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Enhancement of human papilloma virus type 16 E7 specific T cell responses by local invasive procedures in patients with (pre)malignant cervical neoplasia

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It has been suggested that local invasive procedures may alter the natural course of (pre)malignant cervical disease. This could be due to partial excision of the lesions, or *via* induction of cellular immunity against human papillomavirus (HPV) by the local invasive procedures. We studied the influence of local invasive procedures on HPV-16 E7 specific immune responses in patients with different grades of cervical intra-epithelial neoplasia (CIN) and different stages of cervical cancer. Blood was obtained at intake and after invasive procedures from patients with CIN or cervical cancer. Antigen specific T-cell responses were measured by IFN-γ ELISPOT analysis, after stimulation with recombinant HPV-16 E7 protein. As expected, HPV-16 E7 specific IFN-γ T cell responses were more frequent in HPV-16 DNA positive patients compared with that in HPV-16 DNA negative patients (39/50 *vs.* 16/36, ($p = 0.006$, χ^2 test). After invasive procedures, a small number of HPV-16 DNA positive CIN patients, but a considerable proportion of HPV-16 DNA positive cervical cancer patients, showed an enhancement of T cell responses against HPV-16 E7. Induction of T cell reactivity was most pronounced in cervical cancer patients who had undergone previous invasive procedures. Both $CD4^+$ and $CD8^+$ T cells showed E7 specific IFN-γ production upon *in-vitro* stimulation. Our study shows that invasive procedures may enhance HPV-specific cell-mediated immunity in a considerable number of patients with cervical cancer, but in only a minority of CIN patients. Our data indicate that invasive procedures should be considered as possible confounding factors when analyzing the effectiveness of therapeutic immunization studies, especially, when induction of HPV-specific immune responses is used as intermediate end-point.

Key words: cervical cancer; IFN-γ; HPV; $CD4^+$ T cell; $CD8^+$ T cell

Infection with oncogenic human papillomavirus (HPV) plays an important role in cervical carcinogenesis, and HPV DNA can be detected in 90–100% of all cervical cancers.[1,2] The majority of women infected with oncogenic HPV types do not develop cervical intraepithelial neoplasia (CIN) or cervical cancer but clear their HPV infection. The immune system plays an important role herein, as demonstrated by the observation that immunocompromised women, such as AIDS-patients, more often fail to clear an HPV infection and have an increased risk to develop cervical cancer.[3]

The E6 and E7 transforming oncoproteins of HPV play a crucial role in the transformation and maintenance of the malignant phenotype, and therefore, these proteins are the ideal candidates for tumor-specific cervical cancer immunotherapy. Cytotoxic T cell (CTL) and T helper activity specific for E6 and E7 of HPV-16 and HPV-18 (the 2 most common HPV types) have been demonstrated in the peripheral blood of patients with (pre)malignant cervical neoplasia and healthy controls.[4–12] It has been suggested that spontaneous regression of CIN lesions might be associated with cell-mediated immune responses against the E6 and E7 oncoproteins of HPV.[4–11] However, the relative contribution of E6 and E7 specific cellular immune responses to the prevention or the eradication of (pre)malignant cervical neoplasia is debated.[4,11]

Recent reports suggest that the failure to develop a potent T helper type 1 response specific for the oncoprotein E6 and the early protein E2 of HPV might be responsible for the development of high grade CIN lesions and cervical cancer.[7,13,14] The observation that strong proliferative T cell responses specific for HPV-16 E7 peptides are found more often in patients with regression of premalignant cervical lesions[9] also indicates the importance of cell-mediated immune responses against E7 in the prevention of high-grade premalignant cervical neoplasia. Moreover, longitudinal analyses of patients who had cleared an HPV infection showed that these patients had strong HPV-16 E7 induced T-cell responses around the time of viral clearance.[15]

In the clinic, it has been observed that local invasive procedures such as biopsies may alter the natural course of premalignant cervical disease. Partial surgical excision of the lesions and enhancement of HPV antigen presentation resulting from tissue disruption are believed to play a role. This idea of boosting cell-mediated immunity against HPV by local invasive procedures has not been experimentally addressed. The aim of the present study was to explore the influence of local invasive procedures on HPV-16 E7 specific IFN-γ production in peripheral blood mononuclear cells (PBMC) cultures of patients with (pre)malignant cervical neoplasia.

Material and methods

Ethical approval

The study was approved by the local medical ethical committee of the University Medical Center Groningen (UMCG). Written informed consent was obtained from all patients.

Recruitment of patients

CIN and cervical cancer patients were recruited from the outpatient clinic of the Department of Gynecology of the UMCG. Patients were eligible for participation if 2 cervical smears indicated mild, or if 1 smear indicated moderate to severe dysplasia or carcinoma *in-situ*.

In the Netherlands, cervical smears are classified according to a modified Papanicolaou system in which borderline dyskaryosis corresponds well with the Bethesda classification ASCUS, mild

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dyskaryosis with LSIL and moderate and severe dyskaryosis and carcinoma *in situ* with HSIL.[16]

Patients referred to the clinic for cervical cancer were also eligible for participation in the study. From the patients, 40 ml of heparinized and 10 ml of clotted blood was obtained before and after invasive procedures.

Patients with smears showing mild to moderate dysplasia underwent a colposcopically directed biopsy. After CIN grade I was diagnosed, no further therapy was given. From these patients, blood was obtained at intake and 3–6 weeks after biopsy. When CIN grade II/III was diagnosed, the whole transformation zone was excised 4–6 weeks later by loop excision, after which the patients were seen for routine follow-up at the outpatient clinic. From these patients, blood was obtained at intake, 3–6 weeks after biopsy and 3–6 weeks after large loop excision of the transformation zone (LLETZ). In view of the high chances of having CIN II or higher-grade lesions (>90%), patients with 1 smear with severe dysplasia or worse underwent a colposcopy and LLETZ without taking biopsies. From these patients, blood was obtained at intake and 3–6 weeks after excision of the transformation zone.

Patients referred with cervical carcinoma were staged according to the FIGO criteria.[17] Several of the cervical carcinoma patients referred to our clinic already had a previous conization or biopsy in the referring hospital. In general, patients with FIGO stage Ib/IIa were treated by radical surgery and patients with stage IIb–IV were treated with chemoradiation. From the early stage cervical carcinoma patients, blood was obtained at intake, 3–6 weeks after first therapy and 3–6 weeks after eventual adjuvant therapy. From the cervical carcinoma patients receiving chemoradiation, blood was obtained at intake and 8 weeks after chemoradiation.

Obtaining cervical scrapings and establishing HPV-16 status

Obtaining cervical scrapings. At the patient's initial visit, the cervix was scraped with a spatula and brush. The scraped cells were suspended in 5-ml of sterile PBS (pH 7.2) and kept on ice. Approximately 1 ml of the suspension was used for the isolation of DNA.

HPV-16 specific PCR. HPV-16 status was established by HPV-16 specific PCR on DNA isolated from the cervical scrapings and serology of serum for antibodies against HPV-16 L1, E6 and E7. The HPV-16 specific PCR was performed as described by Claas *et al.*[18] and Baay *et al.*[19] HPV-16 primers (product 152 bp) were sense TGCTAGTGCTTATGCAG CAGCAA, antisense ATTTACTGCAACATTGGTAC.

