Immunology and Immunotherapy of high grade cervical lesions and cancer

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Chapter 2

Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16 specific T-cell response

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ABSTRACT

Purpose: To characterize HPV16 E6- and E7-specific T-cell immunity in patients with high-grade squamous intraepithelial lesions (HSIL).

Experimental design: PBMC isolated from 38 patients with HPV16+ HSIL were used to determine the magnitude, breadth and polarization of HPV16-specific T-cell responses by proliferation assays and cytokine assays. Furthermore, HSIL-infiltrating T-cells isolated from 7 cases were analyzed for the presence of HPV16 E6 and/or E7-specific T-cells, phenotyped and tested for the specific production of IFNγ and IL-10 as well as for their capacity to suppress immune responses.

Results: HPV16-specific T-cell responses were absent in the circulation of the majority (~60%) of patients who visit the clinic for treatment of an HPV16+ HSIL lesion. Notably, HPV16-specific T-cell reactivity was predominantly detected in patients returning to the clinic for repetitive treatment of a persistent or recurrent HPV16+ HSIL lesion after initial destructive treatment. The majority (>70%) of these HPV16-specific T-cell responses did not secrete pro-inflammatory cytokines, indicating that most of the subjects, although in principle able to mount an HPV16-specific immune response, fail to develop protective cellular immunity. This notion is sustained by our observation that only 3 HSIL-infiltrating T-cell cultures contained HPV16-specific T-cells, one of which clearly consisted of HPV16 E7-specific regulatory T-cells.

Conclusions: The presence of HPV16-specific T-cells with a non Th1/Th2 cytokine and even suppressive signature in patients with HSIL may affect the outcome of vaccine approaches aiming at reinforcing HPV-specific immunity to attack HPV-induced lesions.

INTRODUCTION

Cervical cancer is preceded by well defined stages of changes in the epithelium known as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL), which are caused by persistent infection with human papillomavirus (HPV). Changes that affect more than one-third of the epithelium are diagnosed as CIN 2 or 3 or high-grade SIL (HSIL). These HSIL have a high chance of progressing to cancer if left untreated (1). Most HSIL are associated with the presence of a high-risk HPV type, in particular HPV type 16 (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).

The key role of the adaptive cellular immune system in the protection against HPV-induced lesions is indicated by the high incidence of persistent HPV-infections and subsequent HPV-related malignancies in immunosuppressed individuals (4) as well as by the fact that only a fraction of infected subjects develop progressing epithelial lesions or cancer (5). Since HPV proteins are foreign to the body, one would expect the immune system to mount a response against these antigens when expressed in the cervical epithelium. Indeed, HPV16 E6, E7 and E2-specific Th1- and Th2-type CD4+ T-cell responses were frequently detected in PBMC cultures of healthy individuals (6-8) and both HPV16-specific CD4+ and CD8+ T cells are able to migrate upon antigenic challenge {van den Hende, 2008 176 /id} showing that successful defense against HPV16 infection is commonly associated with the installment of a systemic effector T-cell response against these viral antigens.

The local microenvironment in an HSIL is associated with an increase in IL-10 production and a decrease in proinflammatory cytokines (10-13), which poses a harsh milieu for the immune system and is likely to affect both the systemic and local immune response. Indeed, patients with HSIL show evidence of non-specific suppression of type 1 T-helper cell cytokine production (11;14) but how this affects the priming and the character of an HPV-specific immune response is only slowly elucidated. So far, the presence of HPV-specific immunity has either been studied in a number of really small cohorts of patients with HSIL (7;15-21), or in somewhat larger cohorts that focused on a highly restricted set of antigenic peptides (22), and/or studies using only one single immunological parameter (23-26) to decide whether a response is present. Unfortunately, huge differences between these studies in design and outcomes complicate the development of a unifying picture on how HPV-specific immunity and HSIL co-evolve.

So-called therapeutic vaccines are being developed for the treatment of individuals who contracted a high-risk type of HPV and were unable to control the viral infection - as dem-

onstrated by the presence of an HPV-induced (pre-)malignant lesion (27-31). A number of therapeutic vaccines have been tested in patients with cervical and non-cervical high-grade genital lesions, but with modest success (32-38). Since these therapeutic vaccines aim at reinstating an effective T-cell response against HPV16 E6 and/or E7 it will become very important not only to know the presence of pre-existing HPV16-specific T-cell immunity in patients with HSIL but also to understand the functionality, as this may bear impact on vaccine efficacy (39;40).

