CHAPTER 2

The detection of circulating Human Papillomavirus (HPV)-specific T cells is associated with improved survival of patients with deeply infiltrating tumors

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ABSTRACT

A detailed analyses of HPV-specific immunity was performed in a large group of patients with HPV-induced cervical cancer (CxCa) in relation to HLA-types and prognostic factors. Patients were HLA-typed and HPV16/18-specific T-cell immunity was assessed by proliferation assay and cytometric bead array using freshly isolated PBMC and by phenotypic analysis of HPV-specific T cells. The results were analyzed in relation to known disease-related HLA-types (DR7, DR13, DR15/DQ06), invasion-depth and size of tumor, lymph node (LN) status and disease free survival.

In total 119 HLA-typed patients with CxCa were analyzed. Patients expressing the HLA-DR13 haplotype were underrepresented as compared to the Dutch population ($p=0.014$), whereas HLA-DR7 was overrepresented in patients with HPV16+ CxCa ($p=0.006$). In 29 of 94 patients (31%) from whom blood could be tested, a proliferative response to HPV16/18 was detected, which was associated with increased numbers of HPV-specific CD4+CD25+ (activated) T cells ($p=0.03$) and HPV-specific CD4+CD25+FoxP3-positive T cells ($p=0.04$). The presence of both FoxP3-positive and negative HPV-specific CD4+CD25+ T cells was significantly correlated ($p=0.01$). Interestingly, the detection of HPV-specific proliferation was associated with invasion depth ($p=0.020$) but not with HLA type, tumor size nor LN status. Moreover, the detection of HPV-specific immunity was associated with an improved disease free survival ($p=0.04$) in patients with deeply infiltrating tumors.

In conclusion, HPV-specific proliferative T-cell response, comprising higher percentages of HPV-specific CD25+ and CD25+FoxP3-positive CD4+T cells, are more frequently detected in patients with deep infiltrating CxCa tumors and associated with an improved survival.

Novelty and impact of the study: Studies on the proliferative T-cell response against the two oncoproteins E6 and E7 of high-risk human papillomavirus type 16 and 18 have shown that the presence of a strong T-cell reaction was associated with protection against disease progression. This notion was sustained by our recent vaccine study in which such a response correlated with the regression of HPV16-induced high-grade lesions. This large prospective study reveals that especially the patients with deep stromal infiltrating cervical tumors display proliferative HPV-specific T-cell responses. Moreover, patients with deeply infiltrating tumors displaying such a HPV-specific proliferative response less often show recurrence of disease, suggesting that the HPV-specific T-cells have a protective effect. This implies that reinforcement of the HPV E6- and E7-specific T-cell response by vaccination may have a positive effect on disease free survival.

INTRODUCTION

Cervical cancer (CxCa) is the second most common cancer in women worldwide (1). It develops as a result of an uncontrolled, persistent infection with a high-risk type of human papillomavirus (HPV), in particular types HPV16 and HPV18 (2). The HPV genome encodes two oncoproteins, E6 and E7, which are constitutively expressed in high-grade cervical lesions and cancer since they are required for the onset and maintenance of the malignant cellular phenotype (3).

As the HPV proteins are foreign to the body one would expect the immune system to respond against these antigens when expressed in the cervical epithelium. Indeed, HPV16-specific Th1- and Th2-type CD4+ proliferative T-cell responses were frequently detected in PBMC cultures of healthy individuals (4-6) and both HPV16-specific CD4+ and CD8+ T cells are able to migrate upon antigenic challenge in exposed healthy individuals (7) showing that successful defense against HPV16 infection is commonly associated with the induction of a systemic effector T-cell response against these viral antigens. This notion is sustained by our most recent study showing that the full regression of HPV16-induced high-grade vulvar lesions is strongly associated with the strength of vaccine-induced HPV-specific immunity against these early antigens, as measured by proliferation and cytokine production (8).

A number of relatively small *in vitro* studies on the presence and function of circulating HPV16- or HPV18-specific T cells in patients with HPV16- or HPV18-induced cervical squamous intraepithelial lesion or cancer have suggested that the development of CxCa is strongly associated with failure to mount a strong HPV-specific type 1 T-helper and cytotoxic T lymphocyte response and the induction of HPV-specific regulatory T cells (5;9-11). Furthermore, studies *in situ* suggested that CD8+ T cells may fail to migrate into the tumor cell nests and when tumors are infiltrated by CD8+ T cells it coincides with the infiltration by CD4+ T cells with a regulatory phenotype, as indicated by the expression of intra-nuclear FoxP3. (12) In addition, half of the tumor-infiltrating T cells express the programmed cell death receptor 1 as a sign of T-cell exhaustion (13). Moreover, the ratio between the tumor-infiltrating CD8+ T cells and co-infiltrating CD4+FoxP3-positive T cells is an independent prognostic factor for overall survival (14), indicating the key role of these different types of T cells in HPV-induced diseases such as cervical cancer.

The limited numbers as well as the response rate of patients analyzed in most of the studies so far precludes the assessment of the role or impact of HPV-specific immunity in

those patients with CxCa who did mount such a response. Therefore, we have performed a prospective study in which the HPV-specific proliferative immune response was measured before surgery of the primary tumor in a large group of patients with HPV16- or HPV18-induced CxCa. The presence or absence of an HPV-specific proliferative immune response – for which we previously showed that it correlated with protective immunity (5;8) – was analyzed with respect to several prognostic factors. These include the different HLA class II-alleles that have been suggested to be associated with protection or risk for HPV-related cervical disease (15-18) as it is believed that some HLA-class II molecules may be better or less well equipped to present the HPV protein-derived peptides to T cells. Furthermore, T-cell immunity in relation to tumor-size, depth of stromal invasion by the tumor, lymph node (LN) status – all known tumor characteristics associated with bad outcome (19) - as well as disease free survival was determined.

