Regulation and subversion of HPV16-specific immunity in cancer patients

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Chapter 3

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Human papilloma virus specific T cells infiltrating cervical cancer and draining lymph nodes show remarkably frequent use of HLA-DQ and -DP as a Restriction Element

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Abstract. Human papillomavirus (HPV)-induced malignancies are frequently infiltrated by lymphocytes. To comprehend the contribution of HPV-specific T cells in this anti-tumor response we developed a method that allowed the analysis of the presence and specificity of cervix-infiltrating and draining lymph node resident T cells in a group of 74 patients with cervical malignancies, 54 of which were induced by HPV16 or HPV18. We detected the presence of HPV16 or HPV18-specific T cells in at least 23 of the 54 HPV-16 or -18 positive patients, and not in the 20 controls. Detailed studies resulted in the identification of 17 novel CD4+ and CD8+ T cell epitopes and their HLA-restriction elements, and also revealed that the HPV-specific immune response was aimed at both E6 and E7 and showed no preferential recognition of immunodominant regions. Unexpectedly, the vast majority of the CD4+ T cell epitopes were presented in the context of the less abundantly expressed HLA-DQ and HLA-DP molecules. Since the identified T cell epitopes constitute physiological targets in the immune response to HPV16 and HPV18 positive tumors they will be valuable for detailed studies on the interactions between the tumor and the immune system. This is crucial for the optimization of cancer immunotherapy in patients with pre-existing tumor-immunity.

Introduction

Cervical cancer is the second most common cancer in women worldwide (1). High risk human papilloma virus (HPV) type 16 and 18 are the cause of cervical cancer in around two thirds of all patients (2, 3). 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 (4).

The tumor-specific expression of these oncoproteins as well as the presence of low levels of circulating E6- and E7-specific T cells detected in the peripheral blood of almost half the patients with cervical cancer (5-11) suggested that they could serve as tumor rejection antigens. However, the existence of circulating HPV-specific T cells does not imply that they contribute to the anti-tumor response. In order to control the disease, these T cells should at least be able to home to the tumor sites. Indeed, a proportion of cervical carcinomas are infiltrated by lymphocytes (12-14) but in-depth knowledge on the specificity of the T cells infiltrating these cervical tumors is still lacking, probably due to relative difficulties in establishing T-cell cultures from tumor tissue. Nonetheless, a few early pioneers were able to isolate HPV-specific tumor infiltrating lymphocytes (TIL) from tumors, resulting in the identification of two single CD8+ T-cell epitopes of HPV16 (15, 16) and two CD4 T-cell epitopes specific for the less prevalent high risk subtypes HPV59 and HPV33 (17, 18). However, larger studies on cervical tissue-infiltrating lymphocytes are urgently needed to comprehend the contribution and role of the HPV-specific adaptive immune response in cervical cancer. In addition, this will allow the rational design of successful immune intervention strategies.

Recent studies showed that two cytokines, IL-7 and IL-15, have a major role in the expansion and survival of CD4+ and CD8+ effector memory T cells. IL-7 provides survival signals for effector T cells (19). IL-15 is a critical growth factor in initiating T-cell divisions, and in contrast to IL-2 - which is generally used to expand TIL cultures - does not limit continued T-cell expansion (20). Furthermore, IL-15 can also act as an antigen-independent activator of CD8 memory T cells (21). Together, IL-7 and IL-15 can expand with very high efficiency effector memory T cells, while central memory T cells are less responsive and naive T cells fail to respond to stimulation with these cytokines (22-24).

In view of the importance of incisively studying the presence, type and specificity of tumor-infiltrating lymphocytes, and inspired by the possibility that the use of IL-7 and IL-15 may promote the expansion of T cells from cervical tissue without the addition of supplementary antigens, we examined a large cohort of 74 patients for the presence of HPV16 and 18 E6 and E7 specific CD4+ and CD8+ T cells in cervical tissue and tumor draining lymph nodes. We detected a HPV-specific T-cell response to 25 different E6- or E7-derived peptides, including 17 novel CD4+ and CD8+ T-cell epitopes, in 43% of all the different HPV16 or HPV18 positive patients. HPV-specific immunity was aimed at both E6 and E7 and showed no preferential recognition of immunodominant regions.

Methods

Subjects. Women presenting with histologically proven cervical neoplasia at the department of Gynaecology of the Leiden University Medical Centre and Haga Teaching Hospital the Hague were enrolled in the CIRCLE study, which investigates cellular immunity against HPV16-positive cervical lesions after providing informed consent. The study design was approved by the Medical Ethical Committees of both hospitals. The subjects were tested for HPV status using HPV16 and HPV18 specific primers on DNA isolated from surgical resection specimens (25). Peripheral blood mononuclear cells (PBMC) for HLA-restriction analysis were obtained from HLA-typed anonymous healthy blood donors after informed consent.

Antigens. A set of overlapping peptides spanning both HPV16 and HPV18 E6 and E7 protein were used for T-cell stimulation assays. HPV16 and HPV18 E6 and E7 consisted of 22-mer peptides overlapping 12 residues. The peptides were synthesized and dissolved as described earlier (6, 8). Recombinant HPV E6 and E7 proteins were produced in recombinant E. coli as described earlier (6). Moreover, a set of overlapping 10-mers (overlapping 9 amino acids) of both HPV16 E6 and E7 was produced to pinpoint the minimal peptide epitope recognized by HPV16-specific T cells.

