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Regulation and subversion of HPV16-specific immunity in cancer patients

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Chapter 4.2

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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

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Abstract. Purpose: To evaluate the impact of a 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, 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 6/6 and 5/6 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 anti-tumor 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.

Introduction

Cervical cancer is the second most common cancer in women worldwide (1), and it is the first cancer recognized by the World Health Organization to be 100% attributable to the infection with a high-risk type of human papillomavirus (HPV) (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 cytotoxic T lymphocyte (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 (10)). Notably, the prophylactic vaccine showed no benefit in women who were already infected with the HPV types covered by this vaccine (reviewed in (10)). Therefore, there is still need for a so-called therapeutic vaccine, 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 (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 peptide 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 (CRPV) outbred rabbit model. Therapeutic vaccination of CRPV infected rabbits with a vaccine consisting of long overlapping peptides covering the entire sequence of the CRPV E6 and E7 proteins in Montanide was able to significantly control wart growth and abrogate latent CRPV infection compared to 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 subcutaneous 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).

In order 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 6 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 9 overlapping E6 peptides and 4 overlapping E7 peptides, which are 25-35 amino-acids long and show an overlap of 10-14 amino acids. These clinical grade peptides cover the complete sequence of HPV16 E6 and E7 proteins (Human Papillomavirus 1997, Los Alamos National Laboratory; <http://hpv-web.lanl.gov>) and were synthesized at the Interdepartmental GMP facility of by the department of Clinical Pharmacy & Toxicology. Synthesis was performed using a CS Bio CS536 solid-phase peptide synthesizer (CS Bio Co., San Carlos, CA, USA) according to the Fmoc-protocol. After purification by HPLC the peptide integrity was checked by mass spectrometry on a VOYAGER DEPRO MALDI-TOF (PE Biosystems, Framingham, USA) and on a QTOF mass spectrometry (Micromass UK Ltd., Wythenshawe, UK) and Edman degradation. The peptides were also sequenced on a HP gas-phase sequencer (Hewlett Packard, Palo Alto, CA, USA), and stored as freeze-dried powder at -20°C until use. At the day of vaccination the 13 peptides (0.3 mg per peptide) are dissolved in DMSO (final concentration is 20%) and admixed with 20 mM phosphate buffer (pH 7.5) and Montanide ISA-51. The 13 overlapping HPV16 E6/E7 peptides were delivered subcutaneously in a dose of 300 μg per peptide in DMSO/20mM phosphate buffer pH 7.5/ Montanide ISA-51 adjuvant (20/30/50 v/v/v) in a final volume of 2.8 ml. This dose was demonstrated to be safe and immunogenic in our first phase I trial (15).

Patients and Vaccination Scheme. Patients who underwent a radical hysterectomy for a histologically confirmed HPV16-positive cervical carcinoma (FIGO stage 1B1) and displayed no signs of metastatic disease were accrued into this phase 2 trial. Eligibility also required the following criteria: (a) performance status of WHO 1-2 and/or Karnofsky score ≥ 60 , (b) pre-treatment laboratory findings of leukocytes $> 3 \times 10^9/\text{L}$, lymphocytes $> 1 \times 10^9/\text{L}$, thrombocytes $> 100 \times 10^9/\text{L}$ and haematocrit $> 30\%$, (c) HIV- and Hepatitis B-seronegative, (d) no radiotherapy, chemotherapy or other potentially immunosuppressive therapy administered within 4 weeks prior to the immunotherapy.

The study design was approved by the Medical Ethical Committee of the Leiden University Medical Center. Following written informed consent the patients were vaccinated 4 times at a 3-week interval. Prior to each vaccination a blood sample (60 ml) was drawn for immunomonitoring as well as for checking the haematological values and organ function markers. In addition, at baseline and 2 weeks after the last vaccination 500 ml blood was isolated. The first patient was vaccinated in November 2004, the last vaccination was given in April

2006. Follow-up extended to 2 years after the start of the study.

Antigens. A set of twenty-two 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 E6₁₃₇₋₁₅₈ and E7_{77-98*}), 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 - E6.4, E7.1 and E7.2). These pools consisted of four 22-mer peptides each of which both peptide pools E6.3 and E6.4 contained peptide E6₁₁₁₋₁₃₂ and peptide pool E7.2 harboured 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 9 amino acids and was used at 10 peptides per pool, resulting in 15 different pools for HPV16 E6 and 9 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 against a common virus. The peptides were synthesized and dissolved as described previously (16).

