ T-cell infiltration is predominantly observed in the subgroup of LN^- patients with circulating HPV-specific T cells. From 50 patients, sufficient amounts of PBMC were available to study the systemic HPV-specific T-cell response using a methodology that detects $CD4^+$ T-cell responses against the HPV early antigens (17, 19, 45). In this group, 24 patients displayed a systemic HPV-specific T-cell response against one or more of the E6 and/or E7 peptide pools, whereas 26 patients failed to produce a detectable T-cell response, a frequency that is in line with our previous findings (17). Examples shown in Fig. 4*A* reveal that some patient PBMC samples show proliferative T-cell reactivity against only a single peptide pool, whereas other samples display a broader response against up to three peptide pools. As illustrated in Fig. 4*B*, IFN-γ production is found in some of the culture supernatants, but this did not depend on the strength of the proliferative response (compare patients 239 and 161 in Fig. 4). Ten of the 24 patients with a systemic HPV-specific immune response produced IFN-γ on stimulation with at least one of the peptide pools. There was no direct relationship between the presence (IR^+) or absence (IR^-) of circulating HPV-specific T cells and the lymph node status of patients ($P = 1.0$) or with the magnitude of tumor-infiltrating T cells (e.g., IR status versus $CD8^+$ T cells; $P = 0.31$).

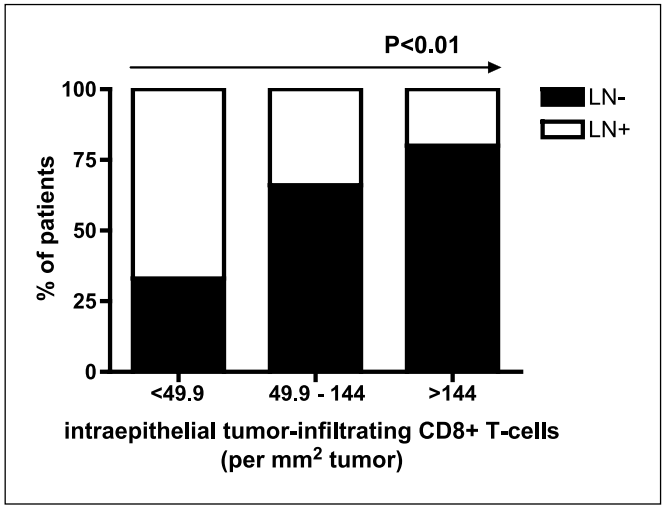

Figure 3. The prognostic value of intraepithelial tumor-infiltrating $CD8^+$ T cells, $CD4^+$ T cells, and regulatory T cells. Patients ($n = 59$) were stratified according to weak infiltration (25 percentile: ≤49.9 intraepithelial $CD8^+$ T cells per mm^2), intermediate infiltration (25–75 percentile), or strong infiltration (75 percentile: ≥144 intraepithelial $CD8^+$ T cells per mm^2) with intraepithelial $CD8^+$ T cells. The percentage of patients with a lymph node metastases of the tumor (LN^+; $n = 23$) or without a lymph node metastases (LN^-; $n = 36$) are indicated per subgroup. The group of patients with weak infiltration consisted of 5 LN^- and 10 LN^+ patients, the group of patients with intermediate infiltration consisted of 19 LN^- and 10 LN^+ patients, and the group of patients with strong infiltration consisted of 12 LN^- and 3 LN^+ patients. There was a significant association between a higher number of infiltrating $CD8^+$ T cells and the absence of lymph node metastases ($\chi^2 = 6.9$; $P < 0.01$).

Therefore, the group of patients was further subdivided according to both the HPV immune status and the lymph node status (Table 2). Considerable differences were found in the groups of patients that displayed a systemic antitumor response. The group of LN^-IR^+ patients displayed a significant higher number of intraepithelial tumor-infiltrating $CD8^+$ T cells than LN^- patients without a concomitant systemic immune response ($P = 0.02$; Table 2). Furthermore, within the group of LN^- patients, not only intraepithelial $CD8^+$ T cells but also the number of $CD8^+CD57^+$ terminally activated effector T cells and regulatory ($Foxp3^+$) T cells as well as $CD8^+/CD4^+$ T-cell ratio were increased in the group of LN^-IR^+ patients ($P = 0.03$; Table 2). These data suggest that, within the group of LN^- patients who in general display a better prognosis, a subgroup can be identified. The patients within this subgroup mount a significantly stronger antitumor response, which