This prospective study reveals that a minority of the patients have circulating HPV-specific T cells that are able to proliferate when stimulated with cognate E6 or E7 antigen. HPV-specific proliferation is more often detected in patients with deep stromal infiltrating tumors and comprises both HPV-specific helper and CD4+CD25+FoxP3-positive T cells. Patients with deeply infiltrating tumors and an HPV-specific proliferative response present less often with recurrent disease.

MATERIAL AND METHODS

Patients

Women presenting with histologically proven CxCa at the Department of Gynaecology of the Leiden University Medical Center (LUMC) were enrolled in the CIRCLE study after signing informed consent. This CIRCLE study investigates cellular immunity against HPV in HPV-induced (pre)malignant lesions and was approved by the Medical Ethical Committee of the LUMC. Patient characteristics are described in Table 1. All patients underwent radical hysterectomy type III and pelvic lymph nodes (LN) were histologically evaluated for the presence of metastatic disease. Sixty percent of the patients received additional radiotherapy in the months after surgery. The subjects were tested for HPV status on DNA isolated from surgical resection specimens (20). Three years and 5-years follow up data were present for 86 % and 60% of the patients.

Blood samples were drawn at the day of and prior to surgery. PBMC were isolated by Ficoll density centrifugation. A proportion of these PBMC was tested directly ex-vivo in a proliferation assay and the remaining cells were cryopreserved in liquid nitrogen. DNA was isolated from granulocytes for the determination of the HLA class I and II type of the patient at the national reference laboratory for histocompatibility testing (LUMC, The Netherlands) by PCR using sequence-specific oligonucleotides and the IMGT/HLA database v2.24.0 (21).

Antigens and Lymphocyte stimulation test (LST)

Pools of overlapping 22-mer peptides spanning the entire HPV16 or HPV18 E6 and E7 proteins were used for the T-cell assays as described previously (6;22). Memory response mix (MRM), consisting of tetanus toxoid (0.75 limus flocculentius/mL; Netherlands Vaccine Institute), sonicated *Mycobacterium tuberculosis* (5 µg/mL; kind gift from Dr. P. Klatser, Royal Tropical Institute) and *Candida* (0.015%; HAL Allergenen Lab) was used as positive control (6;22).

Table 1 Patient group.

	N	%
No. Patients	119	
Mean age*	44,9	
years (range)	25-76	
FIGO stage		
≥ 1b	107	90
2a/b	12	10
HPV type		
HPV16+	77	65
HPV18+	27	23
other types'	7	6
NT or non detectable	8	7
LN metastasis'		
yes	35	29
no	81	68
unknown	3	3
Tumor size		
< 4cm	64	54
≥ 4 cm	44	37
unknown	11	9
Infiltration depth		
≥ 15mm	36	30
< 15 mm	76	64
unknown	7	6

*At time of intervention 'At time of surgery
'Others include HPV type 31(n=2), 33 (n=1) 39 (n=1), 45 (n=2), 69 (n=1)

The capacity of T cells to proliferate on stimulation with the antigen was determined by the lymphocyte stimulation test (LST) as described earlier (4). The average and SD of the 8 medium-only control wells were calculated and the cutoff was defined as this average plus 3 x SD. The stimulation index 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 of the 8 wells had to be above the cutoff value.

Phenotypical T-cell analysis by flow cytometry

PBMC were thawed and seeded in three wells of a 24-well plate (Costar) at a concentration of $1x10^6$ cells/ml in IMDM supplemented with 10% human AB serum (PAA laboratories). The complete set of overlapping 22-mer peptides of HPV16 E6 or E7 were added at final concentration of 5ug/ml. No peptide (medium only) served as negative control.

At the starting point and after 7 days of culture, cells were harvested, washed in cold PBS/5%BSA and stained for the surface markers CD4-APC, CD25-FITC, CD8-PerCP (BD Biosciences) and subjected to the intra-cellular staining protocol for FoxP3-PE (clone PCH101 Ebiosciences) or the isotype control according to the protocol of the manufacturer.

Two well validated clones, the intranuclear FoxP3- expressing clone C148.31 and the FoxP3 negative clone C271.9 (11;23) were used to discriminate between background staining of the cytosol (C271.9) and true high intra-nuclear staining (C148.31) of FoxP3 because we noticed that the official isotype control displayed less background staining than clone PCH101. The samples were measured by flow cytometry (FACS-CALIBUR, BD Biosiences) and evaluated using Cellquest software (BD Biosciences).

Cytokine 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 IFNγ, TNFα, IL-10, IL-4 and IL-2 were determined. According to manufacturer's instructions the proposed detection limit was 20 pg/ml. However, for IFNγ the cut-off value

was set to 50 pg/ml. Positive antigen specific cytokine production was defined as a cytokine concentration above the cut-off value and at least two-fold above the concentration of the medium control (5).