Memory response mix (MRM) consisted of tetanus toxoid (0.75 Limus Flocculentius per ml; National Institute of Public Health and the Environment, Bilthoven, The Netherlands), sonicated *Mycobacterium tuberculosis* (5 µg/ml; kind gift from Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and *Candida* (0.015%, HAL Allergenen Lab, Haarlem, The Netherlands). 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. Interferon- γ (IFN γ) producing HPV-specific T cells were quantified using ELISPOT that was performed as described previously (17-20). Briefly, peripheral blood mononuclear cells (PBMC) were seeded at a density of 2 x 10⁶ cells/well in a 24-well plate (Costar, Cambridge, MA) in 1 ml of ISCOVE's medium (Bio-Whittaker, Verviers, Belgium) enriched with 10% human AB serum (Sigma, St. Louis, MO), in the presence or absence of 5 µg/ml of indicated HPV16 E6- 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 MRM. Following four days of incubation at 37°C, PBMC were harvested, washed and seeded in four replicate wells at a density of 10⁵ cells/well in a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFN γ catching antibody (Mabtech AB, Nacha, Sweden). Further antibody incubations and development of the ELISPOT was performed 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 + 2xSD 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 to non-responders when specific T-cell frequencies were $\geq 1/10,000$ (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).

Proliferative capacity of HPV16-specific T cells by lymphocyte stimulation test (LST). 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 PBMC (1.5 x 10⁵) were seeded into 8-replicate wells of a 96-well U-bottom plate (Costar, Cambridge, MA) to which the indicated peptide pools were added at a final concentration of 10 µg/ml. No antigen served as background control and MRM 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 hours. Then, the cells were harvested onto filters (Wallac, Turku, Finland) using the Micro-cell Skatron harvester (Skatron Instruments AS, Lier, Norway) and counted on the 1205 Betaplate counter (Wallac, Turku, Finland). The average and standard deviation of the 8 medium only control wells were calculated and the cut-off was defined as this average plus 3xSD. The stimulation index (SI) was calculated as the average of tested 8 wells divided by the average of the medium control 8 wells. A positive proliferative response was defined as a stimulation index of at least 3 and the counts of at least 6 out of the 8-wells must be above the cut-off value (6). A vaccine-induced response was defined as a SI which is at least 3-fold higher than the baseline sample.

Cytokine polarization analysis. The supernatants isolated on day 6 of the proliferation assay were subjected to a Th1/Th2 inflammation cytokine bead array (CBA) kit (BD Biosciences, Erembodegem, Belgium). In this array the levels of IFN γ , TNF α , IL-10, IL-5, IL-4 and IL-2 were determined. According to firm prescription the proposed detection limit was 20 pg/ml. However, for IFN γ the cut-off value was set to 100 pg/ml because the standard curve showed linearity starting at a concentration of 100 pg/ml. Positive antigen-specific cytokine production was defined as a cytokine concentration above the cut-off value and >2x the concentration of the medium control (6). A vaccine-induced response was defined as at least a 3-fold increase in the antigen-specific cytokine production in the baseline sample.

Identification of T-cell type using intracellular IFN γ and IL-5 cytokine staining (ICS). PBMC were pre-sensitized for 10 days with a pool of all 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 (TCGF, Zeptomatrix, Gentaur, Brussel, Belgium) and recombinant human IL-15 (PeproTech, London, UK) was added at a final concentration of 10% and 5 ng/ml, respectively on day 1. On day 4, autologous PBMC were thawed and monocytes were allowed to adhere in X-vivo medium (Cambrex, Verviers, Belgium) to 24-well plates (Costar, Cambridge, MA) for 2 hours at 37°C before removal of the non-adhered cells. The monocytes were cultured for 6 days in X-vivo medium with 800 IU/ml recombinant human GM-CSF (Invitrogen, Paisley, UK), 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 pre-sensitized PBMC in the intracellular cytokine staining (ICS). After 1 hour of co-incubation at 37°C, the Golgi-mediated secretion of cytokines was inhibited by the addition of Brefeldin A (Sigma, St. Louis, MO) to a final concentration of 10 µg/ml after which the cells were incubated for an additional 5 hours at 37°C. Then, the cells were harvested, transferred into a V-bottom 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 phosphate buffered saline (PBS) and once with PBS/ Sodium azide (NaAz) 0.02%/Bovine serum albumin (BSA) 0.5%/Saponin 0.1% for permeabilization of the cell membrane. This was followed by incubation in 50 µl PBS/NaAz 0.02%/BSA 0.5%/Saponin 0.1%/Fetal calf serum (FCS) 10% for 10 min on ice. Cells were washed twice with PBS/NaAz

0.02%/BSA 0.5%/Saponin 0.1% and supernatant was removed before 25 µl of PBS/NaAz 0.02%/BSA 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 non-producing 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.