Here we have studied the presence and function of spontaneously induced HPV16-specific T cells in a large group of patients with HPV16+ HSIL. HPV16-specific proliferative T-cell responses were detected in less than half of the patients and the majority of the responses were not associated with the production of IFNγ or other pro-inflammatory cytokines. Notably, even HPV16-specific regulatory T-cells could be isolated from HSIL tissue. The induction of these HPV-specific T-cell responses most likely is the product of surgical treatment with recurrence or persistence of disease.

MATERIALS AND METHODS

Patient inclusion and sample collection

Patients visiting the colposcopy clinic at the department of Gynaecology of the Leiden University Medical Centre or the Haga Teaching Hospital in The Hague were recruited in the CIRCLE study, which investigates cellular immunity against HPV16- positive cervical lesions. The study design was approved by the Medical Ethical Committees of the Leiden University Medical Center and the Haga teaching hospital. Patients were eligible for the current study if they had a histologically proven cervical intraepithelial neoplasia at the time of diagnostic colposcopy or loop electrical excision procedure (LEEP). Notably, patients with chronic HSIL were more motivated to participate in this study hence this group pf patients is somewhat overrepresented in our cohort. Informed consent was obtained from all patients. Seventy ml of blood was drawn on the day prior to LEEP. Serum was obtained and peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood samples by Ficoll (Sigma) density centrifugation for the analysis of HPV-specific T-cell reactivity. In a number of cases tissue from the lesion was obtained for research purposes.

HPV typing

Patients with a HSIL were typed for HPV on paraffin-embedded sections of biopsies using 3 general HPV primer sets (CPI/II [1], MY 9/11[2], GP 5+/6+ [3]) followed by sequencing. Sequencing results were analysed by the NCBI BLAST programme. As controls beta-globine PCR and a blank sample were included (41-43).

Antigens

A set of 22 amino acid long peptides, overlapping by 12 amino acids, and indicated by the first and last amino acid in the sequence of the E6 and E7 protein of HPV16 (e.g. E6.1-22 and the last peptides E6.137-158 and E7.77-98), were used for the screening of T-cell responses. The peptides were mixed into four pools of E6 peptides and two pools of E7 peptides (i.e. E6.1 - E6.4, E7.1 and E7.2). These pools consisted of four 22-mer peptides. Notably, peptide pools E6.3 and E6.4 both contained peptide E6.111-132, whereas peptide pool E7.2 harbored the last five peptides of HPV16 E7. The peptides were synthesized and dissolved as described previously (44). Memory response mix (MRM) consisted of tetanus toxoid (0.75 Limus Flocculentius per ml; Netherlands Vaccine Institute, Bilthoven, The Netherlands), sonicated Mycobacterium tuberculosis (5 μg/ml; kind gift from Dr. P. Klatser, Royal Tropical Institute, Amsterdam, The Netherlands) and Candida (0.015%, HAL Allergenen Lab, Haarlem, The Netherlands). The response to MRM was used as positive control in the assays (8) to confirm the capacity of the antigen presenting cells that are present in PBMC to process and present antigens to memory T-cells.

Proliferative capacity of HPV16-specific T-cells by lymphocyte stimulation test (LST)

The capacity of T-cells to proliferate upon stimulation with the antigen was determined by short-time proliferation assay as described earlier (6;8;45). Briefly, freshly isolated PBMC (1.5×10^5) were seeded into 8-replicate wells of a 96-well U-bottom plate (Costar, Cambridge, MA) to which the indicated peptide pools were added at a final concentration of 10 μg/ml. Medium without antigen served as background control and MRM was taken along as a positive control. The test was conducted in IMDM (BioWhittaker, Verviers, Belgium) containing 10% autologous serum. On day 6, supernatant was harvested for cytokine analysis and subsequently the cells were pulsed with 0.5 μCi [3H]Thymidine (Pelkin Elmer, Boston, USA) per well and incubated for an additional 18 hours. Then, the cells were harvested onto filters (Wallac, Turku, Finland) using the Micro-cell Skatron harvester (Skatron Instruments AS, Lier, Norway) and counted on the 1205 Betaplate counter (Wallac, Turku, Finland). The average and standard deviation of the 8 medium only control wells were calculated and the cut-off was defined as this average plus 3xSD. The stimulation index (SI) was calculated as the average of tested 8 wells divided by the average of the medium control 8 wells. A positive proliferative response was defined as a stimulation index of at least 3 and the counts of at least 6 out of the 8-wells must be above the cut-off value (7).

