Induction of Tumor-Specific CD4+ and CD8+ T-Cell Immunity in Cervical Cancer Patients by a Human Papillomavirus Type 16 E6 and E7 Long Peptides Vaccine

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Abstract

Purpose: The study aims to evaluate the effect of a human papillomavirus type 16 (HPV16) E6 and E7 synthetic long peptides vaccine on the antigen-specific T-cell response in cervical cancer patients.

Experimental Design: Patients with resected HPV16-positive cervical cancer were vaccinated with an overlapping set of long peptides comprising the sequences of the HPV16 E6 and E7 oncoproteins emulsified in Montanide ISA-51. HPV16-specific T-cell immune responses were analyzed by evaluating the magnitude, breadth, type, and polarization by proliferation assays, IFN-γ-ELISPOT, and cytokine production and phenotyped by the T-cell markers CD4, CD8, CD25, and Foxp3.

Results: Vaccine-induced T-cell responses against HPV16 E6 and E7 were detected in six of six and five of six patients, respectively. These responses were broad, involved both CD4+ and CD8+ T cells, and could be detected up to 12 months after the last vaccination. The vaccine-induced responses were dominated by effector type CD4+CD25+Foxp3− type 1 cytokine IFN-γ-producing T cells but also included the expansion of T cells with a CD4+CD25−Foxp3+ phenotype.

Conclusions: The HPV16 E6 and E7 synthetic long peptides vaccine is highly immunogenic, in that it increases the number and activity of HPV16-specific CD4+ and CD8+ T cells to a broad array of epitopes in all patients. The expansion of CD4+ and CD8+ tumor-specific T cells, both considered to be important in the antitumor response, indicates the immunotherapeutic potential of this vaccine. Notably, part of the vaccine-induced T cells display a CD4+CD25+Foxp3− phenotype that is frequently associated with regulatory T-cell function, suggesting that strategies to disarm this subset of T cells should be considered as components of immunotherapeutic modalities against HPV-induced cancers.

Cervical cancer is the second most common cancer in women worldwide (1), and it is the first cancer recognized by the WHO to be 100% attributable to the infection with a high-risk type of human papillomavirus (HPV; ref. 2). HPV type 16 (HPV16) is the most common carcinogenic type and is found in around 50% of invasive cervical tumors worldwide (3, 4).

The occurrence of HPV-induced cancer is strongly associated with failure to mount a strong HPV-specific type 1 T-helper and CTL response (5–7), the lack of CD8+ T cells migrating into the tumor, the induction of HPV16-specific regulatory T cells, and the influx of regulatory T cells into the tumor (8, 9).

Currently, a preventive vaccine, providing protection against HPV16 and HPV18, has been registered. This vaccine is fully protective against persistent infection and the associated development of high-grade genital lesions (reviewed in ref. 10). Notably, the prophylactic vaccine showed no benefit in women who were already infected with the HPV types covered by this vaccine (reviewed in ref. 10). Therefore, there is still a need for a so-called therapeutic vaccine that is able to protect those who are already infected. Not only because the lack of resources among the nations and individuals that have the greatest need will limit the benefits of prophylactic vaccination (reviewed in ref. 10), but also because an estimated 5 million cervical cancer deaths will occur in the next 20 years due to existing HPV infections (11).

All HPV16-induced cervical cancer cells expresses the HPV16 E6 and E7 oncoproteins (3), which makes these proteins excellent target antigens for T cell–mediated immunotherapeutic strategies (12). We have developed a long E6 and E7
peptides vaccine, containing all potential CTL and T-helper epitopes, which may elicit an effective HPV16-specific response able to eradicate existing infections and lesions in human individuals. In a prime boost vaccination scheme, a HPV16 E7 long peptide vaccine induced strong HPV16-specific CD4+ and CD8+ T-cell immunity in mice. In addition, therapeutic vaccination of mice with this vaccine mediated the eradication of established HPV16-positive tumors (13). Subsequently, the long peptide vaccine concept was tested in the cottontail rabbit papillomavirus outbred rabbit model. Therapeutic vaccination of cottontail rabbit papillomavirus–infected rabbits with a vaccine consisting of long overlapping peptides covering the entire sequence of the cottontail rabbit papillomavirus E6 and E7 proteins in Montanide was able to significantly control wart growth and abrogate latent cottontail rabbit papillomavirus infection compared with controls (14). Based on these results a clinical grade synthetic long peptides vaccine, consisting of 13 long peptides covering the HPV16 E6 and E7 antigens, was produced. In a phase I trial, four s.c. injections with the pool of peptides in Montanide ISA-51 in patients with advanced cervical cancer revealed that this vaccine was safe and immunogenic (15).