Antigen presenting cells. Epstein-Barr virus transformed B-cell lines (B-LCL) of the patients were maintained in IMDM (BioWhittaker, Verviers, Belgium) containing 10% Fetal Calf Serum (FCS, PAA laboratories, Pasching, Austria) in 75 cm^2 culture flasks (Greiner bio-one, Alphen aan den Rijn, the Netherlands). Monocytes were generated from peripheral blood lymphocytes as described earlier (26).

Isolation and culture of T cells. Cervical tumor biopsies were obtained from patient uteruses after radical hysterectomy, cervical neoplasia tissue was obtained from Cervical Intraepithelial Neoplasia (CIN) III patients after biopsy. Fresh cervical tissue was minced in to pieces of approximately 1 mm^3 and cultured 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 IL-15 (Peprotech, Rocky Hill NJ, USA). During the first day 5 ng/ml IL-7 (Peprotech) was added to cultures to ensure T cell outgrowth. After 2-3 weeks the specificity of the T cell (TIL, CIN infiltrating lymphocytes (CIL)) cultures was tested and positive cultures were expanded using a mix of irradiated autologous B-LCL and 5 ug/ml cognate peptide.

Lymph nodes were derived from the pelvic region and contained tumor cells, indicative of metastatic cancer. The lymph nodes were cut into pieces

Table 1. HPV16 and 18 resposes detected in infiltrating lymphocytes

HPV Status	Origin	Patient	Age	Cell Type	Stage of disease	Reactivity	SI*	Responding peptides	Responding T cell
HPV16	TIL								
		176	45	squamous	FIGO 1B	E6	80	2	CD4/CD8
		178	40	squamous	FIGO 1B	E7	11	1	CD4
		185	56	squamous	FIGO 3B	E7	6	1	CD8
		192	37	squamous	FIGO 1B				
		194	67	adeno	FIGO 2A	E7	5		
		226	56	squamous	FIGO 1B	E6	3	1	CD4
		229	42	squamous	FIGO 1B				
		230	45	squamous	FIGO 1A				
		246	31	squamous	FIGO 1B				
		265	44	squamous	FIGO 1B	E6	104	2	CD4/CD8
		267	49	squamous	FIGO 1B	E6	109	2	CD4
		271	40	squamous	FIGO 1B				
		281	35	squamous	FIGO 1B				
		283	51	squamous	FIGO 1B				
		308	39	squamous	FIGO 1B				
		312	30	adeno	FIGO 1B				
		331	65	squamous	FIGO 1B	E6	3	2	CD4/CD8
		332	32	squamous	FIGO 1B				
		334	41	squamous	FIGO 1B	E6	5	1	CD8
		338	34	squamous	FIGO 1B				
		340	29	squamous	FIGO 1B				
		343	51	unknown	FIGO 1B				
		344	43	squamous	FIGO 2A				
		363	45	squamous	FIGO 1B				
		369	33	adeno	FIGO 1A				
		371	31	squamous	FIGO 1B				
		372	72	squamous	FIGO 1B				
		390	33	adeno	FIGO 1B	E6/E7	4		
		398	48	squamous	FIGO 1B				
		405	41	squamous	FIGO 2B				
		418	34	squamous	FIGO 1B				
		415	46	squamous	FIGO 1B				
		424	35	squamous	FIGO 1B				
		441	51	squamous	FIGO 1B				
		446	29	squamous	FIGO 1B	E6	4	4	CD4/CD8
	CIL								
		279	60	unknown	CIN3				
		284	36	squamous	CIN2	E7	13	1	CD4
		285	27	squamous	CIN3				
		310	46	squamous	CIN3				
		314	34	squamous	CIN3	E7	11		
		355	47	squamous	CIN3				
		356	26	squamous	CIN3	E7	3.5	1	CD4
	LN								
		148	46	squamous	FIGO 1B	E6/E7	9/3		CD4
		267	49	squamous	FIGO 1B	E6	4		CD4
		271	40	squamous	FIGO 1B	E6/E7	1.5/2		CD4
		427	28	squamous	FIGO 1B	E6	9		CD4/CD8
HPV18	TIL								
		187	43	squamous	FIGO 1B	E6	2	1	CD4
		196	48	adenosquamous	FIGO 2A				
		209	55	squamous	FIGO 1B				
		214	42	adeno	FIGO 1B	E7	15	1	CD4
		228	37	squamous	FIGO 2A	E7	18	1	CD4
		251	39	adenosquamous	FIGO 2A	E7	3		
		261	38	squamous	FIGO 1B				
		335	33	adeno	FIGO 1B				
		378	40	adeno	FIGO 1B	E7	8	1	CD4
	LN								
		151	43	squamous	FIGO 1B	E6/E7	2/3		CD4
HPV16-18-	TIL								
		181	40	squamous	FIGO 1B				
		182	80	squamous	FIGO 2B				
		215	31	squamous	FIGO 1B				
		245	41	squamous	FIGO 1B				
		248	46	squamous	FIGO 2A				
		264	35	adeno	FIGO 1B				
		280	31	squamous	FIGO 1B				
		287	61	carcinosarcome	FIGO 2B				
		289	45	adeno	FIGO 1B				
		292	32	squamous	FIGO 1B				
		324	51	squamous	FIGO 1B				
		353	35	adeno	FIGO 1A				
		373	55	squamous	FIGO 1B				
		377	85	squamous	FIGO 1B				
		381	80	adeno	FIGO 1B				
		384	75	squamous	FIGO 1B				
		414	64	squamous	FIGO 2A				
	CIL								
		348	35	squamous	CIN3				
		354	39	squamous	CIN3				
	LN								
		426	40	squamous	FIGO 1B				

*SI, stimulation index

and incubated for one hour at 37 °C in the presence of collagenase (200 IU/ml, Sigma) and DNAse (50 mg/ml, Sigma), after which the lymph node mononuclear cells were put through a cell strainer (BD, Erebodemgem, Belgium) to obtain a single cell suspension. Separate lymph node mononuclear cell (LMNC) cultures were stimulated with HPV16 or 18 E6 or E7 peptide pools and cultured for 3-5 weeks.