Detection of HPV16-specific CD4+ Foxp3+ T cells. PBMC (1-2 x 10⁶) were cultured for 10 days in medium only, in the presence of 5 µg/ml HPV16 E6 pooled peptides or HPV16 E7 peptides. Then, the cells were harvested and 2 x 10⁵ cells were stained for Foxp3 and CD25 according to the manufacturer (Bioscience). Briefly, the stimulated cells were first stained for surface markers CD25 (anti-CD25 FITC; clone M-A251, BD Pharmingen), CD4 (anti-CD4-APC; clone RPA-T4, BD Pharmingen) and CD8 (anti-CD8 PerCP; clone SK1, BD Pharmingen) before the cells were fixed and permeabilized. Blocking was performed with 2% normal rat serum followed by the addition of anti-human Foxp3

Table 1. Patients characteristics and adverse events observed

ID	Age	T.N.M	Diagnose	Invasion depth	HPV type	Time (mo)	N vacc	Adverse events	Study status	FU (mo)	Clinical status (months)
100	33	1B1.0.0	SCC	9 mm	16	42	4	fever, flu-like symptoms, itching injection site, swelling injection site; burning eyes	completed	27	recurrence (7)
101							0		withdrawn		
102	59	1B1.0.0	SCC	6 mm	16	4	4	fever, flu-like symptoms, itching injection site, swelling injection site, itching neck, back, breast	completed	24	free of disease
103	35	1B1.0.0	SCC	13 mm	16	3	3	fever, swelling injection site, itching fingers and feet	not completed	9	recurrence (0)
104							0		withdrawn		
105	34	1B1.0.0	SCC	8 mm	16	6	4	swelling injection site	completed	13	free of disease
106	58	1B1.0.0	ADC	ND	16	4	3	flu-like symptoms, swelling injection site	not completed	0	Lost in FU
107	33	1B1.0.0	ADC	4.5 mm	16	4	4	swelling injection site	completed	10	free of disease

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; ND, not determined.

(PCH101) antibody or rat isotype IgG2a control. Then the cells were washed and analyzed on the cytometer. As a positive control a previously isolated HPV16-specific CD4+Foxp3+ regulatory T-cell clone (C148.31) and as negative control a HPV16-specific CD4+Foxp3- T-cell clone (C271.9) (8) was used. The fluorescence intensity of these two control clones was used to set the gates for the other samples in which the Foxp3 positivity of the stimulated polyclonal T-cell populations were analyzed. An antigen-induced upregulation of Foxp3 or CD25 was defined as at least twice the percentages of Foxp3 or CD25 positive cells in the medium only control, and a vaccine-induced increase in Foxp3 positive cells was defined as at least a 3-fold increase compared to the percentages of the baseline sample for the same condition.

Results

Safety of the vaccine. In a phase II trial, a total of 6 patients were vaccinated on average 4 months (median 4 months, range 3 to 42 months) after surgically dissection of their histologically proven HPV16 positive cervical carcinoma. The patient characteristics are shown in Table 1. Four of the patients had completed the vaccination regimen of 4 injections and 2 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 prior to 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 10-15 minutes. 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) as well as 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.