HPV-16 specific serology. Presence of IgG antibodies against L1, E6 and E7 proteins of HPV16 were determined by ELISA, as described by Sehr *et al.*[20,21] Briefly, HPV-16 L1, E6 and E7 IgG levels were measured by ELISA, using recombinant proteins fused to glutathione *S*-transferase. Glutathione *S*-transferase without HPV protein sequences was used as specificity control, and any background response to the latter was subtracted to give the HPV-specific OD values.

Triplicate wells were not statistically different to each other (S.D. always <10%) and the results shown represent mean HPV-specific ODs from experiments performed on 2 separate occasions. Cutt-off levels were calculated using sera from German blood bank donors. The cut-off values have been calculated as 2 × (mean + 2 SD) of the values for each antigen. The cut-off OD-values, as defined on the group of German blood bank donors, were as follows: 0.534 (HPV-16 L1), 0.370 (HPV-16 E6, 0.226 (HPV-16 E7).

Isolation of cells

PBMC were isolated from heparinized blood samples by Ficoll (Sigma) density centrifugation. The cells were cryopreserved, stored in liquid nitrogen and thawed by standard procedures to enable simultaneous analysis of the samples taken at different timepoints.

Pathology

Cervical neoplasia was classified according to the most severe lesion found on histological examination of biopsy and LLETZ loop excision specimens, according to international criteria.[16,17] Patients with lesions of adenomatous or adenosquamous cell type were excluded from selection. Tissue samples of patients with squamous cell cervical cancer were derived from biopsies taken during gynecological examination under general anesthesia for clinical staging or from radical hysterectomy specimens in patients with FIGO stage IB/IIA.

Purification and production of HPV-16 E7 protein

The recombinant HPV-16 E7 was produced and purified as follows. The HPV16 E7 gene was cloned in the BamHI site of pET19b, a plasmid that carries an N-terminal His-Tag sequence (Novagen, Madison WI, USA). The plasmid was transformed into *E. coli* BL21 pLysS and E7 was expressed upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG). After overnight culture, the bacteria were pelleted and resuspended in binding buffer (Novagen) containing 6 M ureum. E7 was purified using the Novagen purification system, according to the Novagen protocol for immobilized metal affinity chromatography, in the presence of 6 M ureum during the whole purification process. The pET19b His-Tag sequence contains 10 consecutive histidine residues and binds to divalent cations (Ni^{2+}) immobilized on resin. After unbound proteins were washed away, the target protein was recovered by elution with imidazole. After purification, E7 was dialyzed twice against buffer containing 5 mM Hepes, 20 μM Zn acetate, 5% glycerol followed by dialysis against buffer containing 5 mM Hepes, 20 μM Zn acetate 2 times. E7 was further analyzed for purity on a 12.5% SDS-PAGE gel followed by a silver staining. The proteins were blotted on to PVDF membrane (Immobilon-P; Millipore Corp, Bedford, MA, USA) and E7 was detected with a monoclonal mouse anti-HPV16 E7 antibody (Zymed, South San Francisco, CA, USA). After incubation with alkaline phosphatase-linked secondary antibodies, the blots were stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical). The purity was ≥90%. Finally, after establishing the protein concentration by Lowry, E7 was freeze-dried in aliquots. Before use, the protein was dissolved in DMSO at a concentration of 5 mg/ml.

ELISPOT assay

The ELISPOT assay for the quantification of HPV-16 E7 specific T-cell responses is an adaptation of the ELISPOT assay, as previously described by Schmittel *et al.*[22] We preferred to stimulate the cultures with recombinant E7, to allow natural processing and presentation of the immunodominant epitopes to the cytotoxic and T helper cells.

Cryopreserved PBMC were thawed and seeded at a density of 3×10^6 cells/well of a 12-well plate (Costar) in 2-ml culture medium in the presence or absence of 10 μg/ml purified recombinant protein. The 10-μg/ml E7 protein dilution was first incubated for 1 hr with 500 U/ml Polymixine B (Sigma) for neutralization of possible LPS contamination. The unstimulated PBMC were incubated with medium containing 500 U/ml Polymixin B only.

After 24 hr of incubation at 37°C, PBMC were harvested, washed and seeded in duplo at a density of 2×10^5 cells/well in a precoated ELISPOT plate (Nunc, Silent screen, Rochester, NY, USA), with 15 μg/ml of an IFN-γ catching antibody (1-D1K, MabTech, Nacha, Sweden). For optimal stimulation, the cells were cultured with 10 μg/ml E7 protein (treated with 500 U/ml Polymixin B) or medium with only Polymixin B in the ELISPOT plates for ~20 hr at 37°C. The assay was further performed according to the manufacturer's instructions (Mabtech, Nacha, Sweden). The number of spots was analyzed with a fully automated imaging device (A.EL.VIS, GmbH, Hanover, Germany). The average background in the medium control wells was 28 ± 33 spots. Specific spots were calculated using criteria similar to

other studies.[6] The specific response was calculated by subtracting the mean number of spots $\pm 2 \times$ SD of the medium control wells from the mean number of spots of experimental wells. The response against the HPV-16 E7 protein was considered positive, when the number of specific spots were $\geq 20/2 \times 10^5$ PBMC and the response in the experimental wells was at least 2 times the background level. As a positive control, to establish the vitality of the cells, the PBMC were stimulated with 4 μg/ml ConA. All patients showed strong responses against ConA and there was no difference between the patient groups.

Identification of responders to the invasive procedures

For a variety of reasons, we had material available from 3 patients who did not undergo invasive procedures between 2 blood samplings. These patients had a positive response to the HPV-16 E7 protein, and we were thus able to get an indication of the ''normal'' fluctuation of the IFN-y response as measured by the ELISPOT around base-line.

The first patient had 55 spots per 2×10^5 PBMC in the first blood sample and 64 spots per 2×10^5 PBMC in the second blood sample that was taken 6 weeks later. The second patient had 24 spots per 2×10^5 PBMC in the first blood sample and 26 spots per 2×10^5 PBMC in the second sample. The third patient, who underwent an invasive procedure in the referring hospital, had 26 spots per 2×10^5 PBMC in the first sample and 51 spots in the second sample. In view of these results, and the fact that in literature a 2- to 3-fold induction of antigen specific IFN-γ production is often used to identify someone as a responder to a therapeutic vaccination,[23] a patient was considered to have an increased or decreased response after the invasive procedures, when the number of IFN-γ producing T cells had changed at least 3-fold.

Depletion of PBMC from $CD4^+$ and $CD8^+$ T cells

PBMC were washed and a maximum of 1×10^7 cells was resuspended in 80 μl of buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA). Then 20 μl of MACS CD4 or CD8 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and incubated for 20 min at 4°C. Cells were washed with 1.5 ml buffer and resuspended in 500 μl of buffer. Magnetic separation was performed with the MS columns (Miltenyi Biotec), according to the manufacturers' instructions. Flow cytometric analysis revealed a depletion of $CD4^+$ and $CD8^+$ T cells to less than 1%.