Statistical analyses

The HLA types of CxCa patients were compared for dedicated alleles with a cohort of healthy blood donors derived from the area of Leiden as published by Schipper et al (24). For each of the tested alleles (DRB1*07, 13 or 15) the frequency present in CxCa patients was compared to the frequency in the control cohort by the 2-tailed Fischer's exact test. Note that no information was available about the linkage of DR and DQ alleles in this control group. The relationship between the presence or absence of an HPV-specific immune response and tumor size, LN status or infiltration depth was determined by the two-tailed Mann-Whitney test. The difference in the mean of CD4+CD25+ or CD4+CD25+Foxp3-expressing cells between patients with or without an HPV-specific proliferative response was determined by a two-tailed Mann-Whitney test. The association between the frequencies of both $CD4^{+}CD25^{+}$FoxP3-positive and $CD4^{+}CD25^{high}$Foxp3-negative T cells simultaneously present in PBMC of patients was tested by the Wilcoxon signed rank test. To determine the impact of the immune response on the survival of patients a logrank analysis using graphpad prism software version 4.02 was performed. Furthermore post-hoc analyses using the logrank test were performed to study the impact of immunity on disease free survival in subgroups of patients. Formal testing of these associations of tumor characteristics and the presence of HPV specific immunity was performed with Cox regression using SPSS version 17.0 software package for windows (SPSS inc. Chicago, USA). A p-value <0.05 was considered to be significant.

RESULTS

Patient population

One hundred and nineteen women with cervical cancer (CxCa; Figo stage 1b to 2b) enrolled in the Circle study onto April 2007 were studied. Patient characteristics are shown in table 1. The mean age of these 119 CxCa patients was 44.9 years (range 23.4 to 76.4 years). The majority (>95%) of the patients were from Dutch origin. PBMC used to determine the presence of HPV-specific T-cell immunity were isolated from blood samples drawn at the same day as but prior to surgery. Tumor tissue of all CxCa patients was analyzed for the presence of HPV-DNA. In 77 cases (65%) HPV16 and in 27 patients (23%) HPV18 was detected. Other virus types were only found at a low frequency and in 8 cases no HPV could be detected (Table 1). As >90% of all recurrences occur within 3 years after surgery (25) and follow-up for this period was available for almost all patients (86%), we analyzed the disease-free 3-years survival after surgery which was 81%, which is comparable to the percentage found previously (25). The group of patients for whom 5-years follow-up was available was too small (60%) to allow for a meaningful analysis.

The frequency of HLA alleles associated with risk or protection for CxCa

Three HLA class II alleles have consistently been reported to be related with either a risk (DRB1*07; DRB1*15/DQB1*0602) or with protection (DRB1*13) against cervical (pre-)malignant lesions (15;17;26). In order to confirm these respective associations in the current cohort – as this allows

Table 2. Expression of specific HLA-DR frequencies in CxCa patients.

	CxCa patients				
HLA[§]	**All n=119**	**HPV16+ n=77**	**HPV18+ n=27**	**Controls[˙]**	
DR* 07	30/89	25/52	3/24	459/1937	** 0,0059[˙]
	25%	**32%****	**11%**	**19%**	
DR* 13	21/98	17/60	4/23	669/1686	* 0,014[˙]
	18%*	**22%**	**15%**	**28%**	
DR* 15	35/84	25/52	6/21	414/1208	
	29%	**32%**	**22%**	**26%**	

[§]The frequency of HLA-DR types previously reported to be associated with disease (DR*07, DR*15) or protection (DR*13) were determined. Numbers indicate the number of patients positive/negative for this specific HLA-type within this group.
[˙]Controls are taken form a published Dutch cohort of healthy blood donors (Schipper et al. 1996) [˙] *P*-value, statistical analysis was performed with Fisher's exact test, two-sided.

a more meaningful analysis of a potential relationship between HPV-specific immunity and these disease-associated or protective alleles – the frequency of these described 'risk' or 'protective' alleles was determined in our study group and subsequently compared to the previously published Dutch control population that consists of more than 1622 healthy individuals (24).

Thirty of the 119 CxCa patients (25%) carried the DRB1*07 allele compared to 19.2% in the control group (p=0.13). When only the group of patients with an HPV16-induced tumor was analyzed the frequency of the DRB1*07 allele was significantly increased (p=0.006; Table 2). The DRB1*07 allele seems to be underrepresented in patients with HPV18-induced cancers but the group is too small to allow for a firm conclusion. The frequency of DRB1*15/DQB1*0602 is slightly but not significantly increased in our patient population (Table 2). Similar to previous observations DRB1*13 was found to be underrepresented in the total group of patients with CxCa as only 18% (21 patients) displayed this allele compared to 28.4% in the control population (p=0.014; Table 2).

A third of the patients with HPV-induced cancer mount HPV-specific immunity

In 94 patients with HPV16- or HPV18-induced cervical cancer we determined the presence of circulating HPV-specific T cells by a 7-day lymphocyte stimulation test (LST) (4;6). We have previously shown that this assay is geared towards the detection of predominantly CD4+ T-cell responses (5;6). Freshly isolated PBMC were stimulated with 4 different peptide pools of the E6 and 2 peptide pools of the E7 oncoproteins for HPV16 and HPV18. The immune response to the cognate HPV type was used to determine the response rate (Figure 1a). Only 29 out of 94 (31%) displayed a demonstrable HPV-specific proliferative T–cell response (Figure 1b; LST+). In 16 out of 23 responding HPV16+ patients, the T cells reacted against E6 and in 14 patients against E7. In 7 cases a response to the peptides of both oncoproteins was found. Six of the 18 HPV18+ patients responded to HPV18 peptides. Three responded only to E6 and 3 to the peptides of E7, including 2 patients who responded both to E6 and E7. Notably, this response rate is much lower as previously reported for healthy controls (5;6;22;27).