The synthetic long peptides vaccine induces interferon gamma producing circulating T cells. To study the effect of the vaccine on the immune system, PBMC isolated prior to vaccination, after 2 and 4 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 (Figure 1). In 5 of the 6 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 4 vaccinations had been completed. Figure 1A shows two typical examples (ID102 and ID107) of

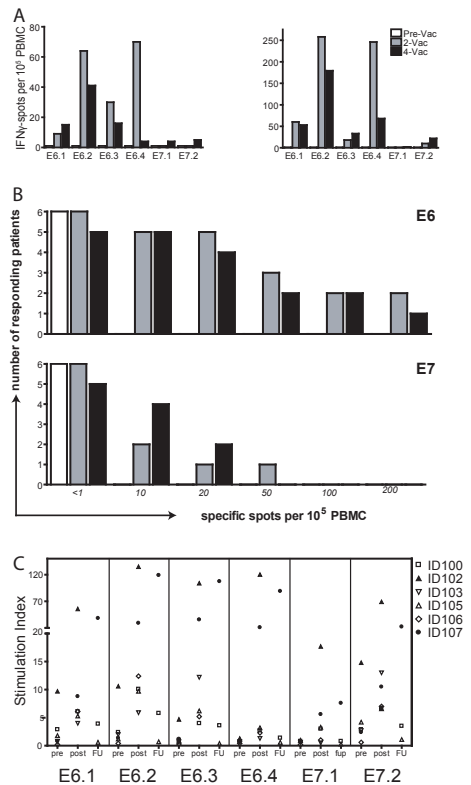


Figure 1. Vaccination with the synthetic long peptides vaccine elicits strong T-cell responses in all patients. A) Two typical examples of the IFN γ -ELISPOT results are shown: ID102 (left) and ID107 (right). The number of specific IFN γ -spots per 10⁵ PBMC obtained after stimulation with the indicated 4 peptide pools covering the HPV16 E6 antigen and the 2 peptide pools of HPV16 E7 is depicted. B) The number of specific spots per 10⁵ PBMC – as a measure of the strength of the HPV16-specific T-cell response – is given for HPV16 E6 (upper graph) and E7 (lower graph). The response prior to vaccination is depicted in white bars, after 2 vaccinations in grey bars and after 4 vaccinations in black bars. Note that the PBMC of patient ID103 were only analyzed prior to the vaccinations and after the second vaccination so that after 4 vaccinations only 5 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 prior to and after the last vaccination (n=6). From patient ID100, ID105 and ID107 also a follow-up (FU) blood sample was obtained at respectively 6, 12 and 10 months after the last vaccination. Each patient is represented by a symbol. In all patients a stimulation index (SI) above 3 was detected against at least 4 peptide pools.

the results obtained in this analysis. No HPV16 E6 or E7 specific IFN γ -producing T cells were detected in the baseline samples while up to 250 specific spots per 10⁵ PBMC were detected after vaccination. The magnitude of the vaccine-induced T-cell response in this patient group is depicted in Figure 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 while the HPV16 E7-