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-5, IL-4 and IL-2 were determined. According

to firm prescription the proposed detection limit was 20 pg/ml. However, for IFNγ the cut-off value was set to 100 pg/ml because the standard curve showed linearity starting at a concentration of 100 pg/ml. Positive antigen specific cytokine production was defined as a cytokine concentration above the cut-off value and >2x the concentration of the medium control (7).

Culture of CIN Infiltrating Lymphocytes

CIN Infiltrating Lymphocytes (CIL's) were isolated and cultured as described previously (46). Briefly, CIL cultures were expanded using a mix of irradiated autologous Epstein-Barr virus transformed B cell lines (B-LCL) and 5 ug/ml cognate peptide in IMDM, supplemented with 10% human AB serum (PAA laboratories, Pasching, Austria), 10% T-Cell Growth Factor (TCGF, Zeptometrix, Buffalo NY, USA) and 5 ng/ml recombinant human IL-15 (Peprotech, Rocky Hill NJ, USA).

Analysis of T-cell specificity

T-cell cultures (25,000-50,000 cells/well) were stimulated with autologous monocytes or irradiated autologous B-LCL's pulsed with their cognate peptide (ID2 HPV16 E7.71-92; ID23 HPV16 E7.51-72; 5 μg/ml) (47) and protein (10 μg/ml) in triplicate wells in a 3-day proliferation assay. After 48 hours supernatant was harvested and stored at -20°C for cytokine analysis. Antigen-specific IFN-γ and IL-10 production was measured by ELISA as described earlier (19).

Detection of CD4+ CD25+Foxp3+ T-cells

HPV16-specific CIL lines were stained 3 weeks after their last antigen-specific activation *in vitro* first for surface markers CD25 (anti-CD25 FITC; clone M-A251, BD Pharmingen), and CD4 (anti-CD4-APC; clone RPA-T4, BD Pharmingen) before the cells were fixed and permeabilized. Blocking was performed with 2% normal rat serum followed by the addition of anti-human Foxp3 (PCH101, eBiosciences, San Diego, CA) antibody or rat isotype IgG2a control. Then the cells were washed and analyzed by flowcytometry. As a positive control a previously isolated HPV16-specific CD4+CD25+Foxp3+ regulatory T-cell clone (C148.31) and as negative control an HPV16-specific CD4+CD25+Foxp3- T-cell clone (C271.9) (47) were used. The fluorescence intensity of these two control clones was used to set the gates for the other samples in which the CD25+Foxp3+ expression of the stimulated polyclonal T-cell populations were analyzed.

HPV16-specific T-cell Suppression Assay

T-cell suppression assay's were performed as described previously (47). Briefly, the CIL lines were co-cultured with allogenic CD4+CD25- responder cells in the presence of 1 μg/ml agonistic anti-CD3 (OKT-3; Ortho Biotech, Bridgewater, NJ) and APC mixture of 5

different B-LCL cell lines. Suppression of the responder cells was analyzed on proliferation and IFNγ production as described previously (47). HPV16 antigen-dependent suppression was measured using a flowcytometry based proliferation assay. Responder cells were labeled with CFSE and co-cultured with PKH -26 labeled CIL lines at a 1:1 ratio. The responder cells were stimulated with a pool of 5 allogenic B-LCLs, the HPV-specific CIL lines were stimulated with 5 ug/ml cognate peptide and autologous B-LCL in the presence of IL-2 (300 IU/ml). After 4 days of culture, the allo-specific proliferation of responder T-cells was analyzed by flowcytometry. HPV-specific CIL lines were treated with 50 μg/ml mitomycin C (Kyowa, Hakko, Japan) for 1 h followed by irradiation (2,000 rad) to prevent proliferation but not effector function.

Statistical analyses

In order to evaluate the effect of a previous treatment for high-grade CIN and persistence of the lesion afterwards on HPV-specific immunity, the patients were divided into two groups. The group of patients with a persistent lesion after treatment consisted of patients who had already undergone a surgical treatment for a HSIL after which the lesion persisted at least for 8 months (range 8-72 months) as indicated by the detection of Pap3a or higher in follow-up smears, or a HSIL at follow-up colposcopy for which these patients all had to undergo a second surgical treatment at the time that blood was drawn for the detection of HPV-specific immunity. Patients without a persistent infection where defined as patients who had no prior treatment before the drawing of blood for the immunological assay. These groups were then subdivided into patients who did show an HPV-specific immune response or in whom no specific immune response was detected and analyzed by a two-sided Fisher's exact test.