Supernatants, obtained at day 6 of these proliferation assays were analyzed for the presence of HPV-specific production of Th1 and Th2 cytokines by cytometric bead array. In only 8 of all 29 patients that showed a HPV-specific proliferative response (28%), this proliferation was

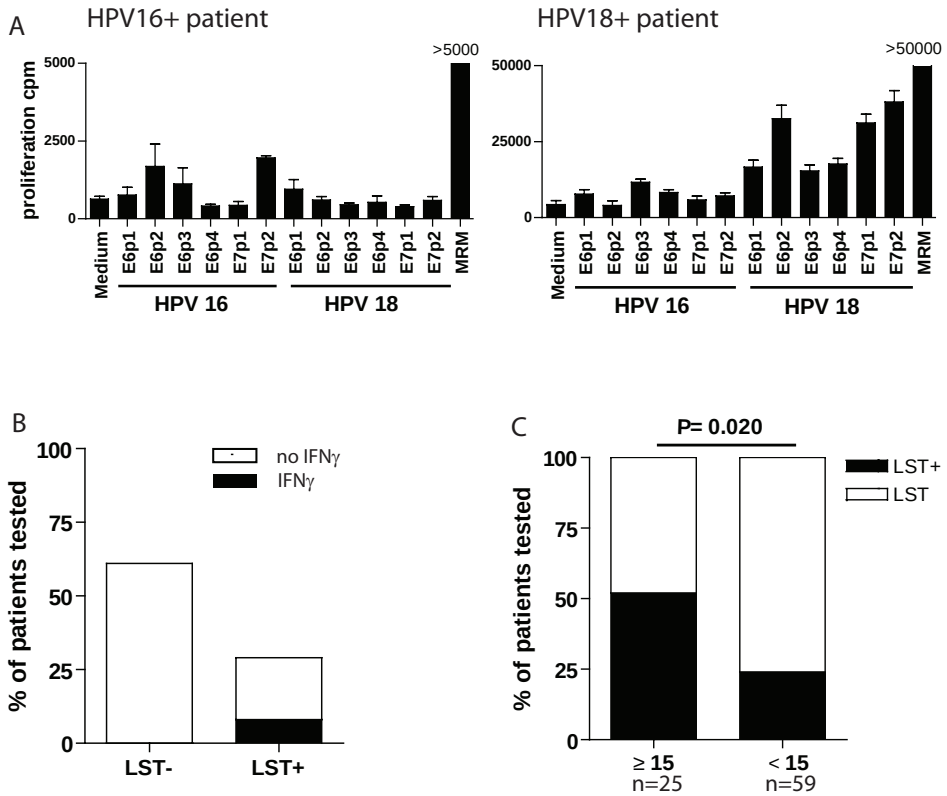

Figure 1 HPV-specific immunity in patients with cervical cancer. (A) PBMC obtained at the day of surgery were stimulated with HPV peptide pools representing either the HPV16 or HPV18 amino acid sequence of the two oncoprotein E6 and E7 (E6 pool 1-4, E7 pool 1-2) or memory response mix (MRM) and after 6 days the proliferation was measured using ^{3}H-thymidine incorporation. Shown is an example of the response detected in an HPV16-positive (left) or HPV18-positive (right) CxCa patient. (B) The percentage of patients (n=65) in whom no HPV-specific proliferative response (LST-) and patients (n=29) who did show an HPV-specific T-cell response (LST+) is depicted. In 8 of these 29 cultures proliferation was accompanied by IFNγ production. (C) The presence of an HPV-specific proliferative T-cell response is associated with deep stromal invasion by the tumor (p=0.02, two-tailed Mann-Whitney). Patients were divided into two groups on basis of the depth of infiltration (≤15mm or >15mm) and the percentage of patients within these groups displaying a systemic HPV-specific proliferative immune responses (LST+) is depicted.

accompanied by the production of detectable amounts of the Th1-effector cytokine IFNγ as depicted in Figure 1b and Table 3. In contrast, IFNγ is detected in two-thirds of the HPV-specific proliferative responses of healthy subjects (4;27). Furthermore, for 3 patients the specific production of IL-10 and in 4 cases TNFα was detected (Table 3). IL-2 was not detected in these cultures which highly likely can be contributed to it's consumption by the proliferating cells as this cytokine was not provided during the culture.

In each assay the positive control recall antigen mix MRM was taken along. This revealed that nearly all of the patients readily responded to bacterial antigens by proliferation and the production of IFNγ (data not shown).

Table 3. Overview of the responses in patients with HPV-specific cytokine production.

						cytokine production**	
patient	**HPV type†**	**Antigen‡**	**peptide pool§**	**SI***	**IFNγ**	**IL-10**	**TNFa**
A	16	E6	121-158	4,7	123	28	25
B	16	E7	51-98	4,6		21	
C	18	E7	1-98	6,7	121		
D	16	E7	51-98	6,4	259		
E	18	E6	41-92	7,6	2943		
		E7	1-62	7,3	2617	64	164
		E7	51-98	8,9	3150	47	67
F	18	E7	51-98	9,2	139		
G	18	E6	1-52	20	3400		56
		E6	41-92	9,5	195		
H	16	E7	51-98	5,8			65
I	16	E7	51-98	6,2	50		
J	16	E6	41-92	7,9	1244		