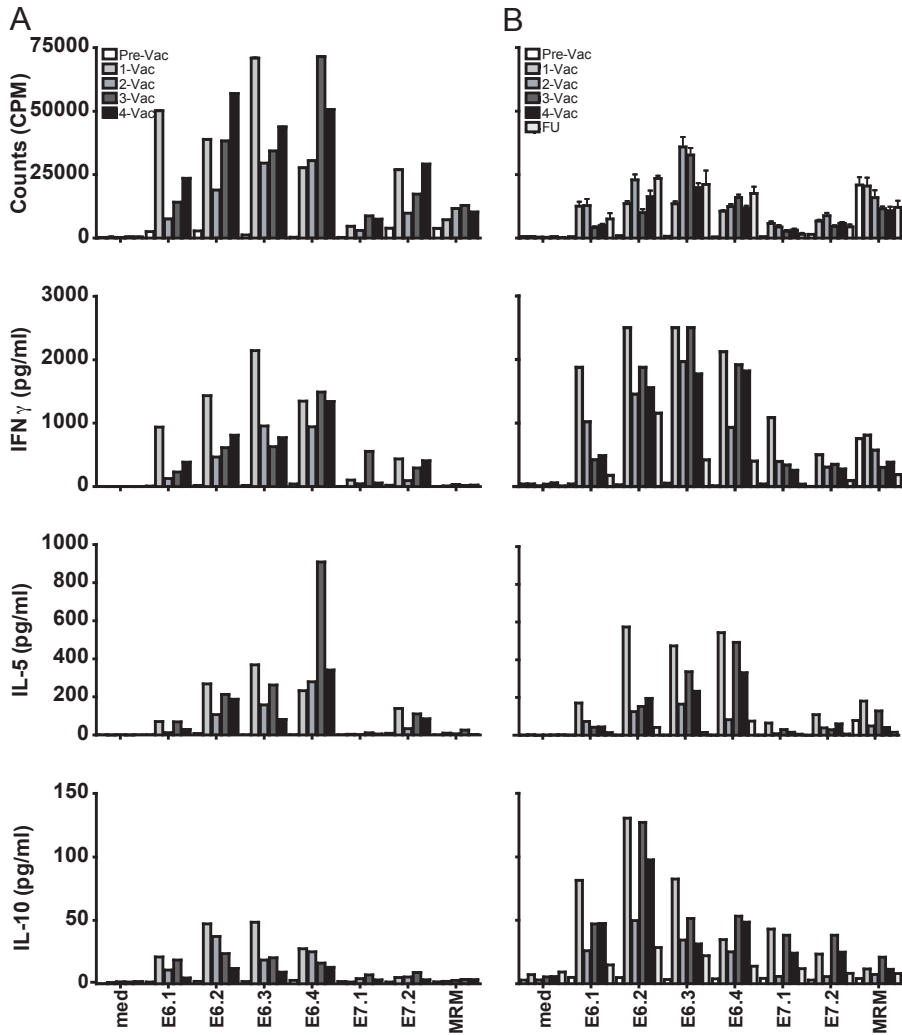


Figure 2: HPV16-specific proliferation is associated with a predominant T-helper type 1 cytokine profile. Two typical examples ID102 (A) and ID107 (B) of the proliferation and associated cytokine production by T cells prior to and after each vaccination are shown. Proliferation is depicted as the mean counts per minute (cpm) \pm SD as tested in the lymphocyte stimulation test and the concentration of the concomitantly produced cytokines is shown in pg/ml as measured in the cytometric bead array. From patient ID107 also the follow-up blood (FU) sample drawn at 10 months after the last vaccination was included. Note that the cytokine concentration not necessarily parallels the magnitude of the proliferative response.

specific response did not exceed 1 per 2000 PBMC (i.e. 50 specific spots). Furthermore, while the HPV16 E6-specific IFN γ -associated immune response already peaked after 2 vaccinations, the IFN γ -associated immune response against HPV16 E7 required more vaccinations. After 2 vaccinations only 2 of the 6 patients responded (ID106 and ID107), while after 4 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 post vaccination 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 MRM, while the T

cells of the other two patients failed to produce IFN γ at both time points (data not shown). In conclusion, in all 6 patients the synthetic long peptides vaccine was able to mount an HPV16 E6-specific T-cell response and in 4 patients the HPV16 E7-specific T cells were enhanced.

Vaccine-induced HPV16-specific T cells proliferate and produce IFN γ 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 tested in a lymphocyte stimulation test (LST) in 8-replicate wells. Based on our cut-off value (SI \geq 3) the PBMC of patient ID102 and ID105 showed a proliferative response

against HPV16 E6 peptide pool 1 to 3 (ID102) and/or HPV16 E7 pool 2 (ID102 and ID105) before vaccination (Figure 1C). The proliferative response of PBMC from patient ID100 to HPV16 E6 peptide pool 1 in the baseline sample was just below cut-off.

After the vaccinations all 6 patients responded to at least 3 of the 4 peptide pools of HPV16 E6 and to one or both peptide pools of HPV16 E7 (Figure 1C). The pre-existing 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 (Figure 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; Figure 2). In three cases (ID100, ID105 and ID107) we could obtain a blood sample 6 to 12 months after the last vaccination in order to measure the HPV16 E6- and E7-specific proliferative response. Even then strong proliferative T-cell responses were observed in patient ID100 and ID107, whereas in patient ID105 the vaccine-induced response had contracted (Figure 1C). In all cases a proliferative response against MRM could be detected in the baseline and post vaccination samples (data not shown).

Supernatants of all samples tested in the LST were isolated at day 6 for the analysis of antigen-specific production of cytokines by cytometric bead array (6). In all 6 patients a vaccine-induced HPV16 E6- and/or E7-specific production of IFN γ was found. The highest detected levels – as measured for a single peptide pool – ranged from 250 pg/ml to >5000 pg/ml. The production of IL-5 frequently coincided with that of IFN γ , but not in all cases. The levels of IL-5 were usually lower and the highest detected levels did not exceed 1000 pg/ml. The production of IL-10 always coincided with IFN γ production, but not the other way round, and peaked at 100 pg/ml. Typical examples (ID102 and ID107) of the proliferative response of PBMC as well as the associated production of cytokines against every peptide pool before the first vaccination and after every following vaccination are depicted in Figure 2. It was noted that the amount of cytokine produced did not always parallel the magnitude of the proliferative response. Importantly, based on the amounts of cytokines detected the overall response was mainly tipped towards a type 1 (IFN γ) and not a type 2 (IL-5 and IL-10) cytokine profile.