RESULTS

Patients and HPV distribution

During a period of 5 years a total of 74 patients with a HSIL were included in this study, 16 patients who were diagnosed with a CIN2 and 58 with a CIN3 (Table 1). The median age was 38 years with a range of 24-68 years. HPV typing revealed that 60 patients (81%) were HPV 16 positive and 2 (3%) were HPV 18 positive. In 11 patients (15%) another HPV type was found (HPV 45, 33, 31) and 1 patient was HPV negative (Table 1).

Patients with HSIL fail to induce a strong HPV16 E6- and E7-specific T-cell response

From 38 HSIL patients (Table 2) freshly isolated PBMC were stimulated with peptides derived from HPV16 proteins E6 and E7 as well as with a mix of common recall antigens

Table 1. HPV distribution in HSIL patients

Patients included	***N* = 74**
Median age (range)	38 (24-68)
Histology CIN	
CIN 2	16
CIN 3	58
HPV typing (%)	
HPV16	60 (81)
HPV18	2 (3)
HPV45	3 (4)
HPV33	1 (1)
HPV31	7 (10)
HPV$^-$	1 (1)

Table 2. Summary of patient characteristics and HPV16-specific immunity

ID	**Age**	**Histology**	**Treatment at time of analysis**	**Previous treatment**	**LST***	**CIL (antigen)** †	**Follow-up**	
							6 mo	**12 mo**
1	28	CIN 2	LEEP		-		Pap 1	Pap 1
2	36	CIN 2	LEEP		-	CIL (E7)	Pap 1	
3	45	CIN 2	LEEP		-		CIN 3	
4	33	CIN 2	LEEP		-		Pap 1	Pap 1
5	40	CIN 2	Conization		-		Pap 2	Pap 2
6	61	CIN 2	LEEP		-			
7	41	CIN 2	LEEP		-		Pap 3a	Pap 2
8	29	CIN 2	LEEP		+		Pap 1	Pap 1
9	35	CIN 2	no treatment		+		Pap 3a	CIN 3
10	43	CIN 2	Conization	LEEP 2×	+		Pap 2	Pap 2
11	35	CIN 3	LEEP		-		Pap 1	Pap 1
12	30	CIN 3	LEEP		-		Pap 1	Pap 2
13	31	CIN 3	Conization	LEEP	+		Pap 1	
14	33	CIN 3	LEEP	LEEP	+		Pap 1	Pap 1
15	31	CIN 3	LEEP		-		Pap 1	Pap 1
16	35	CIN 3	LEEP		-		Pap 1	Pap 1
17	29	CIN 3	Conization		-		Pap 3b	CIN 3
18	61	CIN 3	LEEP		-	CIL	Pap 2	Pap 1
19	27	CIN 3	LEEP		-		Pap 1	
20	43	CIN 3	LEEP		-			Pap 1
21	35	CIN 3	LEEP		-	CIL (E7)		Pap 1
22	25	CIN 3	LEEP		-		Pap 3a	Pap 3a
23	26	CIN 3	LEEP		-	CIL (E7)	Pap 1	Pap 1
24	44	CIN 3	LEEP	LEEP	-		Pap 1	Pap 1
25	35	CIN 3	LEEP		-		Pap 1	Pap 3b
26	41	CIN 3	LEEP		-		Pap 1	
27	51	CIN 3	LEEP		-		Pap 1	
28	41	CIN 3	LEEP	LEEP	+		Pap 3a	
29	42	CIN 3	LEEP		-			
30	34	CIN 3	LEEP	LEEP	+		Pap 3a	Pap 3a
31	52	CIN 3	Conization	LEEP	+		Pap 3a	Pap 1
32	36	CIN 3	Conization	LEEP 2×	+		Pap 2	Pap 2
33	27	CIN 3	LEEP		+	CIL	Pap 1	Pap 1
34	46	CIN 3	LEEP		+	CIL	Pap 1	
35	47	CIN 3	Conization	LEEP	+	CIL	Pap 1	Pap 1
36	45	CIN 3	LEEP		+		Pap 3b	CIN 3
37	34	CIN 3	LEEP		+		Pap 3a	Pap 1
38	39	CIN 3	No treatment	LEEP	+		Pap 1	Pap 1

*Lymphocyte stimulation test; PBMC of patients failed to respond (-) or did proliferate (+) on stimulation with HPV16 E6 and/or E7 peptide pools.
† From this patient, a biopsy from the HSIL lesion was cultured. When the infiltrating T cells were found to react to either HPV16 E6 or E7 in a 3-d proliferation assay, the antigen recognized (E6 or E7) is specified in parentheses. Diagnosis at 6 and 12 mo follow-up by Pap smear or by histology is indicated, when available.