To delineate the T-cell response, induced by the HPV16 synthetic long peptides vaccine in human beings, we have assessed the magnitude, breadth, type, and polarization of vaccine-induced HPV16-specific T cells in a group of six patients, who were vaccinated after surgical removal of their HPV16-positive cervical tumor.

Patients, Materials, and Methods

HPV16 E6 and E7 synthetic long peptides vaccine

The HPV16 vaccine is a long peptide–based vaccine that consists of a pool of nine overlapping E6 peptides and four overlapping E7 peptides, which are 25 to 35 amino acids long and show an overlap of 10 to 14 amino acids. These clinical grade peptides cover the complete sequence of HPV16 E6 and E7 proteins (human papillomavirus 1997, Los Alamos National Laboratory®) and were synthesized at the interdepartmental GMP facility of by the Department of Clinical Pharmacy and Toxicology. Synthesis was done using a CS Bio CS536 solid-phase peptide synthesizer (CS Bio Co.) according to the Fmoc protocol. After purification by high-performance liquid chromatography, the peptide integrity was checked by mass spectrometry on a VOYAGER DE-PRO MALDI-TOF (PE Biosystems) and on a QTOF mass spectometry (Micromass UK Ltd.) and Edman degradation. The peptides were also sequenced on an HP gas-phase sequencer and dissolved as described previously (16).

Antigens

A set of 22–amino acid long peptides, overlapping by 12 amino acids and indicated by the first and last amino acid in the protein E6 or E7 of HPV16 (e.g., E6_1-22, residues 1-22, and the last peptides E6_137-158 and E7_77-94), were used for the screening of CD4+ T-cell responses. The peptides were mixed into four pools of E6 peptides and two pools of E7 peptides (i.e., E6_1-16, E6_17-32, E6_33-48, and E7_49-64). These pools consisted of four 22-mer peptides each, of which both peptide pools E6.3 and E6.4 contained peptide E6_11-32 and peptide pool E7.2 harbored the last five peptides of HPV16 E7. For the analysis of CD8+ T-cell responses pools of 10–amino acid long, overlapping peptides were used. Each peptide displayed an overlap of nine amino acids and was used at 10 peptides per pool, resulting in 15 different pools for HPV16 E6 and nine pools for HPV16 E7 (i.e., E6 p1-p15 and E7 p1-p9). Pools of four 30-mer peptides, with 15 amino acids overlap, spanning the influenza matrix 1 protein of A/PR/8/34 (M1) were used for comparison of the T-cell reaction to a common virus. The peptides were synthesized and dissolved as described previously (16).

Memory response mix consisted of tetanus toxoid (0.75 limus florocellentius/mL; National Institute of Public Health and the Environment), sonicated Mycobacterium tuberculosis (5 μg/mL; kind gift from Dr. P. Klatsner, Royal Tropical Institute), and Candida (0.015%, HAL Allergen Lab.). The response to the pools of influenza peptides and/or MRM was used as positive control in the assays (5).

Immunomonitoring

Analysis of antigen-specific T cells by IFN-γ-ELISPOT.

IFN-γ-producing HPV-specific T cells were quantified using ELISPOT that was done as described previously (17–20). Briefly, peripheral blood mononuclear cells (PBMC) were seeded at a density of 2 × 10^6 cells per well in a 24-well plate (Costar) in 1 mL of ISCOVE’s medium (Bio-Whittaker) enriched with 10% human AB serum (Sigma), in the presence or absence of 5 μg/mL of indicated HPV16 E6-derived or E7-derived 22-mers peptides combined in pools. As a positive control, PBMC were cultured in the presence of indicated pools of influenza A/PR/8/34 M1 protein-derived peptides and/or memory response mix. After 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^6 cells per well in a Multiscreen 96-well plate (Millipore) coated with an IFN-γ-catch antibody (Mabtech AB). Further antibody incubations and development of the ELISPOT was done according to the manufacturer’s instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (BioSys 4000). Specific spots were calculated by subtracting the mean number of spots + 2 × SD of the medium only control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be increased compared with nonresponders when specific T-cell frequencies were ≥1 of 10,000 (17). T-cell frequencies were considered to be boosted by the vaccine when they were at least 3-fold higher than the baseline sample (18–20).