Flow cytometry

Monoclonal antibodies labeled with FITC, PE and PE-cy5 were used for triple color flow cytometry. Cells were incubated with saturating amounts of the antibodies for 30 min on ice, in a volume of 50 μl. After the incubation, the cells were washed 3 times with PBS/1% BSA at 4°C. The cells were kept in the dark at 4°C until analysis. The cells were analyzed with the use of a FACScan (Elite, Coulter, Fullerton, CA, USA).

Statistical analysis

For statistical analysis, SPSS 11.0 software package for Windows was used. Differences between groups in the frequency of positive responses were calculated by a χ^2 test. Only p-values < 0.05 were considered significant.

Results

Correlation between HPV-16 E7 specific T cell responses and HPV-16 DNA status

As expected, crosssectional analysis revealed that in the total patient group, HPV-16 E7 specific IFN-γ production by T cells at any time during the study was more frequent in HPV-16 DNA positive patients as compared with that in HPV-16 DNA negative patients (39/50 *vs.* 16/36, ($p = 0.006$, χ^2 test), pointing to the specificity of our assay. This difference was most pronounced in the cervical carcinoma patients, see Table IV and Figure 2.

In the cervical cancer patients, we did not observe a correlation between the response in the ELISPOT and the antibody levels against HPV-16 L1 and E7. In addition, we could not observe a correlation between the antibody titers against HPV-16 L1 and the HPV-16 E7 specific ELISPOT responses in the CIN patients. As is shown in the tables, only a few CIN patients showed detectable levels of anti E7 antibodies, and therefore, we did not perform further correlation analysis.

Enhancement of HPV-16 E7 specific T cell responses in CIN and cervical cancer patients after (local) invasive procedures

CIN patients. As shown in Table I and Figure 1*a*, HPV-16 E7 specific responses were detected in 2 out of 8 HPV-16 DNA negative CIN I patients at intake. After local invasive procedures, 3 out of these 8 patients responded to E7. Patient 192 shows 50 spots after the invasive procedures. However, we decided to score this patient not to be significantly increased, because the response was not 3-fold above the cut-off level. Therefore, no changes were observed in the E7 specific IFN-γ responses in the HPV-16 DNA negative patients, after invasive therapy.

From the HPV16 DNA positive CIN I patients, 2 out of 4 patients had a T cell response against E7 at intake. After local invasive procedures, 3 out of 4 patients had detectable numbers of IFN-γ producing T cells. One patient showed an increase of the

TABLE I – RESPONSES AGAINST HPV-16 E7 PROTEIN BEFORE AND AFTER INVASIVE PROCEDURES IN CIN I PATIENTS DETERMINED BY IFN-γ ELISPOT

CIN I Pat. ID.	Previous intervention	HPV16 status				First sample response	Interventions	Second sample response	Relative change
		DNA	anti L1	anti E6	anti E7				
89	None	Neg.	Neg.	Neg.	Neg.	**86**	biopsy	**60**	=
127	None	Neg.	Neg.	Neg.	Neg.	**47**	biopsy	**126**	=
183	None	Neg.	ND	ND	ND	(−)	biopsy	(−)	=
209	None	Neg.	ND	ND	ND	(−)	biopsy	(−)	=
84	None	Neg.	Neg.	Neg.	Neg.	(−)	biopsy/cur.	(−)	=
107	None	Neg.	**Pos.**	Neg.	Neg.	(−)	biopsy/cur.	(−)	=
192	None	Neg.	ND	ND	ND	(−)	cur.	**50**	=
6	None	Neg.	ND	ND	ND	(−)	LLetz	(−)	=
					Overall	2/8		3/8	
59	None	**Pos.**	Neg.	Neg.	Neg.	(−)	biopsy	**65**	+
99	None	**Pos.**	Neg.	Neg.	Neg.	(−)	biopsy	(−)	=
124	None	**Pos.**	Neg.	Neg.	Neg.	**70**	biopsy	**49**	=
198	None	**Pos.**	Neg.	Neg.	Neg.	**44**	biopsy	**25**	=
					Overall	2/4		3/4	

Positive responses are indicated in bold. A patient was considered to have an increased or decreased response after the invasive procedures, when the number of IFN-γ producing cells had changed more than 3-fold. (−), no significant response against HPV-16 E7; ND, not done; cur, curettage; +, increase; −, decrease; =, no change.

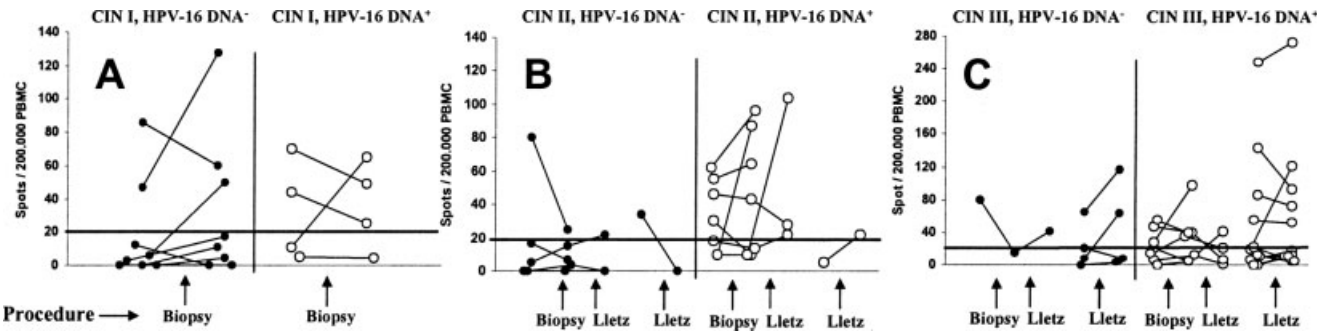

FIGURE 1 – ELISPOT analysis of IFN-γ production induced by recombinant HPV-16 E7 protein in CIN patients of varying grade before and after (local) invasive procedures. The figure shows the number of IFN-γ spot forming cells per 200,000 PBMC, which were obtained before and after (local) invasive procedures. The type of therapy is indicated in the figure. The PBMC were stimulated with 10 μg/ml recombinant HPV-16 E7 protein. The response was considered positive when the number of specific IFN-γ spot forming cells was above 20/200,000 PBMC (indicated by the black line). The filled symbols represent HPV-16 DNA negative patients and the open symbols represent HPV-16 DNA positive patients.