T-cell clones were isolated using limiting dilution according to a protocol adapted from Evans et al (27), replacing IL-2 for 10% TCGF and 5 ng/ml IL-15, and adding 0.5 mg/ml phytohemagglutin (PHA, Murex Diagnostics, Dartford, UK) for T-cell receptor triggering. After limiting dilution T-cell clones were tested for their specificity and maintained in IMDM containing 10% FCS, 10% TCGF and 5 ng/ml IL-15. T-cell clones were expanded using a mix of culture medium, irradiated PBMC from 5 different donors, B-LCL and 0.5 mg /ml PHA.

Analysis of T-cell specificity. T-cell cultures (25,000-50,000 cells/well) were tested on pulsed autologous monocytes or irradiated autologous EBVs for the recognition of HPV16 and 18 E6 and E7 peptides (5 mg/ml) and protein (10 mg/ml) in triplicate in a 3-day proliferation assay. After 48 hours supernatant was harvested and stored at -20°C for cytokine analysis. During the last 16 hours of culture 0.5 mCi/well [^{3}H]thymidine was added to measure proliferation (6). Antigen-specific IFNγ production was measured by ELISA as described earlier (28). In general, stimulation indexes (SI) above 3 were considered to be positive responses. Only in cases that the cultures were screened for HPV specific responses the first time also a SI>2 was considered to be positive, but only if reproducible at least twice.

MHC class II blocking experiments were performed as reported before using murine monoclonal antibodies against HLA-DR (B8.11.2), HLA-DQ (SPV. L3) and HLA-DP (B7/21) (28). Peptide-pulsed APC were incubated with anti-MHC class II antibodies for 2 hours prior to the addition of T cells.

Enumeration of IFNγ producing T cells as measured by intracellular cytokine staining was performed as described earlier (29). Briefly, APC were loaded with cognate peptide or recombinant protein and incubated with T cell cultures. After 1 hour of incubation 10 mg/ml Brefeldin A (Sigma) was added and incubated overnight. Hereafter the cells were fixed with 4% paraformaldehyde (Sigma) and permeabilized with 0.1% Saponin. The samples were subsequently stained with CD4-APC, CD8-PerCP and IFNγ-PE and analysed by flow cytometry. Responses in which the percentage of antigen-stimulated IFNγ-producing T cells exceeded 3 times the control were considered to be positive.

The minimal peptide recognized by CD8 T cells was analysed by IFNγ ELISPOT (6, 8, 26). CD8 T-cell lines were seeded in triplicate wells at a density of 2 x 10^4 on a Multiscreen 96-well plate (Millipore, Etten-Leur, The Netherlands) coated with an IFNγ catch antibody (Mabtech. Nacha, Sweden). The microcultures were stimulated with 1 mg/ml 10-mer peptides and incubated overnight. Analysis of HLA restriction of CD8 T cells was performed using 1 mg/ml 10-mer peptide pulsed PBMC or B-LCL co-cultured with equal numbers of T cells. IFNγ specific spots were stained according to the instructions of the manufacturer (Mabtech). The number of spots was analysed on a fully automated computer assisted video imaging system (BIOSYS).

Results

HPV-specific T cells are present in cervical neoplasia infiltrating lymphocytes. In the current study we analysed the presence, type and specificity of HPV16 and HPV18-specific T cells in cervical neoplastic lesions, which is