Patients and vaccination scheme

Patients who underwent a radical hysterectomy for a histologically confirmed HPV16-positive cervical carcinoma (International Federation of Gynecology and Obstetrics stage IB1) and displayed no signs of metastatic disease were accrued into this phase II trial. Eligibility also

6 http://hpv-web.lanl.gov

www.aacrjournals.org ClinCancerRes2008;14(1)January1,2008179

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Proliferative capacity of HPV16-specific T cells by lymphocyte stimulation test. The capacity of T cells to proliferate upon stimulation with the antigen was determined by short-time proliferation assay as described earlier (5, 21). Briefly, freshly isolated PBMCs (1.5 x 10^6) were seeded into eight-replicate wells of a 96-well U-bottomed plate (Costar) to which the indicated peptide pools were added at a final concentration of 10 μg/mL. No antigen served as background control, and memory response mix was taken along as a positive control. The test was conducted in ISCOVE’s medium containing 10% autologous serum. On day 6, supernatant was harvested for cytokine analysis, and subsequently, the cells were pulsed with 0.5 μCi [3H]thymidine per well and incubated for an additional 18 h. Then, the cells were harvested onto filters (Wallac) using the Micro Cell Skatron Harvester (Skatron Instruments AS) and counted on the 1205 Betaplate counter (Wallac). The average and SD of the eight medium only control wells were calculated, and the cutoff was defined as this average plus 3 × SD. The stimulation index was calculated as the average of tested eight wells divided by the average of the medium control eight wells. A positive proliferative response was defined as a stimulation index of at least 3, and the counts of at least six of the eight wells must be above the cutoff value (6). A vaccine-induced response was defined as a stimulation index, which is at least 3-fold higher than the baseline sample.

Identification of T-cell type using intracellular IFNγ and IL-5 cytokine staining. PBMCs were presensitized for 10 days with a pool of all HPV16 E6, HPV16 E7, or influenza A/PR/8/34 M1 protein peptides of HPV16 E6, HPV16 E7, or influenza A/PR/8/34 M1 protein at a concentration of 2.5 μg/mL per peptide. T-cell growth factor (Zeptometrix) and recombinant human IL-2 (PeproTech) was added at a final concentration of 10% and 5 ng/mL, respectively, on day 1. On day 4, autologous PBMCs were thawed, and monocytes were allowed to adhere in X-vivo medium (Cambrex) to 24-well plates (Costar) for 2 h at 37°C before removal of the nonadhered cells. The monocytes were cultured for 6 days in X-vivo medium with 800 IU/mL recombinant human GM-CSF (Invitrogen), washed, and then pulsed with pools of peptide at a concentration of 5 μg/mL per peptide or pulsed with 10 μg/mL of indicated protein overnight. The next day, these antigen-loaded monocytes were washed and used as APC for the presensitized PBMC in the intracellular cytokine staining. After 1 h of coincubation at 37°C, the Golgi-mediated secretion of cytokines was inhibited by the addition of Brefeldin A (Sigma) to a final concentration of 10 μg/mL, after which the cells were incubated for an additional 5 h at 37°C. Then, the cells were harvested, transferred into a V-bottomed 96-well plate, washed twice with ice-cold PBS, and fixed with 50 μL of 4% paraformaldehyde on ice for 4 min. After fixation, the cells were washed once with cold PBS and once with PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1% for permeabilization of the cell membrane. This was followed by incubation in 50 μL PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1%/FCS 10% for 10 min on ice. Cells were washed twice with PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1%, and supernatant was removed before 25 μL of PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1% containing anti–IFNγ FITC (clone 4S.B3, BD PharMingen), anti–IL-5 PE (clone JES1-39D10, BD PharMingen), anti–CD8 PerCP (clone SK1, BD PharMingen), and anti–CD4-APC (clone RPA-T4, BD PharMingen) was added. After 30 min on ice, the cells were washed, suspended in 50 μL of 1% paraformaldehyde, and analyzed by flow cytometry (22). A positive response was defined as at least twice the percentage of IFNγ-producing CD4+ or CD8+ T cells than in the medium only control, and the response should be visible as a clearly distinguishable population of IFNγ-producing cells separated from the nonproducing cells. A vaccine-induced reaction was defined as at least a 3-fold increase in the percentage of antigen-specific IFNγ-producing T cells of the baseline sample.