(MRM) in a short-term proliferation assay (Figure 1A). We have previously shown that this assay is geared towards the detection of CD4+ T-cell responses (7;8;14;22). HPV16-specific T-cell responses were detected in 15 (39%) of the 38 patients (Table 3). In 6 cases proliferation was detected against E6, in 3 cases to E7 and in 6 cases to both E6 and E7 (Table 3). Analysis of the supernatants of these T-cell cultures for the presence of type 1 and type 2 cytokines revealed the secretion of the Th1 cytokine IFNγ in 6/15 (40%) patients with a pro-

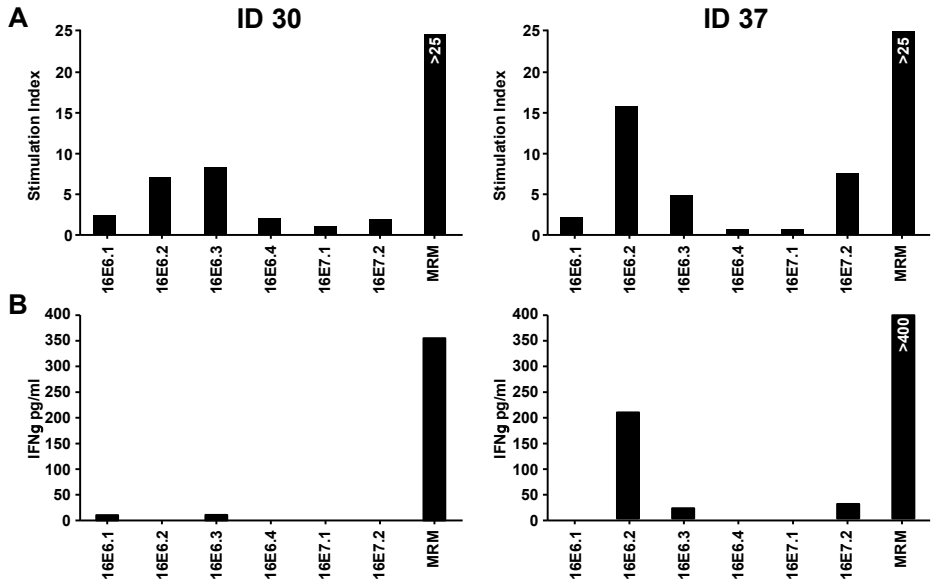


Figure 1. Short-term proliferation assay (LST) and associated IFNg production. (A) Representative examples of the HPV16-specific proliferation following the stimulation of PBMC from two HSIL patients (ID30, ID37). Cultures were tested in a 7 day lymphocyte proliferation assay upon stimulation with HPV16 E6 and E7 peptide pools. ID 30 tested positive for HPV16 peptide pool E6.2 and E6.3; ID 37 was positive for HPV16 peptide pools E6.2, E6.3 and E7.2. (B) Analysis of cytokine production by Cytokine Bead Array showed that only the culture from ID 37 stimulated with peptide pool E6.2 was associated with the production of IFNg, whereas in ID 30 the HPV16-specific T cells did not produce this typical Th1 cytokine.

liferative T-cell response (Figure 1B & Table 3). Occasionally, low levels of TNFα and IL-5 were produced by the HPV16-specific responding cells. Of all the proliferative responses measured (n=31) in these patients, only 9 (<30%) were associated with the production of a pro-inflammatory cytokine (Table 3).

HPV specific T-cell responses are correlated with the persistence/recurrence of HSIL

The majority of HSIL lesions are precancers that are destined to persist (1) and for that reason are surgically removed. As a small percentage of HPV16-positive HSIL may spontaneously regress after a biopsy (48) we hypothesized that the combination of HPV antigens and an invasive (surgical) treatment may deliver sufficient antigenic stimulation and danger signals to activate an HPV16-specific T-cell response. Because a number of our patients (Table 2) had already been surgically treated in the near past for HSIL, the patients were divided into two groups and analyzed with respect to the absence and presence of HPV16-specific immunity. Group 1 (n=28) consisted of those patients who were treated for the first time for an HSIL at the time of testing for HPV-specific immunity, whereas group