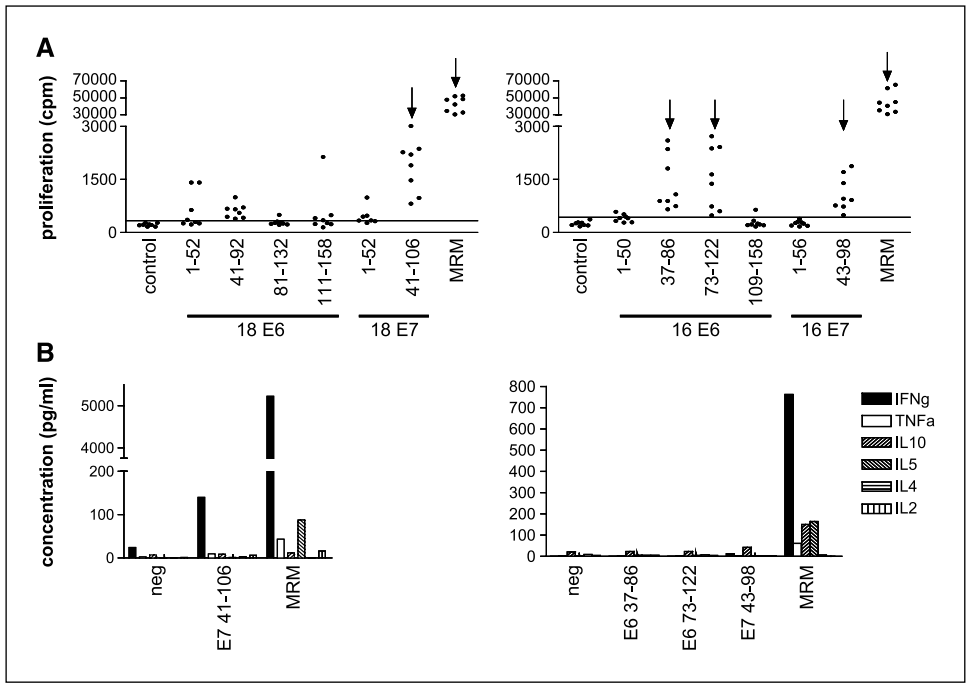

Figure 4. Two representative examples of patient-derived T-cell cultures that recognize HPV-specific peptide pools of HPV16 or HPV18 but display different cytokine profiles. *A,* proliferative responses of patients 239 (*left*) and 161 (*right*). Each *circle* represents the proliferation of an individual microculture (n = 8); supernatants of the positive responses (*arrows*) were tested for cytokine production. *B,* corresponding cytokine levels produced on antigenic stimulation of these microcultures are depicted, indicating that IFN-γ production is found in some but not all of the supernatants isolated from the HPV-specific T-cell proliferative cultures.

is reflected by circulating tumor-specific T cells, a robust $CD8^+$ tumor infiltration, and a higher $CD8^+/CD4^+$ T-cell ratio.

Unexpectedly, the LN^+IR^+ group displayed the lowest numbers of tumor-infiltrating immune cells (Table 2). In addition, this group displayed a significantly lower $CD8^+/CD8^-$ (CD4) ratio (P = 0.003) when compared with the LN^-IR^+ group (Table 2). Furthermore, regulatory T cells residing in the tumor stroma readily outnumbered infiltrating $CD8^+$ T cells, resulting in a low $CD8^+$/regulatory T-cell ratio (Table 2). In contrast, in the group of LN^-IR^+ patients, the number of infiltrating $CD8^+$ T cells surpassed that of regulatory T cells in the stroma despite the fact that in this group also more regulatory T cells were recruited into the tumor. Consequently, the ratio between intraepithelial $CD8^+$ T cells and regulatory T cells in the stroma is higher in the group of LN^-IR^+ than in the LN^+IR^+ group (P < 0.001). There were no significant differences between the groups of LN^- or LN^+ patients without circulating HPV-specific T cells (IR^-).

Discussion

In cervical carcinoma, infiltration of intratumoral lymphocytes has been associated with an improved clinical outcome (10, 11).