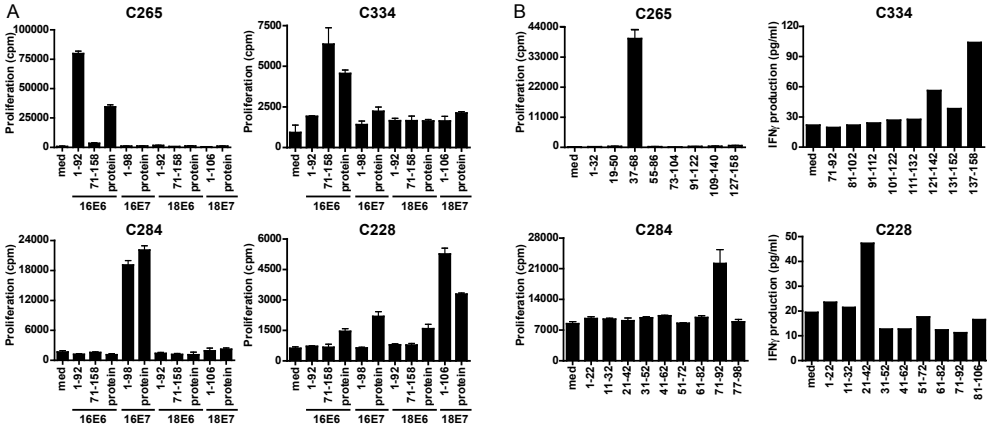

Figure 1. A) Proliferation of initial T-cell cultures isolated from cervical tissue from 4 different patients. All T-cell cultures recognized naturally processed antigen in a 3-day proliferation assay upon stimulation with HPV16 or 18, E6 or E7 peptide pool and recombinant protein. C265 recognized HPV16E6 peptide pool 1-92, C334 HPV16E6 peptide pool 71-158, C284 HPV16E7 peptide pool 1-98 and C228 HPV18E7 peptide pool 1-106. B) Fine mapping of the specificity of bulk cultures using single peptides was measured by proliferation and IFNγ production. C265 responded to stimulation with peptide HPV16E6 37-68, C334 with HPV16E6 peptide 137-158, C284 with HPV16E7 peptide 71-92 and C228 with HPV18E7 peptide 21-42.

the site where HPV-specific T cells encounter their cognate antigen and should exert their effector function. In total 74 patients were analyzed. Cervical tissue was obtained from 61 patients with cervical cancer and from 9 additional patients with CIN III. Minced pieces of tissue were cultured for 2-3 weeks in the presence of a mix of cytokines containing IL-15 and TCGF. To prevent a potential bias in the outgrowth of tumor-specific T cells no exogenous HPV-antigens were provided to these cultures. Within 14-21 days of culture the cytokine expanded T cells were harvested and analysed by FACS. The mean percentage of CD3+ T cells present in these cultures increased from 41% at 2 weeks to 68% at 3 weeks. In general, the culture method did not favour the selective outgrowth of one type of T cell as indicated by the percentage of CD3+CD4+ T cells (34% ± 22%) and CD3+CD8+ T cells (52% ± 22%) at 2 weeks or at 3 weeks (38% ± 21%; 48% ± 24%, respectively). Occasionally, an individual culture showed a more pronounced expansion of either CD4+ or CD8+ T cells (not shown). To analyse the presence of HPV-specific T cells, the cultures were stimulated with autologous monocytes pulsed with different pools of overlapping peptides spanning the E6 and E7 proteins of HPV16 and HPV18, as well as with the respective recombinant proteins. In 19 of the 51 HPV16- or HPV18-positive patients we were able to detect HPV-specific T cells by proliferation (Table 1, Figure 1a). These cultures responded both to peptide and protein loaded monocytes, indicating that the T cells recognized naturally processed antigen. In 8 cultures E6-specific T cells were detected, in 10 cultures the T cells responded to E7 and in one T-cell culture a response to both E6 and E7 was detected. Importantly, no HPV16 or 18 specific T-cell response was detected in HPV16 and 18 negative cervical tissues (n=19), indicating that the observed HPV16- and 18-specific responses were not induced in vitro (Table 1).

Both HPV specific CD4 and CD8 T-cells infiltrate cervical lesions. Following the evaluation of HPV-specific reactivity, the 19 responding T-cell lines were expanded by stimulation with cognate peptide, cytokine mix and feeder cells. Fifteen of these HPV-specific cultures could be sufficiently expanded for further analysis. The fine specificity of the HPV-specific T cells was determined by stimulation of the T cells against each individual peptide of the set of overlapping peptides in short-term stimulation assays. Five cultures recognized 2 or more distinct peptides, whereas the other 10 cultures recognized a single peptide (Figure 1b, Table 1). To assess the type of T cell that responded to antigenic stimulation, the T-cell cultures were stimulated with their cognate peptide and protein antigens and the response was analyzed by intracellular IFNγ staining (Figure 2). The majority of the TIL cultures contained HPV-specific CD4+ infiltrating T lymphocytes (n=13 patients, 13 different peptides recognized), whereas HPV-specific CD8+ infiltrating T lymphocytes were found in 6 cultures. In 9 of the HPV-specific T-cell lines only a CD4+ T-cell response was detected, in 4 T-cell lines both CD4+ T cells and CD8+ T cells reacted and in 2 T-cell lines only a CD8 T-cell response was detected (Table 1, Figure 2).

HLA restriction of tumor infiltrating lymphocytes. The HLA class I and II loci involved in the presentation of HPV peptides to CD8+ and CD4+ T cells were studied using blocking antibodies and partially HLA matched