The 10 patients which in addition to proliferation also produced IFNg, TNF or IL-10 upon HPV-specific stimulation are depicted by a letter (A-J)
†HPV type as found in the tumor by PCR
‡indicates the antigen (E6 or E7) of the HPV type present in the tumor to which the patient responded
§the numbers indicate the first and the last amino-acid of the 22-mer peptide pool of the corresponding antigen to which the PBMC responded by proliferation
*SI=Stimulation index, the fold difference in proliferation when PBMC are stimulated with the indicated peptide pool when compared to PBMC stimulated with medium only
** Specific cytokine production (pg/ml) by PBMC stimulated with the indicated peptide as detected in the pooled supernatant of the 8 replicate test wells;
A specific response was defined as a peptide pool-induced production which was at least twice above background (medium control) and above the cut-off of 20 pg/ml (IL10 and TNFa) or 50 pg/ml (IFNγ)
Only the cultures with a positive cytokine response are shown

The detection of HPV-specific CD4+CD25+FoxP3-positive T cells coincides with that of HPV-specific CD4+CD25+ T cells

Previously we showed that CD4+CD25+Foxp3+ HPV-specific regulatory T cells can be isolated from tumors and lymph nodes of patients with CxCa (11) as well as can be detected in their blood as measured by the co-expression of the intra-nuclear transcription factor FoxP3 and the HPV-specific activation induced upregulation of CD25 ten days after antigen-specific stimulation (28). Although functional assays are the golden standard for the classification of Treg the intra-nuclear transcription factor FoxP3 is the best marker to date(29).Using this approach we determined the frequency of HPV-specific CD4+CD25+FoxP3-positive T cells in 41 patients with HPV16+ CxCa of whom enough PBMC were available. Two previously described HPV-specific regulatory T-cell clones (11;23) were used to set up a stringent gating strategy to ensure the enumeration of $CD25^{high}$ and $FoxP3^{high}$ cells only (Figure 2).

On average 3.4% of the peripheral CD4+ T cells expressed FoxP3 directly *ex-vivo* (Figure 2a), similar to observations in other types of cancer (30). Notably, non-stimulated PBMC cultured for 7 days comprised similar frequencies of FoxP3 expressing CD4+ T cells as PBMC stained *ex-vivo* (Figure 2a), demonstrating that our culture conditions do not induce non-specific FoxP3 expression.