Both HPV16-specific CD4 $^{+}$ and CD8 $^{+}$ T cells were induced by vaccination. The production of mainly IFN γ and IL-5 allowed us to utilize intracellular cytokine analysis to determine the frequency of HPV16-specific CD4 $^{+}$ and/or CD8 $^{+}$ T cells in 10-day pre-sensitized bulk cultures of PBMC isolated either before or after the last

vaccination. In order to analyze HPV16-specific CD4 $^{+}$ T-cell responses, these bulk cultures were stimulated with pools of overlapping 22-mer peptides pulsed APC while pools of overlapping 10-mer peptides pulsed APC were used to analyze the HPV16-specific CD8 $^{+}$ T-cell response. After vaccination all 6 patients displayed circulating HPV16-specific CD4 $^{+}$ T cells that were directed against HPV16 E6. In 5 of these 6 patients, the circulating HPV16-specific CD4 $^{+}$ T cells also responded to APC pulsed with HPV16 E6 protein indicating that these HPV16-specific CD4 $^{+}$ T cells recognized their cognate antigen when processed from the context of whole protein (Figure 3 and Table 2a). In 3 out of the 6 patients also HPV16 E7 specific CD4 $^{+}$ T cells were detected, however only in one patient these cells also recognized the E7 protein (ID106). In all cases an influenza-specific CD4 $^{+}$ T cell could be detected in the post-vaccination sample (data not shown).

HPV16 E6-specific CD8 $^{+}$ T cells were observed in all 6 patients against one to three pools of 10-mer peptides, whereas HPV16 E7-specific CD8 $^{+}$ T cells were detectable in PBMC of 3 patients (Figure 3 and Table 2a). Both the CD4 $^{+}$ and CD8 $^{+}$ T-cell responses directed at HPV16 E6 were stronger and broader than against E7. Notably, while in 3 patients (ID102, ID103

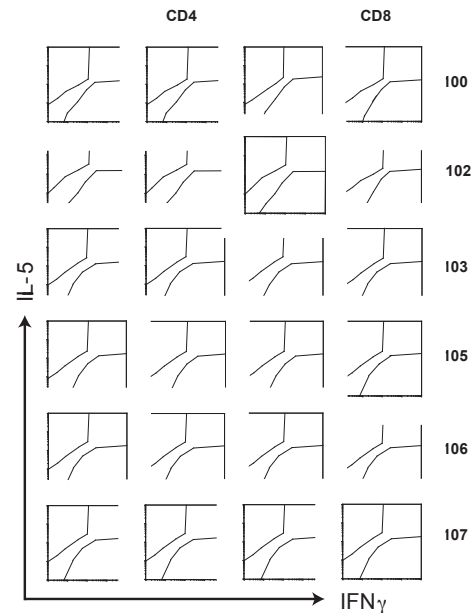


Figure 3. Both CD4 $^{+}$ and CD8 $^{+}$ IFN γ -producing T cells are enhanced after vaccination. Examples of the flow cytometric dotplots used to determine the percentage of IFN γ -producing T cell on the x-axis and IL-5 producing HPV16-specific T cells on the y-axis, present in 10-day pre-sensitized 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 FACS plots of the strongest HPV16-specific CD8 $^{+}$ T-cell response present in each patient (see Table 2) are shown.

Table 2. CD4+ T cells and CD8+ T-cell responses upon stimulation with peptide pools of HPV16