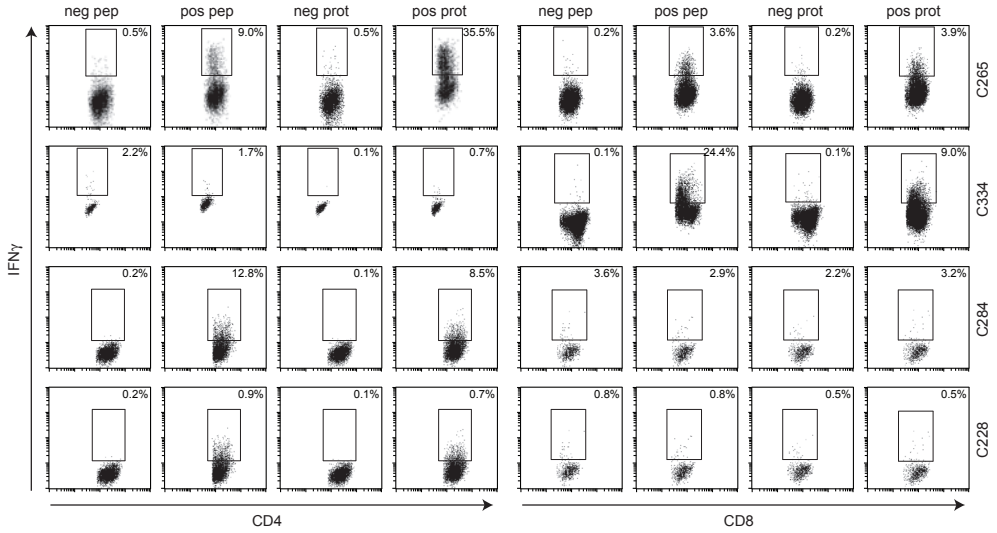

Figure 2. Analysis of the type of T cell responding to HPV antigen as measured by intracellular cytokine staining for IFNγ. For positive peptide and protein, the peptide HPV16E6 41–62 and HPV16E6 protein was used for C265, HPV16E6 protein and peptide 137–158 for C334, HPV16E7 protein and peptide 71–92 for C284 and HPV18E7 protein and peptide 21–42 for C228. Peptides and proteins from HPV counterparts were used as negative controls. C228 and C284 displayed CD4 restricted responses, C334 displayed a CD8 restricted response. The TIL culture of C265 displayed a CD4+ and CD8+ T-cell response which both responded to peptide and protein.

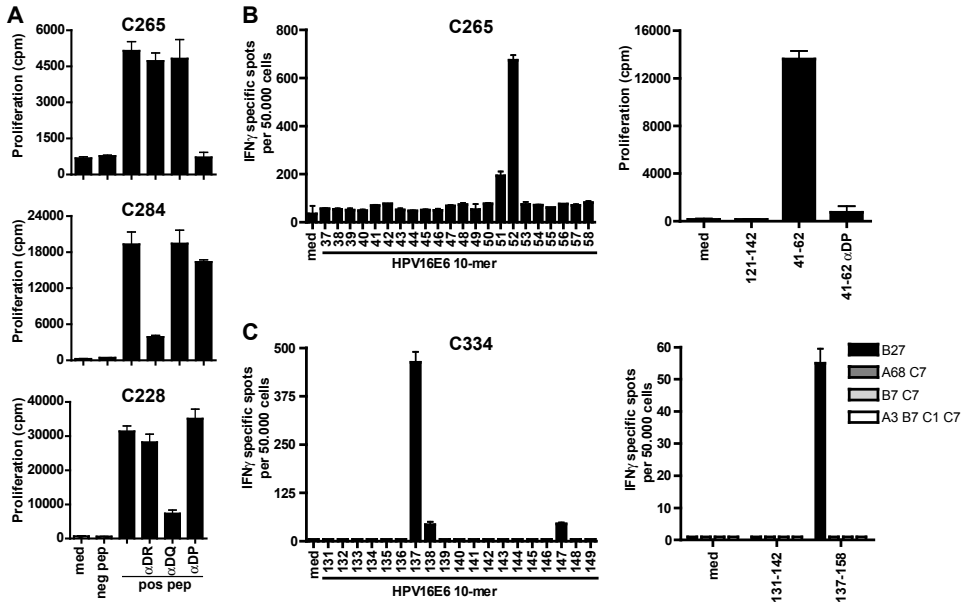

Figure 3. A) Blocking of CD4 restricted responses by HLA class II antibodies in a 3-day proliferation assay. C265 derived T cells were stimulated with peptide loaded autologous B-LCL, C284 derived T cells were stimulated with peptide loaded monocytes that were matched only for HLA-DR12 and C228 derived T cells were stimulated with peptide loaded monocytes, HLA-matched for DQ*0302. B) Finemapping and HLA restriction of TIL cultures. The CD4+ T cells of patient C265 were stimulated with autologous B-LCL pulsed with 10-mer peptides, covering the amino acid sequence of the recognized longer peptide, was tested in an ELISPOT assay. To determine the restriction of these CD4+ T cells they were stimulated with monocytes matched for HLA-DP2 only. C) Similarly, the minimal peptide-epitope recognized by the CD8 T cells of C334 was determined by incubating these T cells with the indicated 10-mer peptides in an ELISPOT assay. The HLA-restriction of C334 CD8+ T-cell response was determined using peptide pulsed PBMC isolated from healthy individuals whom were partially matched with the HLA class I molecules of the patient.

APC isolated from healthy donors. A wide variety of HLA class II molecules were found to be involved in the presentation of the antigens E6 and E7 of HPV16 and HPV18 (Table 2). The use of blocking antibodies against HLA-DR, HLA-DQ and HLA-DP revealed that 3 of the detected responses were restricted by HLA-DR, 3 by HLA-DQ and 3 by HLA-DP (Figure 3a, Table 2). To determine the exact HLA restriction element involved in presentation of the HPV antigen, APC from healthy donors that are matched for only one HLA-allele were used (Figure 3a and 3b right panel). In 6 cases we were not able to exactly determine the restriction element.