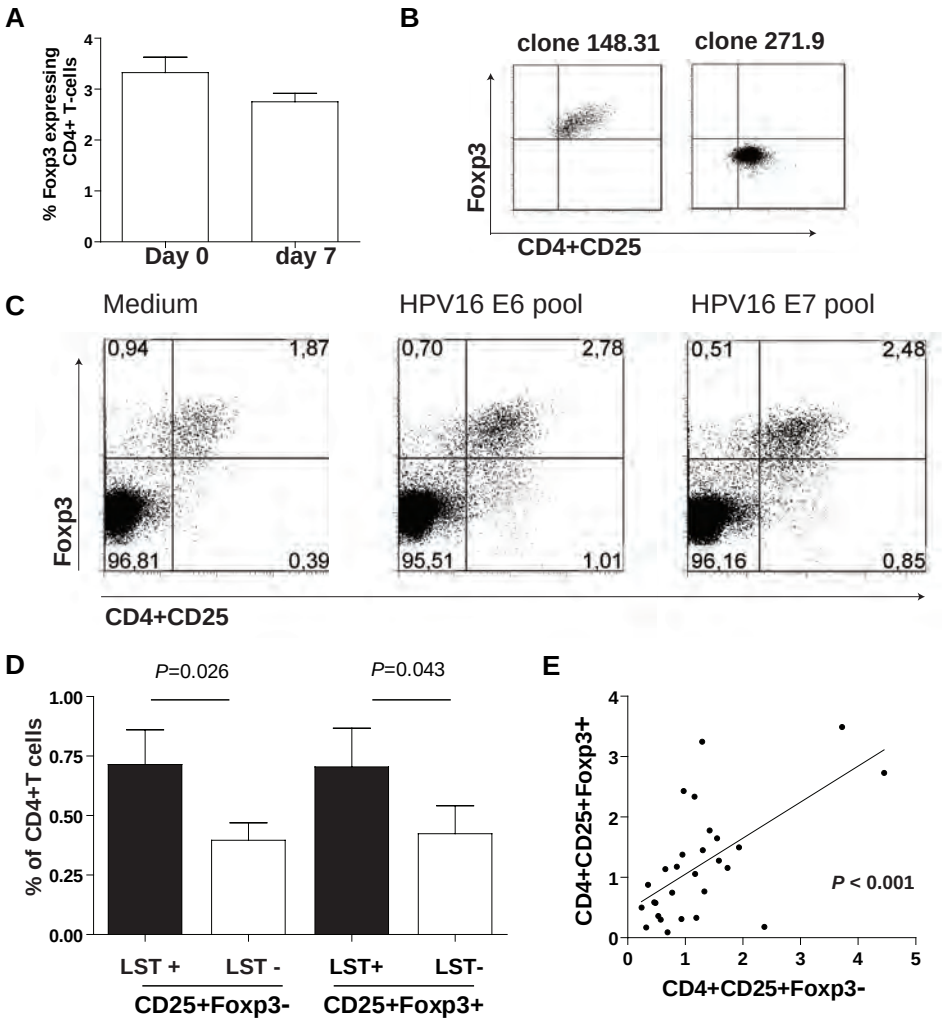

Figure 2 The detection of HPV-specific proliferative responses is associated with higher percentages of HPV-specific FoxP3-positive and-negative CD4+CD25+ T cells. PBMC of HPV16+ CxCa patients isolated before surgery were cultured for 7 days in the absence or presence of the cognate HPV peptides E6 or E7 and stained for the expression of CD25 and the transcription factor FoxP3. (A) On average 3% of the circulating CD4+ T-cells express FoxP3 directly ex-vivo (left bar) and this percentage is not altered when the T cells are cultured for 7 days in medium (right bar). (B) Two HPV-specific T-cell clones, either expressing intranuclear FoxP3 (148.31) or not (271.9) were used to optimize the gating strategy in the experiments. (C) Dot plot example of one HPV16+ patient showing CD4+CD25+ T-cells and CD4+CD25+FoxP3-positive T cells without stimulation (medium) or after stimulation with HPV16 E6 (middle) or E7 (right) as measured by flow cytometry. (D) Increased percentages of HPV-specific CD25+ (activated) and CD25+FoxP3-positive CD4+ T cells are found when patients display specific proliferation in the blood as compared to patients that do not show any T-cell response. The mean (±SEM) percentage of HPV E6- and E7-specific CD4+CD25+ or CD4+CD25+FoxP3-positive T cells is depicted for the group of patients lacking HPV-specific proliferation (LST-; n=27) or displaying HPV-specific T-cell proliferation (LST+; n=14). Means are calculated from stimulated cells after subtraction of PBMC in medium only. P-values were calculated using two-tailed Mann-Whitney. (E) In LST+ patients the detection of both types of HPV-specific CD4+ T-cells is correlated as analyzed by the Wilcoxon signed rank test.

Of the 41 patients tested, 14 displayed an HPV-specific proliferative response while the other 27 patients displayed no detectable proliferative response. Notably, CD4+CD25+FoxP3-positive T cells were already present in non-stimulated cell cultures (medium). The population of $CD25^{high}FoxP3^{high}$ T cells consisted on average of 0.83% (range 0.1-3.0) of total CD4+ T cells, whereas CD4+ T cells with a helper phenotype ($CD4+CD25^{high}$FoxP3-negative) were scarcely present in these control cultures (Figure 2). In the HPV peptides-stimulated cultures, however, both populations are present in the majority of the samples (Figure 2c). Significantly higher numbers of HPV-specific $CD4+CD25^{high}$FoxP3-negative and $CD4+CD25^{high}FoxP3^{high}$ T cells were detected in the HPV-stimulated PBMC cultures of the group of patients whom displayed an HPV-specific proliferative response (Figure 2d, p= 0.03 and p=0.04, respectively), when compared to the group of patients not able to mount an HPV-specific proliferative response. Moreover, the detection of HPV-specific T cells with either a $CD4+CD25^{high}$FoxP3-negative Th-phenotype or with a $CD4+CD25^{high}FoxP3^{high}$ T-cell phenotype was correlated in the patient group displaying HPV-specific proliferative responses ($p<0.001$; Figure 2e). No correlation between the presence of LN metastasis and the numbers of CD4+ $CD25^{high}$FoxP3-negative and $CD4+CD25^{high}FoxP3^{high}$ cells was found.

Notably, although recently CD8+FoxP3+ T cells were detected in the lymph nodes of early stage CxCa patients (31), we could not detect such a population in the PBMC of our CxCa patients (data not shown).

Deep tumor-infiltration of the surrounding tissue is associated with detectable HPV-specific immune responses

In order to assess whether relationships exist between the absence or presence of HPV-specific proliferative responses and the known disease related or protective HLA class II alleles the patients were divided according to immune status and presence or absence of a particular HLA allele (DRB1*07, DRB1*15 or DRB1*13). However, no relationship between the absence or presence of HPV-specific immunity and the presence of these alleles was found.

Known prognostic factors in cervical cancer are LN metastases (present or absent), the size of the tumor (< or ≥ 4cm) and the invasion depth of the tumor in the surrounding cervical tissue (< or ≥ 15mm) (19). These prognostic factors were entered into the Cox proportion hazard model, which revealed a correlation between tumor size and the presence of LN metastases ($p=0.003$) as well as with invasion depth of the tumor ($p=0.002$). However, depth of invasion was not correlated to LN metastasis in this cohort ($p=0.21$).

Subsequently, the presence of an HPV-specific immune response was also analyzed in relation to the LN status in 93 patients, tumor size (n=82) and invasion depth (n=84). Patients were grouped according to the presence or absence of an HPV-specific T-cell response. No differences were found between these groups with respect to tumor size at time of surgery.

In 48% of the patients with nodal metastases HPV-specific T cells were detected, whereas only 27% of the patients without metastasizing tumors displayed an HPV-specific T-cell response ($p= 0.06$; Fisher's exact two sided, data not shown). Interestingly, the group of patients with a deeply infiltrating tumor of at least 15 mm, significantly more often display a detectable T-cell response than patients with less invasive tumors (Figure 1c, $p=0.02$; Fisher's exact two-sided).

Patients with deep infiltrating tumors and HPV-specific immunity display improved survival

The great majority of recurrences occur within 3 years after surgery (25). When the disease-free 3-year survival curves of the CxCa patients are plotted on the basis of the aforementioned prognostic factors or the presence of HPV-specific immunity (Figure 3), the patients with large tumors (≥ 4 cm) are prone to have recurrent disease (Figure 3c, 37% vs 18%, p=0.02). In contrast to patients with less deep stromal invasion relatively more patients with a deeply infiltrating tumor (≥ 15mm) displayed a recurrence of disease within 3 years (Figure 3d 20% vs 35%; p=0.14). Survival was not different when all patients were grouped according to HPV-specific immune status (p=0.45; Figure 3a).