A. Percentages of interferon gamma producing CD4+ and CD8+ T cells																					
Patient	CD4						CD8		CD4						CD8						
	E6.1		E6.2		E6.3		E6.4		E6 prot		E6		E7.1		E7.2		E7 prot		E7		
	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	pre	post	
ID 100	-	0.09	-	0.06	-	0.11	-	0.01	-	0.01	-	0.09 (aa 51-69)	-	-	-	0.03	-	-	-	-	-
ID 102	0.09	0.21	-	0.22	-	0.32	0.12	0.77	-	0.43	-	1.32 (aa 51-69)	-	0.02	0.09	0.33	-	0.03	-	0.35 (aa 51-69)	-
ID 103	-	0.1	0.39	0.55	0.46	1.12	-	0.04	-	0.6	-	0.70 (aa 11-29)	-	-	-	-	-	-	-	-	-
ID 105	-	0.05	-	0.05	-	0.02	-	0.46	-	0.08	-	0.92 (aa 51-69)	-	-	-	-	-	-	-	-	-
												0.83 (aa 61-79)									
ID 106	-	0.45	0.27	1	-	0.64	0.11	4.55	-	2.73	-	0.30 (aa 41-59)	-	0.02	-	1.01	-	0.23	-	0.05 (aa 1-19)	-
												0.83 (aa 51-69)								0.10 (aa 41-59)	
												1.37 (aa 131-149)									
ID 107	-	0.16	-	1.59	-	0.22	-	0.94	-	0.28	-	0.08 (aa 91-109)	-	-	-	0.12	-	-	-	0.21 (aa 71-89)	-

B. Percentages of CD4+CD25+ Foxp3-negative and positive cells																
Patient	CD4+CD25+															
	Foxp3-						Foxp3+									
	pre		post		pre		post		pre		post					
ID 100	E6		0.32		1.96		0.4		1.29		E7		0.68		0.48	
ID 102	E6		-		14.5		-		3.3		E7		0.69		1.81	
ID 103	E6		0.29		0.72		-		2.01		E7		0.38		0.28	
ID 105	E6		0.22		35.4		1.94		3.1		E7		-		1.61	
ID 106	E6		0.39		39.4		1.22		10.2		E7		-		1.99	
ID 107	E6		1.18		13.5		0.87		2.91		E7		0.28		0.62	

NOTE: A) The percentage of IFN-producing T cells upon stimulation with pools of 22-mer peptides (CD4) or pools of 10-mer peptides (CD8) is indicated. For the CD8+ T-cell responses, the number of the first and last amino acid of the HPV16 antigen that is covered by the peptide pool recognized is given. The results of both before vaccination and only the positive responses after the last vaccination are shown. B) The percentages of HPV16 peptide stimulated CD4+CD25+ T cells which do not (Foxp3-) or do express Foxp3 (Foxp3+) after subtraction of the percentage of cells in nonstimulated (medium only) cultures are given. Abbreviations: Pre, before vaccination; Post, after the last vaccination; aa, amino acid.

and ID106) HPV16-specific IFN γ -producing CD4+ T cells could be detected in the baseline samples, HPV16-specific CD8+ T cells were only detected after vaccination (Table 2a). Note that pre-existing CD4+ T-cell responses increased after vaccination (Table 2a).

In conclusion, the synthetic long peptides vaccine is capable of eliciting HPV16-specific CD4+ and CD8+ T-cell responses in all 6 post-operative cervical cancer patients.

Synthetic long peptides vaccination enhances the HPV16-specific CD4+Foxp3+ T-cell subset. We recently reported the existence of HVP16 E6-specific CD4+CD25+Foxp3+ regulatory T cells in patients with HPV16+ cervical cancer (8). Although the synthetic long peptides vaccine was injected with the purpose to enhance CD4+ and CD8+ T-cell effector immunity against the E6 and E7 oncoproteins of HPV16, the presence of such pre-existing E6-specific CD4+CD25+Foxp3+ regulatory 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 5 patients prior to vaccination (Figure 4 and Table 2b). These responses ranged from 0.1 - 1.94% and were predominantly reactive to E6 (4 out of the 5 patients). In all cases, E6- 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 4 of the 6 patients the Foxp3- subset was significantly larger than the Foxp3+ subset of CD4+ T cells (Figure 4 and Table 2b) while in 2 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 towards 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 6 patients who were treated for HPV16-induced cervical cancer and subsequently vaccinated 4 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 immunological assays showed that this vaccine induced broad HPV16 E6-specific T-cell responses in 6/6 patients and HPV16 E7-specific T-cell responses in 5/6 patients. Upon antigenic stimulation these HPV16-specific T cells were able to proliferate and to produce predominantly the T-helper type 1 cytokine IFN γ , 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 employed 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. IFN γ -ELISPOT revealed no pre-existing HPV16-specific immunity while 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 IFN γ explaining why no reactivity was detected by IFN γ -ELISPOT. Furthermore, the proliferation assay detected more responses – associated with IFN γ production – in the post vaccination samples when compared to 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 vs. 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 demonstrated by the assessment of HPV16-specific T-cell responses by intracellular cytokine analysis. This assay,