Table 3. Summary of HPV16-specific T-cell responses in patients with HSIL

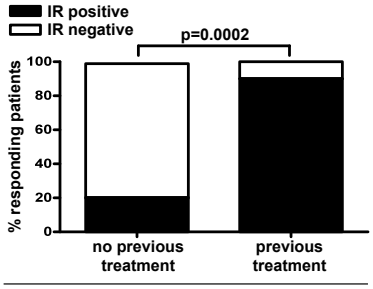
ID	Specificity*	SI †	IFN-γ ‡	Tumor necrosis factor-α ‡	IL-5 ‡
8	E6.4	4	<100	<20	<20
9	E6.2	11	299	<20	<20
10	E6.3	4	167	<20	<20
13	E6.2	4	<100	<20	<20
	E6.3	4	<100	<20	<20
	E7.2	3	<100	<20	<20
14	E6.3	6	<100	<20	<20
	E6.4	10	<100	29.2	<20
	E7.2	3	<100	<20	<20
28	E7.2	6	<100	<20	<20
30	E6.2	7	<100	<20	<20
	E6.3	8	<100	<20	<20
31	E6.2	7	<100	<20	<20
	E6.3	3	<100	<20	<20
	E7.2	6	<100	<20	<20
32	E7.2	13	<100	<20	<20
33	E6.1	5	<100	<20	<20
	E6.2	10	473	<20	31.2
	E6.3	6	<100	<20	<20
	E6.4	10	<100	<20	27.5
	E7.2	8	112	<20	<20
34	E6.1	3	<100	<20	<20
	E6.2	6	<100	<20	<20
	E6.3	11	<100	<20	<20
35	E7.2	7	447	<20	<20
36	E6.2	5	<100	<20	<20
	E7.2	5	<100	<20	<20
37	E6.2	16	210	<20	<20
	E6.3	5	<100	<20	<20
	E7.2	7	<100	<20	<20
38	E6.2	8	781	<20	60

*The peptide pool to which PBMC of the indicated patient proliferated.
† The magnitude of the response indicated by the stimulation index (SI) is shown.
‡ The amount of cytokine produced in the cultures with HPV16-specific PBMC is depicted in pg/mL. Production of IL-4, IL-10, and IL-2 was undetectable in the cultures. Cutoff values are 100 pg/mL for IFN-γ and 20 pg/mL for tumor necrosis factor-α and IL-5. Positive antigen-specific cytokine production was defined as a cytokine concentration above the cutoff value and more than two times the concentration of the medium control (7).

2 (n=10) consisted of patients with HSIL that persisted/recurred after a first destructive treatment and whom were treated again at the time that their HPV-specific immune status was assessed. The latter group displayed a significantly higher response rate to HPV16. The percentage of positive systemic responses in the group with persistent/ recurrent lesions (group 2) was 90% versus 21% in group 1 (p=0.0002, Figure 2). All together our results suggest that a systemic immune response, most often of a non Th1/ Th2 type, develops against HPV16 E6 and/or E7 and is often induced if the lesion persists or recurrs after initial surgical treatment of HSIL.

HPV16 E7-specific regulatory T cells infiltrate cervical HSIL lesions

From seven patients we were able to receive a small piece of cervical tissue for research purposes in order to characterize HPV16-specific T cells present in the HSIL lesion. As part of a larger study we showed that 3 of the 7 of these cervical infiltrating lymphocyte (CIL) cultures contained T-cells which recognized HPV16 E7 (46). From two of these CIL cultures (ID 2 and ID23) sufficient numbers of T cells were obtained for a more in-depth analysis. Upon stimulation with their cognate peptide both CIL cultures produced IFNg



LST positive	6 *(21%)*	9 *(90%)*
LST negative	22 *(79%)*	1 *(10%)*

Figure 2 . HPV16-specific immune responses are more frequently observed in patients with previous treatment of HSIL, than in patients treated for the first time. Of the 28 patients scheduled with a diagnosed HSIL for the first time, only 6 responded (21%) to HPV16 E6 and E7 peptide pools in a short-term lymphocyte stimulation test (LST) whereas a significant proportion of patients with persistence or recurrence of the lesion after initial treatment (90%; p=0.0002) displayed an HPV16-specific immune response (IR).

(Figure 3a). In addition, the culture of ID2 produced low levels of IL-10 and a small population of the CD4+ CIL population co-expressed CD25 and FoxP3, as measured by flowcytometry (Figure 3ab). As we had observed that the presence of HPV16-specific T cells with such a phenotype in cervical carcinoma represented regulatory T cells with the capacity to suppress immune responses (47) we assessed the suppressive capacity of the HPV-specific