Results

Safety of the vaccine. In a phase II trial, a total of six patients were vaccinated on average of 4 months (median, 4 months; range, 3–42 months) after surgical dissection of their histologically proved HPV16-positive cervical carcinoma. The patient characteristics are shown in Table 1. Four of the patients had completed the vaccination regimen of four injections, and two patients stopped earlier because of death due to local recurrence (ID103) and the other (ID106) due to inconvenience after the vaccination. Two patients did not meet the inclusion criteria and were withdrawn before first vaccination. Similarly to what we observed in our phase I studies (15), the adverse events did not exceed grade II toxicity and were temporary. All patients experienced the vaccination as mildly painful. The pain vanished within 10 to 15 min. The local pain was graded lower than grade II according to the common toxicity criteria. Fever (during the first night, not exceeding 40°C and effectively treatable with paracetamol was scored as grade I toxicity), flu-like symptoms (lasting 1–2 days), swelling of the injection site and itching of the injection site (during 1 week), and burning eyes and itching of other skin sites (such as neck, breast, and back, as well as fingers and feet) were observed but did not exceed grade II toxicity. The time of follow-up and the clinical status is given in Table 1. Patient ID100 displayed a local recurrence at 7 months after the last vaccination, and patient ID103 displayed a local recurrence after the third vaccination. In the other patients, no signs of recurrence were found during the time of follow-up.
Vaccine-Induced HPV16-Specific T-Cell Immunity in Cervical Cancer

The synthetic long peptides vaccine induces IFN-γ-producing circulating T cells. To study the effect of the vaccine on the immune system, PBMC isolated before vaccination, after two and four vaccinations were analyzed for the presence of HPV16-specific T cells by IFN-γ-ELISPOT analysis. All patients displayed an HPV16-specific T-cell response after vaccination (Fig. 1). In five of the six patients, HPV16 E6-specific IFN-γ-producing T cells were already detectable after 2 vaccinations and in patient ID105 IFN-γ-producing HPV16 E6-specific T cells were detected after four vaccinations had been completed. Figure 1A shows two typical examples (ID102 and ID107) of the results obtained in this analysis. No HPV16 E6-specific or E7-specific IFN-γ-producing T cells were detected in the baseline samples, whereas up to 250 specific spots per 10^5 PBMC were detected after vaccination. The magnitude of the vaccine-induced T-cell response in this patient group is depicted in Fig. 1B. The overall response against HPV16 E6 was stronger than against HPV16 E7. T-cell frequencies up to 1 per 500 PBMC (i.e., 200 specific spots) were found against HPV16 E6, whereas the HPV16 E7-specific response did not exceed 1 per 2000 PBMC (i.e., 50 specific spots). Furthermore, whereas the HPV16 E6-specific IFN-γ-associated immune response already peaked after two vaccinations, the IFN-γ-associated immune response against HPV16 E7 required more vaccinations. After two vaccinations only, two of the six patients responded (ID106 and ID107), whereas after four vaccinations, also patients ID100 and ID105 displayed an E7-specific response. In all patients, an influenza-specific T-cell response was detected in the baseline and postvaccination sample (data not shown). Only four patients (ID100, ID103, ID106, and ID107) displayed an IFN-γ-associated T-cell response to the positive control antigen mixture memory response mix, whereas the T cells of the other two patients failed to produce IFN-γ at both time points (data not shown). In conclusion, in all six patients, the synthetic long peptides vaccine was able to mount an HPV16 E6-specific T-cell response and, in four patients, the HPV16 E7-specific T cells were enhanced.

Vaccine-induced HPV16-specific T cells proliferate and produce IFNγ and IL-5. To analyze the proliferative capacity of HPV16-specific T cells before and after vaccination as well as during follow-up, PBMC were cultured in a lymphocyte stimulation test in eight replicate wells. Based on our cutoff value (stimulation index ≥ 3), the PBMC of patients ID102 and ID105 showed a proliferative response against HPV16 E6 peptide pools 1 to 3 (ID102) and/or HPV16 E7 pool 2 (ID102 and ID105) before vaccination (Fig. 1C). The proliferative response of PBMC from patient ID100 to HPV16 E6 peptide pool 1 in the baseline sample was just below cutoff.