As we have found that HPV-specific immunity predominantly was detected in patients with deep infiltrating tumors, a subgroup analysis was performed in which the patients were divided according to the presence of an HPV-specific proliferative response and one of the prognostic parameters. These analyses suggested that the presence of HPV-specific proliferative T cells

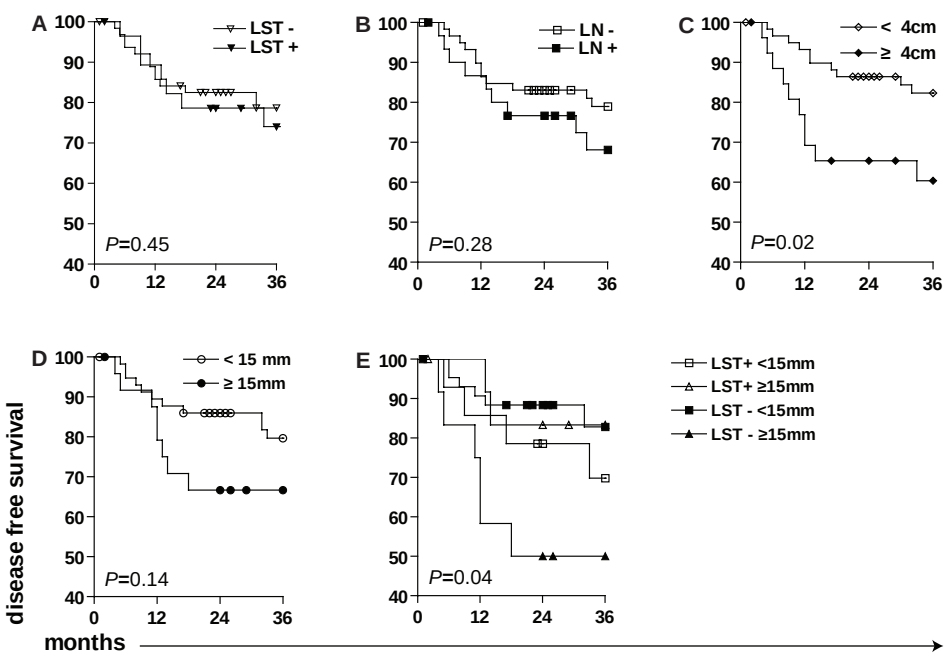

Figure 3. Disease free 3-year survival curves. Disease free survival for the patients is plotted when patients are divided on basis of (A) the presence of an detectable immune response by LST (LST-: open symbols n=64, LST+:closed symbol n=29 (B) the presence or absence of LN metastasis (LN-: open symbols n=60, LN+: closed symbols n=31), (C) a prognostic-defined relevant tumor size (< 4cm: open symbols n=60, ≥ 4cm: closed symbols n=27), or (D) a prognostic-defined relevant depth of stromal invasion (< 15mm: open symbols n=58, ≥ 15mm: closed symbols n=25). (E) Patients are grouped on the presence or absence of an immune response and the depth of infiltration. Notably, the group of patients with deeply infiltrating tumors is divided into 13 patients (open triangles) displaying an HPV-specific proliferative response and 12 patients lacking a detectable response (closed triangles). Symbols represent censored patients. P-values are determined by logrank analysis.

Table 4. Multivariate Cox regression analysis of immune response and invasion depth on risk of recurrence.

Immune response	Infiltration depth	n	HR	95% CI	*p*-value
LST -	< 15 mm*	44	1		
LST -	≥ 15 mm	12	4,33	1,45 - 12,96	0,01
LST +	< 15 mm	14	2,21	0,70 - 6,97	0,18
LST +	≥ 15 mm	13	1.178	0,24 - 5,68	0,84

HR, Hazard ratio; CI, confidence interval; * Reference category
LST- indicates no HPV-specific proliferation by lymphocyte stimulation test
LST+ indicates a HPV-specific proliferation by lymphocyte stimulation test

(LST+) is beneficial in patients with deep (≥ 15mm) infiltrating tumors as the percentage of patients with recurrence was much lower in this group than in those patients without a detectable HPV-specific proliferative response (Figure 3e 18% vs 50%; p=0.043, logrank test). Notably, all patients with invasion of ≥ 15 mm received radiotherapy as additional treatment. Analyses of HPV-specific immunity in relation to LN status or tumor size did not reveal any differences (not shown).

Multivariate analyses of these four groups by Cox proportion hazard analysis confirmed these observations (Table 4). While deep stromal invasion without a demonstrable immune response showed a highly increased disease recurrence risk (HR 4.33, 95% CI 1.45-12.96) as compared to having a less invasive tumor and no immune response, the presence of a detectable HPV-specific proliferative response was associated with a lower risk of recurrence in the patient group with deeply invading tumors (HR 1.18, 95% CI 0.24-5.68).

DISCUSSION

This is the largest prospective study of women with HPV-16 or -18-induced cervical cancer in which the HPV-specific immune response was analyzed in relation to known factors predicting disease prognosis. The PBMC of most (69%) of the 94 tested patients failed to proliferate when stimulated with cognate HPV E6 or E7 peptides *in vitro*. Furthermore only in a limited number of patients HPV-specific T cell responses were associated with the production of IFNγ. HPV-specific immunity - in patients who showed an HPV-specific proliferative response - consisted of circulating FoxP3-negative and –positive CD4+C25+ T cells. This large study confirms previous findings in small groups of patients (5;32-34). Moreover, it allows a more definitive conclusion with respect to the response rate and the type of the T-cell response to the tumor-specific HPV E6 and E7 antigens in patients with CxCa as well as scrutiny of the relationship between HPV-specific immunity and tumor characteristics or survival.