TABLE II – RESPONSES AGAINST HPV-16 E7 PROTEIN BEFORE AND AFTER INVASIVE PROCEDURES IN CIN II PATIENTS DETERMINED BY IFN-γ ELISPOT

CIN II Pat. ID.	Previous intervention	HPV16 status				First sample response	Interventions	Second sample response	Interventions	Third sample Response	Relative change
		DNA	anti L1	anti E6	anti E7						
30	None	Neg.	Neg.	Neg.	Neg.	(−)	Biopsy	(−)	Lletz	**22**	=
140	None	Neg.	ND	ND	ND	(−)	Biopsy	(−)	LLetz	(−)	=
159	None	Neg.	ND	ND	ND	(−)	Biopsy	(−)	LLetz	(−)	=
128	None	Neg.	ND	ND	ND	**80**	Biopsy/LLetz	**25**	None		=
154	None	Neg.	Neg.	Neg.	Neg.	**34**	LLetz	(−)	None		=
190	None	Neg.	ND	ND	ND	(−)	LLetz	(−)	None		=
					Overall	2/8		1/6		1/3	
42	None	**Pos.**	**Pos.**	Neg.	Neg.	**55**	None	**64**	Biopsy	NA	=
14	None	**Pos.**	**Pos.**	Neg.	Neg.	**62**	Biopsy	**96**	LLetz	NA	=
70	None	**Pos.**	Neg.	Neg.	Neg.	(−)	Biopsy	**87**	LLetz	NA	+
73	None	**Pos.**	Neg.	Neg.	Neg.	**46**	Biopsy	**43**	LLetz	**28**	=
135	None	**Pos.**	Neg.	Neg.	Neg.	(−)	Biopsy	(−)	LLetz	**104**	+
139	None	**Pos.**	Neg.	Neg.	Neg.	(−)	Biopsy	(−)	LLetz	**22**	=
212	None	**Pos.**	Neg.	Neg.	Neg.	**30**	Biopsy	(−)	LLetz	NA	=
153	None	**Pos.**	Neg.	Neg.	Neg.	(−)	LLetz	**22**	None		=
					Overall	4/8		6/8		3/3	

Positive responses are indicated in bold. A patient was considered to have an increased or decreased response after the invasive procedures, when the number of IFN-γ producing cells had changed more than 3-fold. (−), no significant response against HPV-16 E7; ND, not done; NA, not available; +, increase; −, decrease; =, no change.

response after the invasive procedures, but the remaining patients showed no change.

As shown in Figure 1*b* and Table II, 6 HPV-16 DNA negative and 8 HPV16 DNA positive CIN II patients were analyzed longitudinally in the ELISPOT assay. At intake, 2 out of 6 HPV-16 DNA negative CIN II patients showed a positive IFN-γ response against the HPV-16 E7 protein in the ELISPOT assay. In 5 of these patients, the invasive procedures had no effect on the T cell response. In 1 patient, the invasive procedures resulted in a decrease of the numbers of T cells that produced IFN-γ. Although patient 30 becomes positive (22 spots/2 $\times$ 10^5 PBMC) at its third sample, we decided to interpret this result conservatively and score the response in this patient not to be changed as compared with that of the pre-treatment response.

From the 8 HPV16 DNA positive CIN II patients, 4 responded against E7 at intake. In 6 patients, no effect of the invasive procedures on the T-cell response was observed, whereas in 2 patients, the invasive procedures resulted in an increase of the IFN-γ response. As was also observed in the CIN I patients, patient 139 and 153 became positive after the invasive procedures (22 spots/2 $\times$ 10^5 PBMC). We interpreted these results conservatively and considered the response equal as compared with that of the pre-treatment response.

Six HPV16 DNA negative and 18 HPV16 DNA positive CIN III patients were analyzed longitudinally for their T-cell response against HPV-16 E7 (Table III and Fig. 1*c*). At intake, 3 out of 5 HPV-16 DNA negative CIN III patients showed a positive IFN-γ response against the HPV-16 E7 protein. After the first local invasive procedure, 2 out of 6 patients responded to the E7 protein. One patient displayed an increase in the number of IFN-γ producing T cells and in 4 of the 6 patients, the invasive procedures had no effect on the IFN-γ production.

Nine out of 18 HPV16 DNA positive CIN III patients responded to E7 in the ELISPOT assay at intake. After the first local invasive procedure, again 9 out of these 18 patients responded, of which 2 patients displayed an increase in the number of IFN-γ producing T cells. From 3 patients, samples were available after the second local invasive procedure. One of these patients showed an increase in the number of IFN-γ producing T cells in her third sample as compared with that of the first sample at intake and the second sample after the first local invasive procedure. However, after the invasive procedures, the response was not increased at least 3-fold and therefore this patient was scored as no change. Taken together, in 16 patients the invasive procedures had no effect on the IFN-γ producing T cells and in 2 patients, an increase in their response was observed.

Cervical cancer patients. Out of 16 HPV-16 DNA negative cervical cancer patients, 6 responded against HPV-16 E7 in the ELISPOT assay at any time during the study, whereas 16 of 20 HPV-16 DNA positive patients responded.

TABLE III – RESPONSES AGAINST HPV-16 E7 PROTEIN BEFORE AND AFTER INVASIVE PROCEDURES IN CIN III PATIENTS DETERMINED BY IFN-γ ELISPOT

CIN 3 Pat. ID.	Previous intervention	HPV16 status				First sample response	Intervention	Second sample response	Intervention	Third sample response	Relative change
		DNA	anti L1	anti E6	anti E7						
39	None	Neg.	Neg.	Neg.	Neg.	**79**	Biopsy	(−)	LLetz	**42**	=
5	None	Neg.	ND	ND	ND		LLetz	(−)	None		
56	None	Neg.	**Pos.**	Neg.	Neg.	(−)	LLetz	(−)	None		=
71	None	Neg.	Neg.	Neg.	Neg.	(−)	LLetz	**54**	None		+
79	None	Neg.	Neg.	Neg.	Neg.	**21**	LLetz	(−)	None		=
216	None	Neg.	ND	ND	ND	**65**	LLetz	**117**	None		=
					Overall	3/5		2/6		1/1	
35	None	**Pos.**	Neg.	Neg.	Neg.	(−)	Biopsy	(−)	LLetz	(−)	=
37	None	**Pos.**	ND	ND	ND	**28**	Biopsy	**98**	LLetz	NA	+
113	None	**Pos.**	ND	ND	ND	(−)	Biopsy	(−)	LLetz	NA	=
131	None	**Pos.**	**Pos.**	Neg.	Neg.	**20**	Biopsy	(−)	LLetz	**42**	=
178	None	**Pos.**	ND	ND	ND	**47**	Biopsy	**40**	None	(−)	=
182	None	**Pos.**	**Pos.**	Neg.	Neg.	(−)	Biopsy	**35**	LLetz	**20**	=
304	None	**Pos.**	ND	ND	ND	**54**	Biopsy	**40**	LLetz		=
18	None	**Pos.**	Neg.	Neg.	Neg.	(−)	LLetz	(−)	None		=
114	None	**Pos.**	Neg.	Neg.	Neg.	**55**	LLetz	**52**	None		=
122	None	**Pos.**	Neg.	Neg.	Neg.	(−)	Lletz	(−)	None		=
126	None	**Pos.**	ND	ND	ND	**22**	LLetz	(−)	None		=
163	None	**Pos.**	Neg.	Neg.	Neg.	**143**	LLetz	**93**	None		=
176	None	**Pos.**	ND	ND	ND	**85**	LLetz	**72**	None		=
177	None	**Pos.**	ND	ND	ND	**248**	LLetz	**272**	None		=
191	None	**Pos.**	Neg.	**Pos.**	Neg.	(−)	LLetz	**121**	None		+
195	None	**Pos.**	**Pos.**	Neg.	Neg.	(−)	LLetz	(−)	None		=
197	None	**Pos.**	**Pos.**	**Pos.**	Neg.	(−)	LLetz	(−)	LLetz	NA	=
215	None	**Pos.**	Neg.	Neg.	**Pos.**	(−)	LLetz	(−)	None		=
					Overall	9/18		9/18		2/4	

Positive responses are indicated in bold. A patient was considered to have an increased or decreased response after the invasive procedures, when the number of IFN-γ producing cells had changed more than 3-fold. (−), no significant response against HPV-16 E7; ND, not done; NA, not available; +, increase; −, decrease; =, no change.