After the vaccinations all six patients responded to at least three of the four peptide pools of HPV16 E6 and to one or both peptide pools of HPV16 E7 (Fig. 1C). The preexisting proliferative responses of ID102 were boosted by the vaccine, whereas this was not the case for the E7-specific proliferative response of ID105. A close examination of the proliferative response after each vaccination showed that vaccine-induced T-cell responses were already measurable after one vaccination, but it also revealed that the magnitude of these responses was subject to variation (Fig. 2). T cells to some peptide pools proliferated stronger after one vaccination than after multiple vaccinations, whereas the proliferative response to other peptide pools was relatively constant in time (e.g., ID102, pool E6.1 and ID107 E6.4, respectively; Fig. 2). In three cases (ID100, ID105, and ID107), we could obtain a blood sample 6 to 12 months after the last vaccination to measure the HPV16 E6-specific and E7-specific proliferative response. Even then, strong proliferative T-cell responses were observed in patients ID100 and ID107, whereas in patient ID105 the

### Table 1. Patient characteristics and adverse events observed

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>T.N.M</th>
<th>Diagnose depth</th>
<th>HPV</th>
<th>Time (mo)</th>
<th>N vacc</th>
<th>Adverse events</th>
<th>Study status</th>
<th>FU (mo)</th>
<th>Clinical status (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>33</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>9 mm</td>
<td>16</td>
<td>42</td>
<td>4</td>
<td>Completed</td>
<td>27</td>
<td>Recurrence (7)</td>
</tr>
<tr>
<td>101</td>
<td>59</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>6 mm</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>Fevers, flu-like symptoms, itching injection site, swelling injection site; burning eyes</td>
<td>Completed</td>
<td>24</td>
</tr>
<tr>
<td>102</td>
<td>35</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>13 mm</td>
<td>16</td>
<td>3</td>
<td>3</td>
<td>Fever, swelling injection site, itching neck, back, breast</td>
<td>Not completed</td>
<td>9</td>
</tr>
<tr>
<td>104</td>
<td>34</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>8 mm</td>
<td>16</td>
<td>6</td>
<td>4</td>
<td>Swelling injection site</td>
<td>Withdrawn</td>
<td>13</td>
</tr>
<tr>
<td>106</td>
<td>58</td>
<td>1B1.0.0</td>
<td>ADC</td>
<td>not determined</td>
<td>16</td>
<td>4</td>
<td>3</td>
<td>Flu-like symptoms, swelling injection site</td>
<td>Withdrawn</td>
<td>0</td>
</tr>
<tr>
<td>107</td>
<td>33</td>
<td>1B1.0.0</td>
<td>ADC</td>
<td>4.5 mm</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>Swelling injection site</td>
<td>Completed</td>
<td>10</td>
</tr>
</tbody>
</table>

NOTE: The cervical cancer tumor was histologically diagnosed as squamous cell carcinoma or adenocarcinoma. The time period (in months) between the treatment of cervical cancer and the first vaccination is given. Patients were scheduled to receive a total of four vaccinations; the number of vaccinations is given. All adverse events of the vaccine are temporarily, and systemic side effects like fever and flu-like symptoms were readily treated with paracetamol. The swelling at the injection site was not painful and probably due to the adjuvant Montanide ISA-51 in the vaccine. The follow-up of these patients after the last vaccination is indicated in months. Patient ID103 died 9 mo after the last vaccination because of local recurrence. The clinical status is given, and the time period (in months) after the last vaccination between brackets. Abbreviations: SCC, squamous cell carcinoma; ADC, adenocarcinoma; N vacc, number of vaccinations; FU, follow-up.
Synthetic long peptides vaccination enhances the HPV16-specific CD4+ and CD8+ T-cell responses in all six postoperative cervical cancer patients.