Table 2. Summary of the number of tumor-infiltrating immune cells in groups of patients stratified according to the absence (LN^-) or presence (LN^+) of lymph node metastases and circulating HPV-specific T cells

	LN^-		LN^+	
	IR^- (n = 17)	IR^+ (n = 14)	IR^- (n = 9)	IR^+ (n = 10)
$CD8^+$ T cells*	90 ± 65	200 ± 145	129 ± 190	58 ± 46
$CD8^-$ ($CD4^+$) T cells	42 ± 29	62 ± 46	54 ± 75	61 ± 69
$CD3^+CD8^+CD57^+$ T cells	9.8 ± 12	13 ± 15	8.4 ± 16	5.6 ± 8.5
$CD3^-CD57^+$ NK-like cells	4.4 ± 6.7	3.1 ± 2.5	3.8 ± 5.9	2.0 ± 2.2
Intraepithelial $Foxp3^+$ T cells †	7.5 ± 7.1	15 ± 9.5	10 ± 15	7.1 ± 5.8
Stromal $Foxp3^+$ T cells	100 ± 69	136 ± 69	100 ± 44	104 ± 42
$CD8^+/CD8^-$ (CD4) T-cell ratio ‡	2.3 ± 1.3	4.4 ± 4.5	2.6 ± 2.1	1.5 ± 1.6
CD8/intraepithelial regulatory T-cell ratio ‡	27 ± 47	28 ± 40	51 ± 112	9.7 ± 11
CD8/stromal regulatory T-cell ratio ‡	1.3 ± 1.3	1.8 ± 1.6	1.2 ± 1.3	0.4 ± 0.2

*Cell densities of only the intraepithelial tumor-infiltrating T cells (expressed by cell number/mm^2 ±SD) were enumerated because of previous reports stating that the infiltration of tumor cell nests displays the strongest correlation with prognosis (10, 11, 29–31, 33).

† The number of both intraepithelial and stromal tumor-infiltrating regulatory ($Foxp3^+$) T cells was quantified because there is no evidence that either location is more important.

‡ The ratio of intraepithelial $CD8^+$ and intraepithelial $CD8^-$ ($CD4^+$) T cells was determined as well as the ratio between intraepithelial $CD8^+$ T cells and regulatory ($Foxp3^+$) T cells.

In the present study, we did a detailed analysis of the immune response in patients with HPV-induced cervical cancer. Although infiltration of most TIL subtypes positively correlated with each other, intraepithelial $CD8^+$ T cells were the only subtype that correlated with a lack of pelvic lymph node spread and, therefore, might have a better prognosis. This observation is consistent with other studies, indicating that strong intraepithelial $CD8^+$ T-cell infiltration is associated with a favorable course of disease in colorectal cancer (29, 30), ovarian cancer (33, 46), and endometrial carcinoma (31). In addition, we showed that failure of $CD8^+$ T cells to substantially infiltrate the tumor results in a significantly lower $CD8^+/CD4^+$ T-cell ratio in patients with lymph node metastases when compared with patients without metastatic disease or to normal cervical tissue. Similar observations were made in colorectal and ovarian cancer (32, 33). Importantly, recently, we detected and isolated HPV-specific $CD4^+$ Th1 cells as well as HPV-specific $CD4^+Foxp3^+$ regulatory T cells from several lymph nodes and tumors derived from patients with cervical cancer,[6] indicating that part of the cervical tumor-infiltrating $CD4^+$ T cells can suppress local immunity. The number of regulatory T cells in cervical carcinoma was directly examined by detection of Foxp3, serving as specific marker for at least a subset of regulatory T cells (34). In healthy cervices, such regulatory T cells were almost absent. In contrast, cervical tumor tissue was strongly infiltrated by $Foxp3^+$ T cells (Fig. 2). We did not find a direct correlation between the presence of regulatory T cells and the lymph node status of patients, but patients without such metastases displayed a significantly more favorable intraepithelial $CD8^+$/regulatory T-cell ratio. As expected, the strongest correlation was found when the $CD8^+$/regulatory T-cell ratio was determined with $Foxp3^+$ T cells present in the tumor stroma, where also most of the $CD4^+$ T cells reside (Fig. 1). This suggests that a robust $CD8^+$ T-cell infiltration can overcome the negative effects of tumor resident $CD4^+$ regulatory T cells, resulting in a failure of the tumor to metastasize to the lymph nodes. A recent comprehensive study of the percentage of $CD8^+$ T cells, $CD4^+$ T cells, and regulatory T cells in both tumor-draining lymph nodes and tumor of mice suggests that this concept may indeed be true. In comparison with lymph nodes, $CD8^+$ T cells are underrepresented in the tumor when compared with $CD4^+$ T cells and regulatory T cells (47). Releasing the breaks on T cells by anti-CTLA-4 therapy strongly increased the number of tumor-infiltrating $CD8^+$ T cells, resulting in a significant increase of the $CD8^+$/regulatory T-cell ratio, and this correlated with tumor rejection. Interestingly, a therapy-induced decrease in this ratio was observed in the tumor-draining lymph node (47).