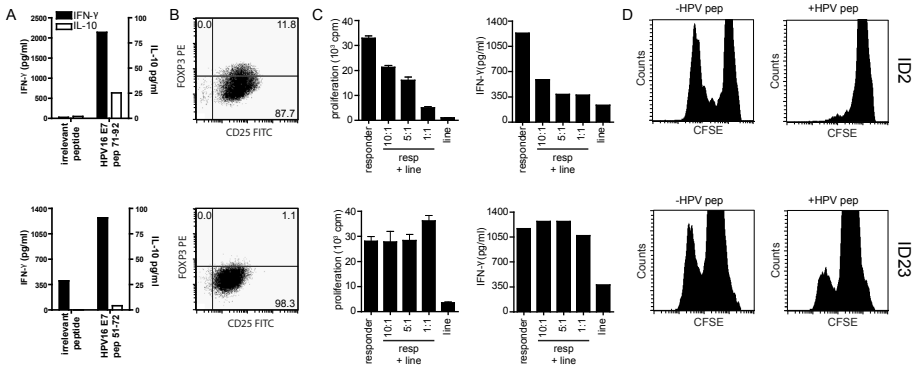


Figure 3. HPV16-induced HSIL lesions can be infiltrated by HPV16-specific regulatory T cells. Cervical infiltrating lymphocyte (CIL) cultures of ID2 and ID23 contained T-cells which recognized HPV16 E7 (46), and were analyzed for (A) their capacity to produce IFNγ and IL10 upon stimulation with cognate antigen, (B) the co-expression of the with regulatory T-cell associated markers CD25 and Foxp3. (C) Their capacity to suppress CD4+CD25- responder T-cells, cultured with the HPV-specific CIL lines at indicated different ratios, was measured by proliferation (left) and IFNγ production (right). T-cells of ID2 suppressed both proliferation and INFγ production. (D) The antigen-dependency of the HPV16 E7-specific regulatory T cells of ID 2 to exert their suppressive function was shown in a CFSE-based proliferation assay (47). Left and right panels depict responder cell proliferation in the absence or presence of their cognate peptide respectively.

CIL cultures in a classical suppression assay (Figure 3c). The CIL culture of patient ID2 was able to suppress both proliferation and IFNg production of CD4+CD25- responder T cells, whereas the CIL culture of patient ID23 did not contain such suppressive capacity. In order to prove that the HPV16-specific T cells were responsible for this suppressive effect, a CFSE-based suppression assay was performed in which the suppressive action of the CIL culture in the presence and absence of HPV16 peptide was tested (47). As expected, the CIL culture of patient ID2 almost completely suppressed the proliferation of the responder cells (85%) dependent on stimulation with HPV peptide, whereas the CIL culture of patient ID23 did not show substantial suppression (32%) when stimulated with its cognate HPV peptide (Figure 3d). These data demonstrate that HPV-specific regulatory T cells not only are present in patients with cervical carcinoma, but can already develop earlier during the malignant transformation of a persistently HPV16 infected cervix.

DISCUSSION

In this study we show that a systemic proliferative T-cell response against HPV16 is often absent in the majority of patients who visit the clinic for treatment of an HPV16+ HSIL lesion. In a number of cases HPV16-specific T-cell reactivity can be detected in the form of proliferative responses which are not associated with HPV16-specific secretion of the pro-inflammatory Th1 or Th2 signature cytokines. Importantly, HPV16-specific T-cell reactivity is predominantly detected in patients returning to the clinic for repetitive treatment of an HPV16+ HSIL lesion because of persistence or recurrence of the lesion after initial destructive treatment. This was not the case in patients visiting the clinic for a first treatment of their HPV16 HSIL lesion (Figure 2). The observation that these responses lack a clear pro-inflammatory signature indicates that this type of immune activation should not be regarded as beneficial, but rather as a reflection of the fact that most of the subjects, although in principal able to mount an HPV16-specific T-cell response, fail to develop a cellular immune response that is associated with protection against HPV-induced lesions (7;18;22;25). This notion is sustained by our data showing for the first time that already at this pre-malignant phase a population of regulatory T cells, which specifically recognize HPV16 E7 antigen, arises and infiltrates the HPV16+ HSIL lesion (Figure 3).