Vaccination with the synthetic long peptides vaccine elicits strong T-cell responses in all patients. A, two typical examples of the IFNγ-ELISPOT results are shown: ID102 (left) and ID107 (right). The number of specific IFNγ-spots per 10^6 PBMC obtained after stimulation with the indicated four peptide pools covering the HPV16 E6 antigen and the two peptide pools of HPV16 E7 is depicted. B, the number of specific spots per 10^6 PBMC, as a measure of the strength of the HPV16-specific T-cell response, is given for HPV16 E6 (top) and E7 (bottom). Response before vaccination (white columns), after two vaccinations (gray columns), and after four vaccinations (black columns). Note that the PBMC of patient ID103 were only analyzed before the vaccinations and after the second vaccination; thus, after four vaccinations, only five patients could be analyzed. C, proliferative responses upon stimulation with the indicated peptide pools of HPV16 E6 and E7 are depicted as a stimulation index for each individual patient before and after the last vaccination (n = 6). From patients ID100, ID105, and ID107, also a follow-up blood (FU) sample was obtained at respectively 6, 12, and 10 mo after the last vaccination. Each patient is represented by a symbol. In all patients a stimulation index above 3 was detected against at least four peptide pools.

Fig. 1. Vaccination with the synthetic long peptides vaccine elicits strong T-cell responses in all patients. A, two typical examples of the IFNγ-ELISPOT results are shown: ID102 (left) and ID107 (right). The number of specific IFNγ-spots per 10^6 PBMC obtained after stimulation with the indicated four peptide pools covering the HPV16 E6 antigen and the two peptide pools of HPV16 E7 is depicted. B, the number of specific spots per 10^6 PBMC, as a measure of the strength of the HPV16-specific T-cell response, is given for HPV16 E6 (top) and E7 (bottom). Response before vaccination (white columns), after two vaccinations (gray columns), and after four vaccinations (black columns). Note that the PBMC of patient ID103 were only analyzed before the vaccinations and after the second vaccination; thus, after four vaccinations, only five patients could be analyzed. C, proliferative responses upon stimulation with the indicated peptide pools of HPV16 E6 and E7 are depicted as a stimulation index for each individual patient before and after the last vaccination (n = 6). From patients ID100, ID105, and ID107, also a follow-up blood (FU) sample was obtained at respectively 6, 12, and 10 mo after the last vaccination. Each patient is represented by a symbol. In all patients a stimulation index above 3 was detected against at least four peptide pools.
T cells in cervical cancer patients (8) brings forward the possibility that vaccination might also result in activation and expansion of this regulatory T-cell subset. Therefore, PBMC isolated before the first and after the last vaccination were tested for the presence of HPV16-specific CD4+CD25+Foxp3+ T cells. PBMC were stimulated with either HPV16 E6 or E7 peptides and rested for 10 days, as this allows the measurement of stably Foxp3-expressing T cells (23), which are specific for HPV16. As a control, PBMC were cultured without antigen. HPV16-specific CD4+CD25+Foxp3+ T cells were found in five patients before vaccination (Fig. 4 and Table 2B). These responses ranged from 0.1% to 1.94% and were predominantly reactive to E6 (four of the five patients). In all cases, E6-specific and/or E7-specific CD4+CD25+Foxp3- T-cell responses of similar magnitude were detected. After vaccination, both the HPV16 E6-specific subset of Foxp3- and Foxp3+CD4+ T cells were expanded. In four of the six patients, the Foxp3- subset was significantly larger than the Foxp3+ subset of CD4+ T cells (Fig. 4 and Table 2B), whereas in two patients (ID100 and ID103), the percentage of HPV16 E6-specific CD4+CD25+Foxp3- and CD4+CD25+Foxp3+ T cells was similar after vaccination. In case of HPV16 E7, only small increases in both the percentage of CD4+CD25+Foxp3- and Foxp3+ T cells were found after vaccination. Moreover, the balance between these two subsets of E7-specific T cells was not tilted toward one or the other. These data suggest that therapeutic vaccination not only results in the induction of HPV16-specific CD4+ T-helper cells (CD4+CD25+Foxp3-) but can also induce/boost HPV16-specific CD4+CD25+Foxp3+ T-cell populations.

**Discussion**

We have assessed the cellular immune response of six patients who were treated for HPV16-induced cervical cancer and subsequently vaccinated four times with a synthetic long peptides vaccine covering the complete sequence of the oncoproteins of HPV16 E6 and E7 emulsified in the mineral oil–based adjuvant Montanide ISA-51. Utilization of several complementary immunologic assays showed that this vaccine induced broad HPV16 E6-specific T-cell responses in six of six patients and HPV16 E7-specific T-cell responses in five of six patients. Upon antigenic stimulation, these HPV16-specific T cells were able to proliferate and to produce predominantly the T-helper type 1 cytokine IFNγ, which is a hallmark for the HPV16-specific immune response in healthy subjects (6).
Notably, vaccination resulted in the enhancement of HPV16-specific CD4+ and CD8+ T-cell reactivity in all cases. Both types of HPV16-specific T cells have been implicated in the protection against progressive disease (5–7). Furthermore, vaccine-induced HPV16-specific T-cell reactivity could be measured up to 12 months after the last vaccination.