In case of patient C265 HPV-specific CD4+ and the CD8+ T cells both responded to the same peptide (Figure 2). In order to discriminate between these two T-cell responses, T-cell clones were established through limiting dilution. Unfortunately, only CD4+ T-cell clones were obtained and, as such, only the HLA class II-restriction element could be established. Therefore, it was only possible to determine the peptide recognized and restriction in 5 other HPV-specific CD8 T-cell cultures (Table 2). As an example, Figure 3 shows the determination of the 10-mer peptide-epitope and restriction of the CD8 T-cell response (Figure 3c) of the TIL culture obtained from patient C334. This response was restricted by HLA-B27 as this CD8 T-cell culture responded only upon stimulation with HLA-B27 matched peptide loaded APC and not with other partially HLA class I matched APC from other donors (Figure 3c). One patient (C265) displayed a CD8+ T-cell response to two different epitopes, and 2 patients (C176 and C334) responded to the same HLA-B27-restricted CTL epitope (Table 2).

HPV-specific T cells in tumor draining lymph nodes. Tumor draining lymph nodes are the site where HPV-specific T cells are primed and activated and, therefore, the HPV-specific T-cell response was also studied in the tumor draining lymph nodes from 6 different cervical cancer patients. Single cell suspensions of lymph node mononuclear cells (LNMC) were isolated from cervical patients displaying metastases in their lymph nodes. We were not able to directly detect HPV specific responses ex vivo in freshly isolated LNMC (data not shown). Therefore, LMNC were first expanded by one round of in vitro stimulation with HPV16 or 18 E6 and E7 peptide pools. In 4 cases the LNMC responded to HPV16 and in 1 patient an HPV18 response was detected by proliferation and IFNg production (Table 1, Figure 4A). Similar to the TIL cultures, patients with HPV16-positive tumors reacted only to HPV16 whereas the patient diagnosed with an HPV18-positive cervical cancer reacted only against HPV18. No response to either HPV16 or HPV18 was detected in the LMNC from an HPV16/18-negative

Table 2. T-cell epitopes recognised by cervical cancer patients

	novel	epitope recognised	restriction	origin	patient	% response with HLA-class II blocking §			Matched for	% of max response||	Matched for	% of max response||	Matched for	% of max response||
						anti-DR	anti-DQ	anti-DP						
CD4	*	HPV16E6.11-32	DP17	LN	C148	113	91	-2	DP0401 DP17	100	DP0401	18		
	*	HPV16E6.11-32	DP1401	LN	C271	100	103	10	DP0201 DP1401	100	DP1401	194		
		HPV16E6.11-32	DP1401	LN	C427	101	100	0	DP1401	100	DP0101	2		
	*	HPV16E6.37-68	DP0201	TIL	C226	92	82	-1	DP0201 ‡	100				
		HPV16E6.52-61	DP0201	TIL	C265	90	88	4	DP0201 DP4001	100	DP0201	59		
		HPV16E6.55-86	unknown †	LN,TIL	C267									
		HPV16E6.61-82	DP0101 and DP1401	LN	C427	141	146	-16	DP0101	127	DP1401	100		
	*	HPV16E6.71-92	DP0101	LN	C427	100	94	15	DP0101	100	DP1401	0		
	*	HPV16E6.73-105	DP4	LN	C148	123	110	1	DP0401 DP17	100	DP0401	680		
		HPV16E6.73-105	unknown †	LN,TIL	C267									
		HPV16E6.91-112	DR15 or DQ*05	TIL	C331	134	114	115	DR1 DR15 DQ*0501 DQ*06	100	DR15 DQ*06	35	DR1 DQ*0501	5
		HPV16E6.91-122	unknown †	LN	C267									
	*	HPV16E6.101-122	DQ*06	LN	C427	65	-3	96	DQ*0402 DQ*06	100	DQ*0402	-2	DQ*06	59
		HPV16E6.101-122	DQ*06	TIL	C446	112	33	130	DQ*06 ‡	100				
		HPV16E6.121-142	DP0201 or DQ*05	TIL	C265	70	99	88	DR1 DR16 DR2 DQ*05 DP0201 DP4001	100	DQ*0501 DP0201	97		
		HPV16E6.121-142	unknown †	TIL	C187									
	*	HPV16E6.129-138	DR7	TIL	C176	10	113	140	DR7	100	DR7	75	DR1	8
	*	HPV16E7.21-42	DR4	TIL	C178	8	94	62	DR15	9	DR4	100		
	*	HPV16E7.51-72	DP1901	CIL	C356	98	127	1	DP1901	99	DP1401	29		
	*	HPV16E7.76-86	DR12	CIL	C284	18	101	84	DR4 DR12	100	DR12	135		
	*	HPV18E6.51-72	DQ*0301	LN	C151	89	35	103	DQ*0301	100	DQ*0501	-2		
	*	HPV18E6.71-92	DQ*0501	LN	C151	51	-3	46	DQ*0301	1	DQ*0501	100		
	*	HPV18E7.1-32	DQ*0302, DQ*0308	TIL	C214	42	14	71	DR4 DQ*0302	166	DR4 DQ*0308	100	DR4	6
		HPV18E7.1-32	unknown †	TIL	C378									
	*	HPV18E7.21-42	DQ*0302	TIL	C228	90	22	112	DQ*0302 DQ*0602	100	DQ*0302	161		
CD8	*	HPV16E6.13-22	HLA-B7	TIL	C446				A3	0	A1 A3 B35 Cw4 Cw7	1	A3 B7 Cw7	89
		HPV16E6.29-38	HLA-A2	LN	C427				A2	147	B14	-7	Cw8	-4
		HPV16E6.52-61	HLA-B57	TIL	C331				A1 B57 Cw6	100	A1 Cw6	0	A31 B57 Cw6	116
		HPV16E6.52-61	unknown †	TIL	C265									
		HPV16E6.129-138	unknown †	TIL	C265									
	*	HPV16E6.137-146	HLA-B27	TIL	C176				B27	93	B13 Cw1 Cw6	2		
		HPV16E6.137-146	HLA-B27	TIL	C334				B27	100	A68 Cw7	0	A3 B7 Cw1 Cw7	1
	*	HPV16E6.149-158	HLA-B14	LN	C427				A2	0	B14	100	Cw8	0
		HPV16E7.11-19	HLA*0201	TIL	C185				A2	100	A3	0		