In a previous study, in which the immune response to HPV16 E6 and E7 was studied in patients with cervical carcinoma, we were able to detect an HPV16-specific proliferative response in about half of all patients, but only in 1 of the 8 tested subjects with an HPV16+ HSIL (7). Similar observations regarding the low frequency of responders were made in small cohort studies performed by others (18;22). The reason why fewer patients with HSIL mounted an immune response to E6 and E7 in comparison to cervical cancer patients at that time remained unclear. Our current study of a large group of 38 HPV16+ HSIL patients

confirms that the majority of HSIL patients fail to mount an HPV16 E6/E7-specific T-cell response. The absence of an HPV16-specific immune response in patients with HSIL and the presence of such responses in about half of the patients with cancer previously led us to hypothesize that the long-term presence of the HPV16 E6 and E7 antigens in a developing tumor may eventually trigger the induction of a CD4+ T-cell response (7). Here, we identified a larger group of HPV16+ HSIL patients which was able to mount an HPV16 E6/E7-specific immune response. Importantly, most patients within this group suffered from a persistent/recurrent HSIL lesion and had already been treated by destructive treatment before this immunological analysis was performed. This suggests that the HPV16 E6/E7-specific immune response detectable in the circulation of cervical cancer patients has developed as part of long term exposure to the HPV16 antigens in the persistent/recurrent HSIL in combination with the danger signals delivered by the previous invasive treatment. This notion is sustained by our observation that the cytokine profile of the HPV16-specific immune response in persistent/recurrent HSIL patients is similar to that of what we observed in cervical cancer patients (7) as well as the fact that the majority of established high-grade CIN lesions will evolve towards cervical carcinoma when left untreated (1). As such, the group of patients with recurrent/persistent HSIL may reflect our 'missing link' with respect to the absence of HPV16 E6/E7-specific immune responses in first time diagnosed HSIL patients and the presence of these responses in patients with cervical cancer. As yet, it is unclear whether either the previously given destructive treatment, the persistence of the lesion, and as such HPV antigens, or the combination of both is responsible for the activation of the HPV16-specific proliferative response. Notably, a previous study in HSIL patients showed that activation of HPV16 E7-specific immunity shortly after a local invasive procedure occurred only in 2 out of 18 patients (26). In that study immunity was measured by IFNγ ELISPOT while we showed that most of the HPV16-specific proliferative responses detected in our group of patients were not associated with the production of IFNγ (Figure 1, Table 3). The former study, thus, may have underestimated the response rate after local invasive procedures. Interestingly, a history of a long period (15-51 months) of persistent HPV16 infection is also associated with the activation of HPV16 E7-specific immunity, albeit that these responses are weak considering the fact that an indirect measurement consisting of a highly sensitive cellular bio-assay for IL-2 production was needed to detect these responses (23). Taken together, these studies build a strong case supporting the idea that local danger signals and long-term exposure to sufficient amounts of antigen are key in the development of the weak and dysfunctional immune responses observed in HSIL patients.

The number of circulating, regulatory T cells as defined by CD4+CD25^{high} T cells (49) or CD4+CD25^{high} CTLA4+ T cells (50), is increased in patients with HSIL when compared to healthy controls. In addition, immunohistochemical analysis of HSIL lesions not only revealed that HSIL lesions represent immunosuppressive environments (13) but also that

immune cells possessing a suppressive phenotype – as defined by CD25+TGFβ+ and CD4+TGFβ+ immune cells – may infiltrate such HSIL lesions (12). However, the specificity of these circulating and HSIL-infiltrating regulatory T-cells was never determined. Previously, we showed that the CD4+ subset of T cells infiltrating cervical carcinomas consisted of HPV16-specific regulatory T cells able to suppress proliferation and cytokine production of responder cells (47). Similar functional analyses of the T-cell populations infiltrating HSIL not only showed that indeed fully functional regulatory T cells can infiltrate premalignant cervical lesions but also that these regulatory T-cells exerted their action upon recognition of their cognate HPV16-specific antigen (Figure 3). Interestingly, a higher number of circulating CD4+CD25^{high} CTLA4+ regulatory T cells coincides with the presence of HPV16-specific T cells in the blood of patients with HSIL (50) indicating that such responses may co-evolve. Recently, we have developed an assay to measure the percentage of HPV16-specific regulatory T-cells in the circulation of patients with cervical cancer (27). Similar analyses in patients with HSIL may reveal to which extent the detected HPV16-specific responses lacking a clear cut pro-inflammatory signature (Figure 2 and Table 3) may actually represent HPV16-specific regulatory T cells. The lack of PBMC precluded such an analysis in the current group of patients but this question will be addressed in a new study.

The detection of HPV16-specific T cells with a non Th1/Th2 cytokine and even immune suppressive signature in patients with HSIL bears implications to immunotherapeutic vaccine approaches aiming at reinforcing HPV-specific immunity to attack HPV-induced lesions. Recently, we showed that such a vaccine also activates/boosts an unwanted pre-existing HPV16-specific T-cell repertoire in cervical cancer patients (27) suggesting that strategies to overrule or eliminate the responses of these subsets of T-cells in cancer patients should be considered for immunotherapeutic strategies against HPV-induced cervical lesions.

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