As demonstrated in Table IV and Figure 2, the invasive procedures for the cervical carcinoma patients were rather diverse. In 15 of the HPV-16 DNA negative patients, the invasive procedures had no effect on the IFN-γ response, but in 1 patient the response was decreased after the invasive procedures. In the HPV-16 DNA positive patients, no effect of the invasive procedures was observed in 11 patients, 5 patients showed an increase of their IFN-γ production and 1 patient showed a decrease of her IFN-γ response.

Before intake, approximately two thirds of the patients had already undergone a local invasive procedure in the referring hospital. Interestingly, the HPV-16 DNA positive patients who had previously undergone local invasive procedures showed strong induction of IFN-γ producing T cells after the subsequent invasive procedures in our hospital (Table IV and Fig. 2). Four of these patients showed an induction of more than 3-fold in their number of IFN-γ producing T cells after invasive procedures. In the 14 HPV-16 DNA negative cervical cancer patients, who had previously undergone invasive procedures, none showed a significant change in their IFN-γ production.

Of the HPV-16 DNA positive patients, 4 patients appeared to have residual tumor or metastasis after completion of therapy (Table IV: ID numbers 46, 137, 144, 298; open triangles Fig. 2). At intake, PBMC cultures from 3 of these 4 patients showed specific T cell responses after stimulation with HPV-16 E7. Intriguingly, these 3 patients showed a strong increase in the number of cells producing IFN-γ after invasive procedures, with 1 patient displaying a continuous rise in the HPV-16 E7 induced IFN-γ production, during progressive disease (patient 137).

Contribution of $CD4^+$ and $CD8^+$ T cells to HPV16 E7 induced IFN-γ production

To investigate which cells contribute to the IFN-γ production in the ELISPOT assay, we depleted the PBMC of 5 randomly selected HPV16 DNA positive and 2 randomly selected HPV-16 DNA negative CIN and cervical cancer patients of $CD4^+$ and $CD8^+$ T cells. After depletion the cell-suspensions were stimulated with the E7 protein. FACS analysis demonstrated that after depletion the percentage of $CD4^+$ or $CD8^+$ T cells dropped below 1%. In the CD4 depleted cell-suspension, the percentage of $CD8^+$ T cells was on average 44.1% ± 15.7% and in the CD8 depleted cell-suspension, the percentage $CD4^+$ T cells was on average 71.8% ± 13.1%.

As shown in Figure 3, both CD4 and CD8 depleted cultures of the HPV-16 DNA positive patients show a positive IFN-γ response against HPV-16 E7 and the response is increased as compared with the unseparated PBMC. In general, the responses in the CD4 depleted cultures were higher as compared with that of the CD8 depleted cultures. The 2 tested HPV-16 DNA negative patients displayed a low response against the HPV-16 E7 protein.

Taken together, these results indicate a mixed response of both $CD4^+$ and $CD8^+$ T cells in the response against the E7 protein.

Discussion

In our study, we demonstrate that in a considerable number of HPV-16 DNA positive cervical cancer patients, local invasive procedures strongly enhance HPV-16 E7 induced T cell responses. Especially, those cervical cancer patients who already underwent local invasive procedures in the referring hospital showed the strongest enhancement of HPV-16 E7 specific IFN-γ T cell responses, probably as a result of priming by the first invasive procedures and boosting by the second invasive procedures.

In CIN patients, the enhancement of T cell responses by invasive procedures, as measured by HPV-16 E7 specific IFN-γ production, was observed in only a small minority and was not that pronounced as in the cervical cancer patients. This might be explained by the following reasons: (*i*) In CIN patients the anti-

TABLE IV – RESPONSES AGAINST HPV-16 E7 PROTEIN BEFORE AND AFTER INVASIVE PROCEDURES IN CERVICAL CANCER PATIENTS DETERMINED BY IFN-γ ELISPOT

CxCa Pat. ID.	Stage	Previous interventions	HPV16 status				First sample response	Interventions	Second sample response	Interventions	Third sample response	Interventions	Remarks	Relative change
			DNA	anti L1	anti E6	anti E7								
20	IA1	None	Neg.	Neg.	Neg.	**Pos.**	(−)	Lletz	(−)	Vag. Hyst.	NA			=
146	IIB	None	Neg.	ND	ND	ND	(−)	EUA, biopsy, RT	**33**	biopsy, wm.	NA			=
194	IB1	biopsy	Neg.	ND	ND	ND	(−)	wm.	(−)	None				=
91	IB1	con./biopsy	Neg.	Neg.	Neg.	Neg.	(−)	wm.	(−)	None				=
321	IB1	con./biopsy	Neg.	ND	ND	ND	(−)	wm.	(−)	None				=
133	IB1	con.	Neg.	Neg.	Neg.	Neg.	**24**	None	**26**	EUA, wm.	(−)			=
189	IB1	biopsy	Neg.	ND	ND	ND	(−)	LD/RC	(−)	None				=
204	IB1	Lletz	Neg.	Neg.	Neg.	**Pos.**	(−)	biopsy	(−)	wm./RC	NA			=
269	IIA+B	biopsy	Neg.	ND	ND	ND	(−)	EUA, biopsy	(−)	RC	NA	EUA	Diabetic type 2	−
160	IIB	biopsy	Neg.	Neg.	Neg.	Neg.	(−)	EUA, RC	**39**	Laporotomy	NA		metastasis	=
38	IIB	biopsy	Neg.	Neg.	Neg.	Neg.	**25**	stage	(−)	RC	NA		liver metastasis	=
33	IIB	biopsy	Neg.	Neg.	Neg.	Neg.	(−)	EUA/stage, RC	(−)	Biopsy, wm.	NA			=
115	IIB2	biopsy	Neg.	Neg.	Neg.	Neg.	(−)	EUA, RC	(−)	LLetz	(−)			=
218	IIB	biopsy	Neg.	Neg.	Neg.	**Pos.**	(−)	EUA, Lletz, RC	(−)	biopsy				=
152	IIB	biopsy	Neg.	Neg.	Neg.	Neg.	**91**	EUA, RC	**30**	biopsy	NA			=
43	IIB	biopsy	Neg.	ND	ND	ND	(−)	wm.	**20**	None				=
						Overall	3/16		5/16		0/2			
27	IA1	None	**Pos.**	**Pos.**	Neg.	Neg.	**34**	Lletz	(−)	Utarus expiration				=
108	IA1	None	**Pos.**	**Pos.**	**Pos.**	Neg.	**101**	LLetz	**21**	None	NA	Vag. Hyst.		=
26	IA1	None	**Pos.**	Neg.	Neg.	Neg.	**28**	LLetz	**89**	None				=
202	IA1	None	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**26**	biopsy/RC	(−)	None				=
106	IB1	None	**Pos.**	Neg.	Neg.	Neg.	**110**	LLetz	**113**	None	NA	wm.		=
10	IB1	None	**Pos.**	**Pos.**	**Pos.**	**Pos.**	(−)	LLetz	**25**	wm./RC	(−)			=
16	IIB	None	**Pos.**	**Pos.**	**Pos.**	**Pos.**	**102**	biopsy/RC						
344	IB	biopsy/con.	**Pos.**	ND	ND	ND	**52**	None	ND	RC	NA			=
186	IB1	biopsy	**Pos.**	Neg.	Neg.	**Pos.**	**22**	EUA, LLetz	**176**	wm./RC	NA			=
102	IB1	biopsy	**Pos.**	ND	ND	ND	(−)	wm./RC	(−)	None	(−)			−
222	IB	biopsy	**Pos.**	**Pos.**	**Pos.**	Neg.	(−)	biopsy/LLetz	**20**	wm.	(−)			=
298	IB	biopsy	**Pos.**	ND	ND	ND	(−)	biopsy	(−)	RC	NA	EUA	Metastasis, terminal	=
46	IIA	biopsy	**Pos.**	Neg.	**Pos.**	**Pos.**	**22**	LLetz/RC	**253**	biopsy	NA	wm.	Residual tumor	=
175	IIA	biopsy	**Pos.**	**Pos.**	Neg.	**Pos.**	**26**	None	**51**	wm.	NA			=
207	IIA	biopsy	**Pos.**	ND	ND	ND	(−)	wm.	**29**	None				=
130	IB2	con.	**Pos.**	ND	ND	ND	**28**	wm.	None	RC	**124**			=
137	IB/II	biopsy	**Pos.**	Neg.	**Pos.**	**Pos.**	**28**	LD, CT, RT	**38**	EUA, biopsy	**150**	wm.	Still high tumor load	=
144	IIB	biopsy	**Pos.**	Neg.	**Pos.**	**Pos.**	**65**	RC	**137**		NA	EUA	Residual tumor	=
220	III	biopsy	**Pos.**	ND	ND	ND	(−)	RT	(−)	LLetz	(−)			=
161	IV	biopsy	**Pos.**	Neg.	**Pos.**	**Pos.**	(−)	Biopsies	(−)	LD	NA	Tumor stage 4, Palliatine ther.		=
						Overall	13/20		11/17		2/6			