Previously, we have developed a number of assays to measure, quantify, and type spontaneously induced HPV16-specific T-cell responses (5, 6, 8, 17–21) and used these techniques to measure the HPV16-specific T-cell response upon vaccination. In general, the results of these different assay techniques were comparable. However, in a number of cases, slightly different results were obtained. IFNγ-ELISPOT revealed no preexisting HPV16-specific immunity, whereas the short-term proliferation assay did. However, in the latter assay, proliferation was accompanied by the production of either no or very low levels of IFNγ, explaining why no reactivity was detected by IFNγ-ELISPOT. Furthermore, the proliferation assay detected more responses associated with IFNγ production in the postvaccination samples when compared with ELISPOT. This can be explained in several ways. First, the levels of cytokines detected in the supernatant can be either the net result of the production by many cells producing low levels or that of a few cells producing high levels, the latter resulting in only few spots and thus a negative score in the ELISPOT analysis. Furthermore, the prolonged incubation time (7 days proliferation versus 4 days ELISPOT) is highly likely to contribute to the enhanced sensitivity of the proliferation assay. The use of fresh PBMC over thawed PBMC is not likely to affect the analyses (24). This was also shown by the assessment of HPV16-specific T-cell responses by intracellular cytokine analysis. This assay, set up to reveal whether CD4+ and/or CD8+ T cells responded to the vaccine, required a 10-day expansion period in vitro before the analysis was done. As a result both preexisting and broad T-cell responses after vaccination were observed similar to what was seen in the proliferation assay. However, the fact that not all of the preexisting HPV16-specific T-cell reactivities were detected by

![](image1.png)

**Fig. 3.** Both CD4+ and CD8+ IFNγ-producing T cells are enhanced after vaccination. Examples of the flow cytometric dot plots used to determine the percentage of IFNγ-producing T cells on the x axis and IL-5 producing HPV16-specific T cells on the y axis present in 10-d presensitized PBMC isolated after vaccination by intracellular cytokine analysis are depicted. For each patient, the medium control, the strongest HPV16 E6-specific peptide response (see Table 2), and the reactivity against the natural protein of HPV16 E6 are shown. In addition, the fluorescence-activated cell sorting plots of the strongest HPV16-specific CD8+ T-cell response present in each patient (see Table 2) are shown.
either one of the assays indicates that the detection of this low frequency preexisting T cells is less accurate.

Although high amounts of both HPV16 E6 and E7 peptides were injected, a more pronounced immune response was mounted against HPV16 E6. Previous studies in which subjects were vaccinated with either HPV16 L2E6E7-fusion protein (TA-CIN; ref. 18) or recombinant vaccinia viruses expressing the two oncoproteins of both HPV16 and HPV18 (TA-HPV; refs. 19, 20) also resulted in more T cells against E6 than to E7. This suggests that antigenic competition can affect the magnitude of vaccine-induced T-cell responses to some of the injected antigens. One study reported no differences in the response rate against E6 and E7 (11), but here the number of responding T cells were not quantified, excluding the possibility to reveal differences in the magnitude of the response. Importantly, the number of E7-specific T cells can be increased to the level of the E6-specific response by injecting the E6 and E7 antigens in two different locations (15).

All six patients in our study showed a proliferative response to E6 and E7 after vaccination. Based on the results of the IFN\(\gamma\)-ELISPOT and intracellular IFN\(\gamma\) analysis, the response to E6 was always associated with the production of IFN\(\gamma\), whereas in four of six cases INF\(\gamma\) was produced when PBMC were stimulated with E7. When compared with other studies with similar or larger group sizes, in which, based on IFN\(\gamma\) production, a response rate between 40% and 75% is observed (11, 18–20), the long peptides vaccine is somewhat more effective in inducing HPV16-specific immunity (six of six responding patients). Not only the response rate but also the breadth of the response is greater after peptide vaccination when compared with vaccine trials in which either TA-CIN, TA-HPV, or both were injected. In these trials we analyzed, similar to the analysis of the current study, the HPV16-specific immune response by stimulating PBMC with four different pools of E6 peptides and two pools of E7 peptides by IFN\(\gamma\)-ELISPOT (18–20). Of the total of 23 subjects that responded in the former trials, 16 displayed a response to one peptide pool and only five patients (\(~\)21%) responded to three or more peptide pools. In comparison, in the current study all patients reacted to three or more peptide pools.