† HPV-specific T cells were lost during culture. ‡ Patient is homozygous for the indicated HLA-type. § Response was calculated by subtracting the negative control and divided by the response without blocking antibody. || Response was calculated by subtracting the negative control and devided by the autologous response. * Novel epitope

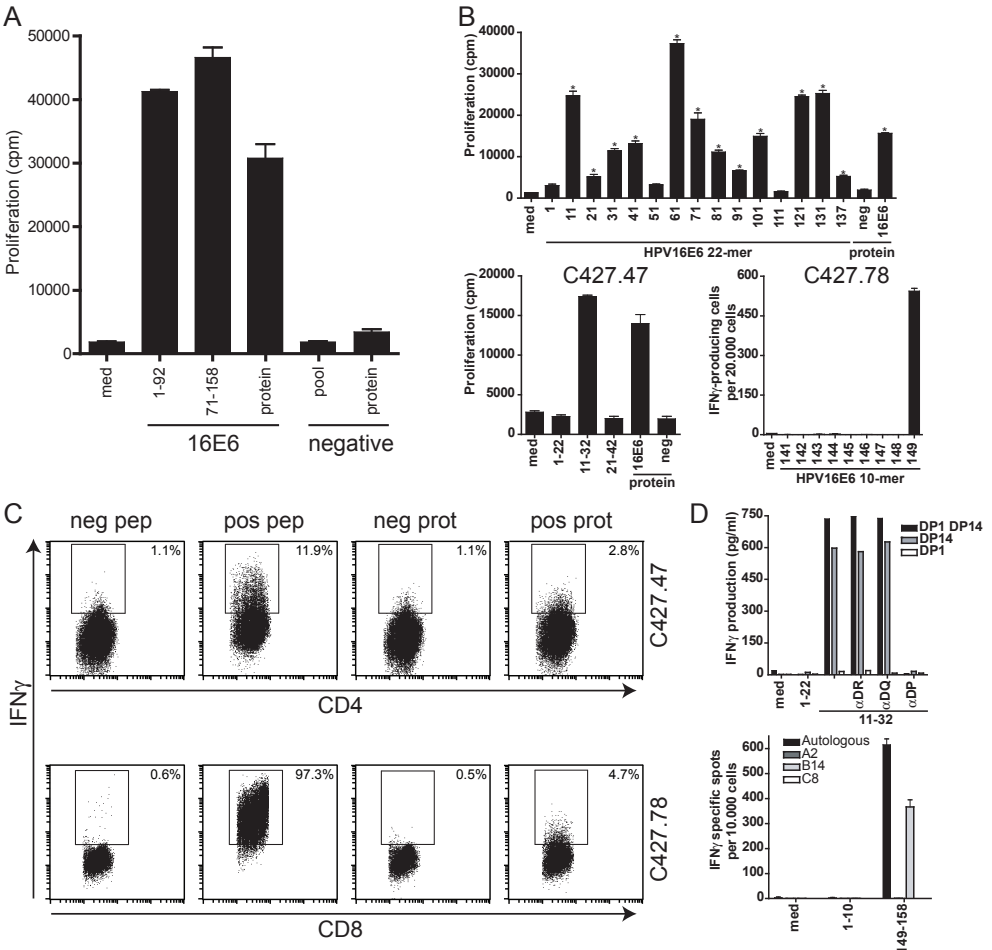

Figure 4. Analysis of T-cell reactivity present in tumor draining lymph node of C427. A) Reactivity of T-cell cultures after 3 weeks after stimulation with HPV16E6 peptide pulsed autologous B-LCL measured in a 3-day proliferation assay. B) Upper panel: recognition pattern of the T cell culture upon stimulation with autologous B-LCL pulsed with single 22-mer peptides. Lower panels: charting of the minimal epitope recognized by T cell clones that were derived from this initial LNMC culture. CD4 T-cell clone C427.47 was stimulated and tested in a 3 day proliferation assay (left panel). The CD8 T cell clone C427.78 was tested in an IFNγ ELISPOT assay (right panel). C) The type of T cell responding was determined by intracellular cytokine staining. HPV16E6 peptide 11-32 (upper panel) and peptide 137-158 (lower panel) were used as positive peptides. HPV18E7 peptide and protein were used as negative controls. D) The restriction element was analyzed using HLA class II blocking antibodies on partially matched B-LCL for class II (C427.47, upper panel) and on partially matched B-LCL for HLA class I (C427.78, lower panel), indicating that the CD4+ T cell response was restricted by HLA-DP14 and the CD8+ T cells by HLA-B14.

patient, despite the fact that the LNMC were stimulated with HPV16 and HPV18 peptides in vitro (Table 1).