Positive responses are indicated in bold. A patient was considered to have an increased or decreased response after the invasive procedures, when the number of IFN-γ producing cells had changed more than 3-fold. (−), no significant response against HPV-16 E7; ND, not done; NA: not available; +, increase; −, decrease; =, no change, wm; wertheim, RC, radio and chemotherapy, LD, lymphedectomy, RT, radio therapy; CT, chemotherapy; EUA, examination under anesthesia.

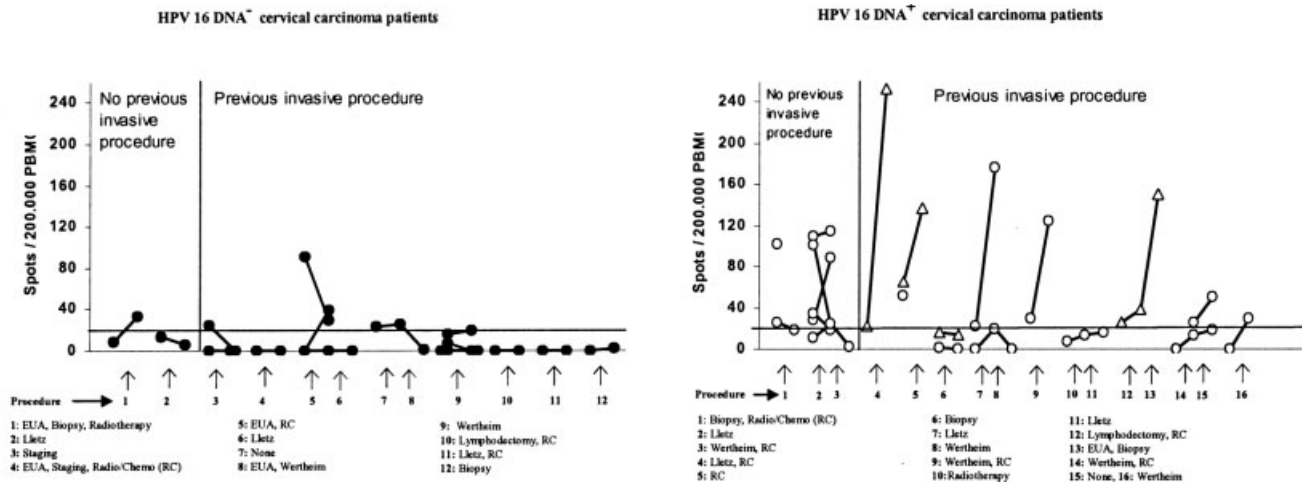

FIGURE 2 – Cervical carcinoma patients show an increase of HPV-16 E7 induced IFN-γ production after invasive procedures. The figure shows the number of IFN-γ spot forming cells per 200,000 PBMC, which were obtained before and after (local) invasive procedures. The type of therapy is indicated in the figure. The PBMC were stimulated with 10 μg/ml recombinant HPV-16 E7 protein. The response was considered positive when the number of specific IFN-γ spot forming cells was above 20/200,000 PBMC (indicated by the black line). The filled symbols represent HPV-16 DNA negative patients and the open symbols represent HPV-16 DNA positive patients. The open triangles represent the patients with a residual tumor or metastasis after completion of therapy.

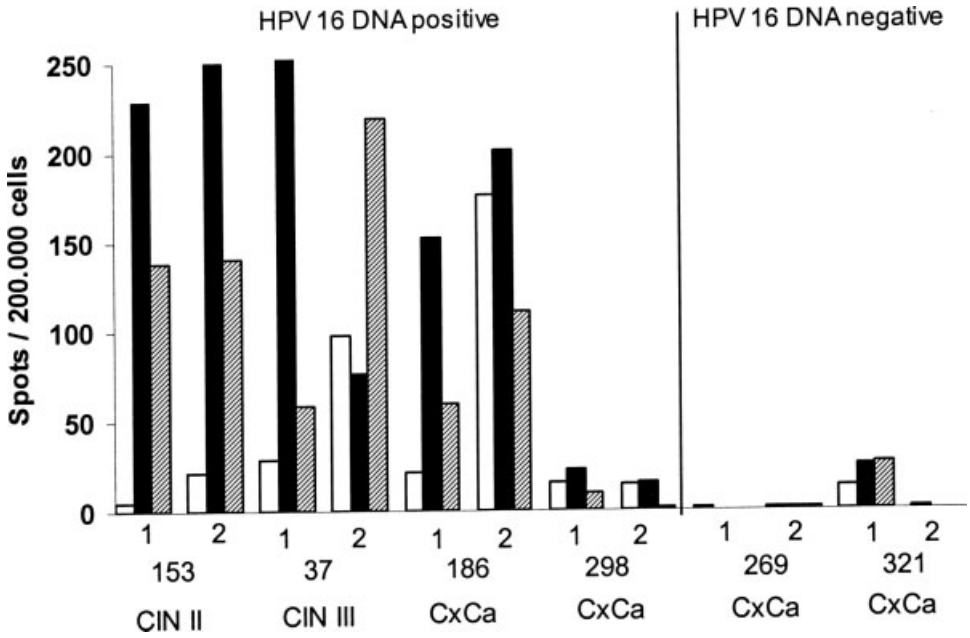

FIGURE 3 – Influence of CD4 and CD8 T cell depletion on IFN-γ production in response to HPV-16 E7 protein in the ELISPOT assay. The figure shows the number of IFN-γ producing cells per 2×10^5 PBMC (white bars), 2×10^5 $CD4^+$ depleted cells (black bars) or 2×10^5 $CD8^+$ depleted cells (hatched bars). CIN: Cervical Intraepithelial Neoplasia. CxCa: Cervical Cancer. 1: Sample obtained before invasive procedures. 2: Sample obtained after invasive procedures.