In addition to the local immune response, we also analyzed systemic antitumor immunity. For this, we exploited the T-cell response against the tumor-specific antigens E6 and E7 encoded by HPV. Previous studies indicated that the expression of E6 and E7 in cervical carcinoma results in the induction of a detectable HPV-specific proliferative $CD4^+$ T-cell response in approximately half of all cases (17–19, 22, 23, 48). Here, circulating HPV-specific proliferative T cells were detected in 24 of 50 cases. There was no direct correlation between the presence of HPV-specific systemic immunity and lymph node metastases, which were present in approximately half of the 24 patients displaying HPV immunity. However, subdivision according to both lymph node status and HPV immune status revealed an intriguing picture.

First, the higher number of $CD8^+$ T cells, higher $CD8^+/CD4^+$ T-cell ratio, and higher $CD8^+$/regulatory T-cell ratio that was found in the group of LN^- patients, when compared with the group of LN^+ patients, could be attributed to those LN^- patients displaying a concomitant systemic tumor-specific immune response (LN^-IR^+; Table 2). The number of infiltrating $CD8^+$ T cells was higher in this subgroup of patients than in the LN^- patients without such a response. Strikingly, $CD8^+$ T-cell infiltration in the LN^-IR^- patient group was comparable with that of LN^+ patients. In ovarian, endometrial, and colorectal cancer, strong intraepithelial $CD8^+$ T-cell infiltration has a favorable prognostic effect (29–31, 33, 46), suggesting that especially the LN^-IR^+ subgroup of lymph node–negative cervical cancer patients will display a better disease course.

Second, we observed that the most extreme differences occurred among the patients with a positive HPV immune status. Whereas in the LN^- patient group the presence of circulating HPV-specific T cells was associated with higher numbers of infiltrating T cells (LN^-IR^+), the opposite was observed in the LN^+ patient group (Table 2). The LN^+IR^+ group displayed the lowest number of intratumoral $CD8^+$ T cells and the lowest $CD8^+$/regulatory T-cell ratio. These data suggest that the presence of circulating HPV-specific T cells not necessarily reflects a proper antitumor response and implies that the presence of tumor cell metastases in the draining lymph nodes may direct the development of unwanted tumor-specific T-cell responses that can counteract the recruitment of effector cells in the tumor. Indeed, HPV16 E6-specific $CD4^+Foxp3^+$ regulatory T cells isolated from tumor-positive lymph nodes can suppress the action of antigen-specific T cells at a one-to-one ratio.[6] Notably, ratios of one to one and even two to one (regulatory T cells/$CD8^+$ T cells) are present in the tumors of LN^+ patients (Table 2).

In conclusion, our results show that the presence of intraepithelial $CD8^+$ T cells infiltrating tumor cells is associated with the lack of tumor metastases in the draining lymph nodes of cervical cancer patients. Because the absence of lymph node metastases is strongly associated with a better prognosis, patients with strong $CD8^+$ T cells infiltrating tumor cells are likely to display an improved clinical outcome in cervical cancer. Moreover, our data suggest that a subgroup of patients within the cohort of LN^- patients, who may experience the relatively best course of disease, exists. In view of our current data on the relationships among lymph node status, systemic antitumor immunity, and tumor infiltration, studies evaluating immunocorrelations between vaccine-induced tumor-specific T-cell responses and clinical outcome should include an examination of the constitution of the local immune response.

Acknowledgments

Received 9/13/2006; revised 10/30/2006; accepted 11/1/2006.

Grant support: Netherlands Organization for Scientific Research Zon/Mw 920.03.188 (S.H. van der Burg and G.G. Kenter) and 917.56.311 (S.H. van der Burg).

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We thank all the patients who have participated in this study and F. Prins for his excellent help in the analysis of tissue sections by confocal microscopy.

[6] S.H. van der Burg et al. Cervical cancer is associated with the presence of CD4+ regulatory T-cells specific for the high risk human papillomavirus E6 oncoprotein, submitted for publication.

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