In addition to the HPV16-specific CD4+ T-cell responses, all six patients also displayed circulating HPV16 E6-specific IFN\(\gamma\)-producing CD8+ T cells after vaccination. Furthermore, we detected HPV16 E7-specific CD8+ T cells in three of the six
An HPV16-specific CD8+ T-cell response to E6 or E7 was only detected in case of a concomitant CD4+ T-cell response to the same antigen (Table 2A). In contrast, only 5 of 32 analyzed patients injected with TA-HPV showed evidence of vaccine-induced HPV16/18-specific CD8+ T-cell immunity (25, 26). The injection of E6E7 fusion protein in ISCOMATRIX resulted in the induction of HPV16-specific CD8+ T-cell responses in 5 of 15 patients (11). It is not clear whether the CD8+ T-cell responses of these earlier reported trials were directed against E6 or E7. In two other trials with TA-CIN or TA-HPV, the CD8+ T-cell response was not measured to all possible CD8+ T-cell epitopes but to three predefined HPV16 E7 HLA-A*0201-restricted peptides. Only 1 of the 17 patients analyzed showed a vaccine-induced response to one of the E7 peptides (19, 27). In these studies, the lack of a good response rate of CD8+ T cells against E7 is possibly related to the failure to induce E7-specific CD4+ T-cell immunity (19, 20). Other vaccines specifically aiming at the induction of HPV16-specific CD8+ T cells to one or two predefined HLA-A2–restricted E7 peptides reported higher response rates (28–30).

We observed that vaccination not only resulted in the boost of HPV16-specific CD4+CD25+Foxp3+ T cells but also enhanced the HPV16 E6-specific CD4+CD25+Foxp3+ T-cell population (Table 2B). Based on our recent study, in which we isolated

![Fig. 4.](image) Vaccination results in the enhancement of HPV16-specific CD4+CD25+Foxp3+ T cells. The flow cytometric dot plots depict the CD4+CD25+Foxp3+ and CD4+CD25+Foxp3+ T-cell population in PBMC after 10 d of stimulation with E6 or E7. As a control, 10-d cultured nonstimulated PBMC are shown. The percentages of positive cells, before and after vaccination, are given in the quadrants. ND, not done.
HPV16 E6-specific CD4+CD25+Foxp3+ T cells from cervical cancer patients and showed that these T cells were able to suppress the proliferation and cytokine production of other T-helper type 1 cells after stimulation with their cognate HPV16 E6 antigen (8). Although no formal proof is given in this way, one may assume that also the vaccine-induced HPV16 E6-specific Foxp3+ T cells are able to exert suppression on other T cells. Interestingly, although the percentage of HPV16-specific CD4+CD25+Foxp3+ T cells readily outnumbered their Foxp3− counterparts in four patients, two patients (ID100 and ID103) displayed a lower number of HPV16-specific CD4+CD25+Foxp3+, the magnitude of which was comparable with that of the HPV16-specific CD4+CD25+Foxp3− T-cell subset. These latter two patients were the ones who progressed during (ID103) and after vaccination (ID100). It is highly likely that also the other therapeutic HPV vaccines (11, 18–20) will stimulate HPV-specific CD4+CD25+Foxp3+ T cells. The presence and vaccine-induced increase of HPV16-specific regulatory T cells indicate that strategies to eliminate or disarm regulatory T cells should be considered for immunotherapeutic strategies against HPV16-induced cancers.

In conclusion, the HPV16 E6 and E7 synthetic long peptides vaccine is able to vigorously enhance the number and activity of HPV16-specific CD4+ and CD8+ T cells to a broad array of epitopes in all vaccinated patients. The expansion of both types of HPV16 (tumor)–specific T cells indicates the potential of this vaccine for the immunotherapy of HPV16-induced progressive infections, lesions, and malignancies.

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References


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Induction of Tumor-Specific CD4+ and CD8+ T-Cell Immunity in Cervical Cancer Patients by a Human Papillomavirus Type 16 E6 and E7 Long Peptides Vaccine