genic load of the E7 oncoprotein is lower as compared with that in cervical cancer patients. Moreover, in cervical cancer patients the basement membrane is disrupted, making viral antigens more accessible for presentation to the immune system. (*ii*) Patients with CIN had less aggressive and less frequent invasive procedures as compared with those of the cervical cancer patients, while they also had more ''disease'' for a longer period in time. Our observation that an enhanced systemic anti HPV immunity by local invasive procedures does not occur frequently point to the possibility that, with regard to the clinically observed spontaneous regression of CIN lesions, perhaps an enhancement of local (innate) immune responses may be involved. Therefore, future studies should focus on the effects of invasive procedures on local (innate) immuneresponses.

For the present study, we decided to focus our analysis on the T-cell response against E7, because in 2 separate studies it was shown that a potent cellular immune response against E7 *in-vitro* and *in-vivo* was related to viral clearance or (spontaneous) regression of the premalignant lesion.[9,24]

Our results on the possible enhancement of HPV-16 E7 induced T cell responses by local invasive procedures may shed new light on studies that indicated a relation between spontaneous regression of CIN lesions and cellular immune responses against HPV-16 E6 or E7.[9,13] For instance, in the study by Kadish *et al.,* patients were, on average, included 2 months after abnormal cervical cytology and diagnostic biopsy.[9] In these patients, another biopsy was taken at intake, for comparison with the end-point biopsy at 12 months. Therefore, the repeated biopsies in a relative short period of time may have enhanced the cell-mediated immune response against HPV in these patients and, apart from the possible effect of partial surgical excision by taking biopsies, subsequently increased the ''spontaneous'' regression of these premalignant lesions.[9]

In line with previous crosssectional studies on HPV-16 E7 induced IFN-γ production of T cells from patients with (pre)malignant cervical neoplasia,[8,15] we also observed that HPV-16 E7 induced IFN-γ production indeed is associated with a positive HPV-16 DNA status. Our crosssectional data seems in contrast with recent studies by Welters *et al.* and de Jong *et al.*, who showed low frequencies of CIN III and cervical cancer patients responding to E6 or E7 with T helper cell IFN-γ production.[7,14] This discrepancy might be explained by the fact that in our assay we measured IFN-γ, that is produced by T helper and CTL, whereas Welters *et al.* and de Jong *et al.* have measured CD4 T cell responses only.

Also in HPV-16 DNA negative patients, HPV-16 E7 specific responses were observed, although not of the magnitude and frequency observed in the HPV-16 DNA positive CIN and cervical cancer patients. A more detailed analysis showed that the HPV-16 negative CIN I patient 127 and the CIN III patient 71, with a strong enhancement of T cell reactivity after invasive procedures, were positive for HPV-58 and HPV 33, respectively. These 2 viruses are phylogenetically closely related to HPV-16.[25] Therefore, it is reasonable to hypothesize that the HPV-16 E7 specific responses in HPV-16 DNA negative individuals is resembling T cell memory and or cross reactivity to closely related HPV viruses.

The lack of a correlation between the antibody levels and the ELISPOT results is not surprising, because these 2 tests measure humoral and cellular immunity. A limitation of serology is the fact that it is not the most accurate method to establish current HPV infection. However, serology gives information on previous HPV infections and systemic humoral immunity.

Intriguingly, in 3 of the 4 HPV-16 DNA positive cervical carcinoma patients with residual tumor or metastasis after completion of therapy, an increase of E7 induced IFN-γ T cell responses was observed, indicating that in these patients the presence of a relatively high cellular immune response against HPV-16 E7 was not capable to prevent tumor growth. The strong response against HPV-16 E7, as observed in these cervical cancer patients, might be the result of continuous stimulation of the immune system because of the presence of a high antigenic load of the E7 oncoprotein. Accordingly, our results indicate that the E7 specific T cell IFN-γ production in cervical cancer patients might be a marker for the severity of the lesion, more than reflecting the capability of the host to control tumor growth. However, we only measured E7 induced IFN-γ production by T cells in the peripheral blood and no other cytokines or immune effector mechanisms like IL-4 or granzymes.

The depletion experiments suggest that both $CD4^+$ and $CD8^+$ T cells contribute to the response against the E7 protein. The fact that we observe potent $CD8^+$ T cell responses is in line with studies by Todd *et al.*, who showed $CD8^+$ T cell responses against HPV-16 E7 in HPV-16 positive high-grade vulval intraepithelial neoplasia patients.[26,27]

Interestingly, depleting the PBMC from $CD4^+$ T cells or $CD8^+$ T cells resulted in a strong increase of the IFN-γ production as compared with that of the undepleted PBMC. Three mechanisms might be involved in this increase.

1. It has been shown that in PBMC of cancer patients, the levels of regulatory T cells are increased.[28] Regulatory T cells are capable of suppressing the antigen specific response of CTL.[29] Therefore, depleting $CD4^+$ T cells will also deplete the regulatory T helper cells, which may lead to strong $CD8^+$ T cell responses. Indeed, our preliminary results show increased levels of CD4/CD25 positive T cells in the PBMC of cervical cancer patients, which is an indication for increased levels of regulatory T cells (data not shown).
2. Depleting the PBMC from CD4 or CD8 can dramatically influence the relative proportion of the other cell subsets. After depleting the PBMC from their CD4 T cells, flowcytometric analysis showed that the % CD8 T cells on average increased from 19.8% ± 8.2% to 44.1% ± 15.7%. After CD8 depletion, the % of CD4 T cells increased on average from 48.8% ± 13.6% to 71.8% ± 13.1%. Also, the relative proportion of the monocytes was increased. We kept the number of cells in the wells constant for 200,000 cells, irrespective of the number of T cells. Therefore, the increase of the IFN-γ production in the CD4 depleted population can be partly explained by the relative increase of the CD8 T cell population. However, it is reasonable to suggest that depletion of regulatory T cells, is not a major factor in the induction of the responses after the CD8 depletion. In those cultures the increase might be due to enriched populations of responder cells.
3. Because of the change in the ratio of antigen presenting cells (monocytes and B cells) *versus* T cells, antigen presentation might have been more efficient, leading to enhanced activation of the antigen specific T cells.

Taken together, the depletion experiments do not reveal the relative involvement of the CD4 and CD8 T cells, but it is reasonable to assume that both CD4 and CD8 T cells are involved in the T cell response against the HPV-16 E7 protein.

In summary, our study shows that invasive procedures may enhance HPV-specific cell-mediated immunity in a considerable number of patients with cervical cancer. However, this induction is only found in a small minority of CIN patients. The continuous stimulation of the immune system due to the high expression of the oncoprotein E7 and the more frequent and aggressive invasive therapy in cervical cancer patients might explain this difference. Our data strongly indicate that invasive procedures such as biopsies should be considered as confounding factors when analyzing the effectiveness of therapeutic HPV immunization studies, in which induction of HPV-specific immune responses are used as intermediate end-points.