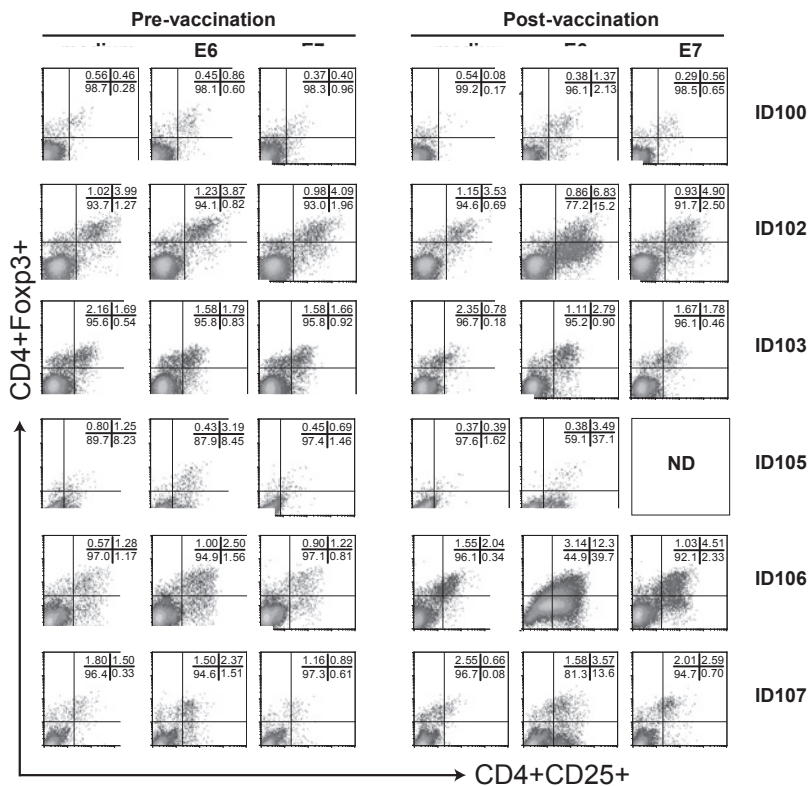


Figure 4: The flow cytometric dotplots depict the CD4+CD25+Foxp3- and CD4+CD25+Foxp3+ T-cell population in PBMC after 10-days of stimulation with E6 or E7. As a control 10-day cultured non-stimulated PBMC are shown. The percentages of positive cells, before and after vaccination, are given in the quadrants. ND, not done.

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 performed. As a result both pre-existing 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 pre-existing HPV16-specific T-cell reactivities were detected by either one of the assays indicates that the detection of this low frequency pre-existing 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) (18) or recombinant vaccinia viruses expressing the two oncoproteins of both HPV16 and HPV18 (TA-HPV) (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 6 patients in our study showed a proliferative response to E6 and E7 after vaccination. Based on the results of the IFN γ -ELISPOT and intracellular IFN γ analysis, the response to E6 was always associated with the production of IFN γ whereas in 4 out of 6 cases IFN γ was produced when PBMC were stimulated with E7. When compared to other studies with similar or larger group sizes, in which – based on IFN γ production – a response rate between 40-75% is observed (11, 18-20), the long peptide vaccine is somewhat more effective in inducing HPV16-specific immunity (6 out of 6 responding patients). Not only the response rate, but also the breadth of the response is greater after peptide vaccination when compared to 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 4 different pools of E6 peptides and 2 pools of E7 peptides by IFN γ 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 3 or more peptide pools. In comparison, in the current study all patients reacted to 3 or more peptide pools.

In addition to the HPV16-specific CD4+ T-cell responses, all 6 patients also displayed circulating HPV16 E6-specific IFN γ -producing CD8+ T cells after vaccination. Furthermore, we detected HPV16 E7-specific CD8+ T cells in 3 of the 6 patients (Table 2). 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 2). In contrast, only 5 out 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 out 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 in total 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 2 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 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 prove 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, while in 4 patients the percentage of HPV16-specific Foxp3- T cells readily outnumbered their Foxp3+ counterparts, 2 patients (ID100 and ID103) displayed a lower number of HPV16-specific CD4+CD25+Foxp3-, the magnitude of which was comparable to 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 HPV-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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