Interestingly, the presence of circulating HPV-specific proliferative T cells was associated with deep infiltration of the tumor in the surrounding normal tissue (p=0.02). Recently, we reported that surgery-mediated tissue destruction of lesions was strongly associated with the induction of HPV-specific immunity in patients with HPV16+ HSIL (10). Together these data suggest that the induction of HPV-specific immunity in patients with HPV-induced established cervical neoplasia is the result of local destruction of normal tissue and contact with antigen presenting cells therein, allowing the presentation of HPV antigen and the activation of naïve

or memory HPV-specific T cells. Possibly, this normally does not occur because of the tumor-induced lack of mobility and tolerogenic phenotype of DC within cervical tumors (35;36). The fact that tumor size is not related to the induction of HPV-specific immunity can be explained because generally tumors increase in size by growing into the lumen of the cervix.

Because the number of patients within our cohort was too low to perform an unselected HLA-association study, we chose to study the frequency of only three HLA class II alleles which have consistently been reported to be related with either a risk or with protection against cervical (pre-) malignant lesions. The combination of the two HLA class II alleles DRB1*13-DQB1*0603 was found to be protective in 9 out of 19 studies reviewed by Hildesheim (17). It is unclear which allele is important since DRB1*1301 and DQB1*0603 are in linkage disequilibrium. In our cohort DRB1*13 was also found to be underrepresented (p=0.014) when compared to a large group of Dutch controls (24). Furthermore, the allele DRB1*07 was found to be associated with an increased risk for the development of CIN lesions in Dutch patients (15). Here, we show that this allele is also present at a significantly higher frequency (p=0.006) in our group of patients with HPV16+ CxCa sustaining the observation that DRB1*07 is associated with a risk to develop HPV16-induced malignancies. The mechanism through which this may operate may relate to the absence or type of CD4+ T-cell response induced but we did not find a relationship between HPV-immunity and these HLA types in our cohort of cancer patients. The events -associated with protection or failure- possibly occur already at an early stage of the infection and as such, are no longer demonstrable in patients with cancer.

The size and design of our study, in which patients were followed up after surgery, allowed us to study the impact of HPV-specific immune responses in relation to disease-free survival at 3 years. We did not find a direct correlation between survival and the presence of circulating tumor-specific T cells. A similar study in patients with melanoma also failed to detect a direct correlation (37), albeit that there only the numbers and not the function of the tumor-specific T cells was studied. Here, the presence of HPV-specific T cells was determined by function (proliferation). Interestingly, subgroup analyses revealed that those patients with deeply infiltrating tumors – who in general display a low survival rate (67%, Figure 3d) – and circulating HPV-specific T cells able to proliferate when stimulated with E6 or E7 antigen, displayed a better 3-year disease free survival (82%) than patients lacking such an immune response (50%; p=0.043). Also, Cox proportion hazard analysis of these four subgroups showed a strong reduction of the risk of disease recurrence, in that the hazard ratio decreased from 4.3 to 1.2, when patients with deeply infiltrating tumors displayed an HPV-specific proliferative response (Table 4). Notably, both groups of patients comprised predominantly FIGO stage 1b tumors and all patients had received additional treatment with radiotherapy, excluding this as a variable. A recent study suggests that the presence of HPV-specific immunity reflects disease severity and is not associated with increased 3-year disease free survival in a small group of 32 patients (32).This is likely related to the stage of disease, as the group studied consisted mainly of patients with a more advanced stage of disease (FIGO 2b-3b), whereas we studied patients with an earlier stage (FIGO 1b-2b) of disease. We find an increase in proliferative responses in patients with LN metastasis when the subgroup analyses were performed on the basis of absence or presence of LN metastasis. It is described that T cells within tumor-draining metastasized LN might display a more suppressive phenotype suggesting that these suppressive T cells may suppress tumor-specific immunity (31). However, we did not observe differences in the phenotype of the PBMC of patients with LN metastatic

disease or without metastatic disease. Survival benefit of the presence of a HPV-specific response thus may be very specific for the group of patients with deeply infiltrating tumors.

The picture that emerges from these data is that during progression of tumors, as evidenced by the deeper infiltration of tumors in the normal surrounding tissue, HPV antigens can be taken up and presented by antigen presenting cells most likely in the less suppressive context of the adjacent tissue. This results in activation of HPV-specific T cells, defined by their capacity to either proliferate or suppress HPV-specific T cells. The latter is also supported by others who report that HPV-specific immunity coincides with enhanced levels of regulatory T cells (38;39). Also in melanoma the presence of circulating antigen-specific regulatory T cells has been reported (40).

A key effect of HPV-specific regulatory T cells is the suppression of proliferation and the production of cytokines by effector cells, including IFNγ (11). The low number of HPV-specific responses associated with the production of IFNγ suggests that such responses may have been suppressed. However, the detection of HPV-specific proliferation in 31% of the patients implies that if suppression occurs this is not always strong enough to suppress T cell function completely and HPV specific T cells can have a beneficial antitumor effect as the 3-year disease free survival of patients with deeply infiltrating tumors displaying an immune response is better than those without such a response.

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