References

1. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189:12–9.
2. Bekkers RL, Massuger LF, Bulten J, Melchers WJ. Epidemiological and clinical aspects of human papillomavirus detection in the prevention of cervical cancer. Rev Med Virol 2004;14:95–105.
3. Bouwes Bavinck JN, Berkhout RJ. HPV infections and immunosuppression. Clin Dermatol 1997;15:427–37.
4. Eiben GL, Velders MP, Kast WM. The cell-mediated immune response to human papillomavirus-induced cervical cancer: implications for immunotherapy. Adv Cancer Res 2002;86:113–48.
5. Man S. Human cellular immune responses against human papillomaviruses in cervical neoplasia. Expert Rev Mol Med 1998;1998:1–19.
6. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, Geluk A, van Meijgaarden KE, Franken KL, Ottenhoff TH, Fleuren GJ, Kenter G, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 2001;91:612–8.
7. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, Franken KL, Drijfhout JW, Fleuren GJ, Kenter G, Melief CJ, Offringa R, et al. Frequent display of human papillomavirus type 16 E6-specific memory T-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003;63:636–41.
8. Bontkes HJ, de Gruijl TD, van den Muysenberg AJ, Verheijen RH, Stukart MJ, Meijer CJ, Scheper RJ, Stacey SN, Duggan-Keen MF,

Stern PL, Man S, Borysiewicz LK, et al. Human papillomavirus type 16 E6/E7-specific cytotoxic T lymphocytes in women with cervical neoplasia. Int J Cancer 2000;88:92–8.
9. Kadish AS, Timmins P, Wang Y, Ho GY, Burk RD, Ketz J, He W, Romney SL, Johnson A, Angeletti R, Abadi M. Regression of cervical intraepithelial neoplasia and loss of human papillomavirus (HPV) infection is associated with cell-mediated immune responses to an HPV type 16 E7 peptide. Cancer Epidemiol Biomarkers Prev 2002;11:483–8.
10. Nakagawa M, Stites DP, Palefsky JM, Kneass Z, Moscicki AB. CD4-positive and CD8-positive cytotoxic T lymphocytes contribute to human papillomavirus type 16 E6 and E7 responses. Clin Diagn Lab Immunol 1999;6:494–8.
11. van der Burg SH, de Jong A, Welters MJ, Offringa R, Melief CJ. The status of HPV16-specific T-cell reactivity in health and disease as a guide to HPV vaccine development. Virus Res 2002;89:275–84.
12. Warrino DE, Olson WC, Knapp WT, Scarrow MI, D'Ambrosio-Brennan LJ, Guido RS, Edwards RP, Kast WM, Storkus WJ. Disease-stage variance in functional CD4(+) T-cell responses against novel pan-human leukocyte antigen-D region presented human papillomavirus-16 E7 epitopes. Clin Cancer Res 2004;10:3301–8.
13. Nakagawa M, Stites DP, Patel S, Farhat S, Scott M, Hills NK, Palefsky JM, Moscicki AB. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. J Infect Dis 2000;182:595–8.
14. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, Kenter G, Offringa R, van der Burg SH. Human papillomavirus type 16-positive cervical cancer is associated with impaired $CD4^+$ T-cell immunity against early antigens E2 and E6. Cancer Res 2004;64:5449–55.
15. de Gruijl TD, Bontkes HJ, Walboomers JM, Stukart MJ, Doekhie FS, Remmink AJ, Helmerhorst TJ, Verheijen RH, Duggan-Keen MF, Stern PL, Meijer CJ, Scheper RJ. Differential T helper cell responses to human papillomavirus type 16 E7 related to viral clearance or persistence in patients with cervical neoplasia: a longitudinal study. Cancer Res 1998;58:1700–6.
16. Bulkmans NW, Rozendaal L, Snijders PJ, Voorhorst FJ, Boeke AJ, Zandwijken GR, van Kemenade FJ, Verheijen RH, van Groningen K, Boon ME, Keuning HJ, van Ballegooijen M, et al. POBASCAM, a population-based randomized controlled trial for implementation of high-risk HPV testing in cervical screening: design, methods and baseline data of 44,102 women. Int J Cancer 2004;110:94–101.
17. Finan MA, DeCesare S, Fiorica JV, Finan MA, DeCesare S, Fiorica JV, Chambers R, Hoffman MS, Kline RC, Roberts WS, Cavanagh D. Radical hysterectomy for stage IB1 vs IB2 carcinoma of the cervix: does the new staging system predict morbidity and survival? Gynecol Oncol 1996;62:139–47.
18. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. Am J Pathol 1989;135:703–9.
19. Baay M, Lardon F, Vermorken JB, Verhoeven V, Avonts D, Van Royen P, Wouters K, Van Damme P, Van Marck E. HPV in cervix and vagina. Sex Transm Infect 2004;80:249–50.
20. Sehr P, Muller M, Hopfl R, Widschwendter A, Pawlita M HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase. J Virol Methods 2002;106:61–70.
21. Sehr P, Zumbach K, Pawlita M. A generic capture ELISA for recombinant proteins fused to glutathione *S*-transferase: validation for HPV serology. J Immunol Methods 2001;253:153–62.
22. Schmittel A, Keilholz U, Bauer S, Kuhne U, Stevanovic S, Thiel E, Scheibenbogen C. Application of the IFN-γ ELISPOT assay to quantify T cell responses against proteins. J Immunol Methods 2001;247:17–24.
23. Mazzaferro V, Coppa J, Carrabba MG, Rivoltini L, Schiavo M, Regalia E, Mariani L, Camerini T, Marchiano A, Andreola S, Camerini R, Corsi M, et al. Vaccination with autologous tumor-derived Heat-shock protein gp96 after liver resection for metastatic colorectal cancer. Clin Cancer Res 2003;9:3235–45.
24. Hopfl R, Heim K, Christensen N, Zumbach K, Wieland U, Volgger B, Widschwendter A, Haimbuchner S, Muller-Holzner E, Pawlita M, Pfister H, Fritsch P. Spontaneous regression of CIN and delayed-type hypersensitivity to HPV-16 oncoprotein E7. Lancet 2000;356:1985–6.
25. Shah KV, Howley PM. Papillomaviruses. In: Fields BN, Knipe DM, Howley PM, eds. Fields virology. Philadelphia: Lippincott-Raven Publishers, 1995.2077–110.
26. Todd RW, Roberts S, Mann CH, Luesley DM, Gallimore PH, Steele JC. Human papillomavirus (HPV) type 16-specific $CD8^+$ T cell responses in women with high grade vulvar intraepithelial neoplasia. Int J Cancer 2004;108:857–62.
27. Todd RW, Steele JC, Etherington I, Luesley DM. Detection of $CD8^+$ T cell responses to human papillomavirus type 16 antigens in women using imiquimod as a treatment for high-grade vulval intraepithelial neoplasia. Gynecol Oncol 2004;92:167–74.
28. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 2003;9:606–12.
29. Aandahl EM, Michaelsson J, Moretto WJ, Hecht FM, Nixon DF. Human $CD4^+$ $CD25^+$ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. J Virol 2004;78:2454–9.