T-cell clones isolated from these LNMC cultures were characterized with respect to their fine specificity and HLA-restriction element. CD4+ T-cell reactivities were found to 10 different peptides, 7 of which were not detected in the TIL cultures. Three of these epitopes were restricted by HLA-DQ and the other 4 by HLA-DP. In addition, one HLA-A*0201-restricted and one HLA-B14-restricted CD8+ T cell epitope was identified (Table 2). Figure 4 shows an example of the analysis of a LNMC culture. After one round of stimulation the LNMC cultures specifically responded to APC loaded with pools of HPV16E6 peptides or recombinant protein (Figure 4A). Analysis of the reactivity against single peptides showed recognition of a broad repertoire of peptides (Figure 4B) and the CD4+ and CD8+ T-cell clones isolated from this culture recognized their cognate antigen when naturally processed from recombinant protein (Figure 4C). The restriction was further determined using HLA class II blocking antibodies and APC form partially matched donors (Figure 4D).

Taken together, the analysis of both TIL and tumor-draining lymph node cells revealed that in 23 of the 54

different HPV16 or HPV18 positive patients a specific T-cell response to in total 25 different E6- or E7-derived peptides can be detected. Notably, 13 CD4+ T-cell peptide-epitopes were restricted by HLA-DQ or HLA-DP, 3 by HLA-DR and in 6 cases we were not able to distinguish between HLA-DQ/DP and HLA-DR (Table 2). Of the CD8+ T cell responses found, 2 were restricted by HLA-A, 4 by HLA-B and 2 were undetermined (Table 2).

Discussion

The HPV16 encoded oncoproteins E6 and E7 can serve as tumor rejection antigens in animal models (30, 31) suggesting that they may also serve as target antigens for tumor-infiltrating lymphocytes in cervical cancer, but this has never been systematically analyzed in a large group of patients. We were able to establish a high number of TIL and CIL cultures reactive against HPV16 and HPV18, which are the HPV types most prominently associated with cervical cancer (2, 3). The cytokine mix used ensured the outgrowth of both CD4 and CD8 T cells without an overt preference for the expansion of either type of T cell. In the course of our study 19 TIL cultures were established from patients diagnosed with a tumor positive for an HPV type other than HPV16 or HPV18. None of these cultures reacted to stimulation with the E6 or E7 antigens from HPV16 or HPV18. Notably, TIL and CIL from HPV16-positive patients did not respond to E6 and E7 of HPV18 and vice versa (Table 1). Therefore, the observed HPV-specific T-cell responses in the TIL and CIL of HPV16- or HPV18-positive patients are not the result of in vitro induced T-cell responses but a reflection of the anti-tumor response in vivo. Recently, we showed that this protocol was also successful in the expansion of TIL cultures from a small cohort of patients with ovarian cancer (32).

Similar numbers of TIL cultures responded to E6 and E7 (Table 1). Identification of the cognate peptide-epitopes and HLA-restriction elements of the HPV-specific immune responses revealed that HPV-specific immunity was not restricted to a specific immunodominant region but was aimed at all domains of the E6 and E7 oncoproteins (Table 2), suggesting that both HPV E6- and E7-specific T cells will contribute to the anti-tumor response. Strikingly, our analysis revealed that the great majority of the HPV-specific CD4+ T cell responses were restricted by HLA-DQ or DP (13/16) and not by HLA-DR (Table 2). This was unexpected because HLA-DR is the most abundant HLA class II molecule on the cell surface of APC (33) as well as on cervical cancer cells with de novo HLA class II expression (34). Furthermore, in other tumor antigens most of the CD4+ T cell epitopes identified are presented in the context of HLA-DR (80/93; see database on http://www.cancerimmunity.org). However, in cervical cancer there seems to exist a more prominent role for HLA-DQ and HLA-DP restricted T cells, arguing that strategies, incorporating computer algorithms, to identify functional T-cell responses against HPV should not be focused on HLA-DR only (35, 36). The basis for this unusual frequent use of HLA-DQ and –DP warrants further investigation.

In 7 patients an HPV-specific CD8+ T-cell response was detected, while 23 tumor-infiltrating lymphocyte cultures were HPV-specific. Considering the fact that CD8+ T cells were readily expanded from the tumour biopsies this outcome suggests that patients fail to induce an HPV-specific CD8+ T-cell response, that HPV-specific CD8+ T cells are not able to home to the tumours, or that these T cells have inherent limitations in their capacity to proliferate in vitro either due to exhaustion or because they are dysfunctional (37). The HPV-specific CD8+ T cells that could be expanded from tumours were shown to recognize 3 novel HLA-B7, HLA-B14 and HLA-B27 restricted CD8 T cell epitopes and confirmed the that HLA-A*0201-restricted tumor-infiltrating CD8+ T cells could recognize the HPV16 E7.11-20 epitope (15, 16), albeit that stronger reactivity was observed against the peptide sequence 11-19 (data not shown). In addition, CD8+ T cells reactive to the HLA-B57 restricted epitope HPV16E6.52-61 were detected. Based on the detection of HLA-B57-restricted HPV16E6.52-61-specific CD8+ T cells in the peripheral blood of healthy subjects it has been suggested that this CTL epitope may play an important role in clearing HPV16-infection (38, 39). The detection of HPV16E6.52-61-specific CD8+ T cells in tumours indicate that they may also contribute to the anti-tumour response.

In cervical cancer patients the circulating HPV16-specific CD4+ T cells fail to produce detectable quantities of IFNγ when stimulated with their cognate antigen (5). Here, we show that most of the tumour-infiltrating cultures contain HPV-specific CD4+ T cells which can produce .IFNγ, albeit low concentrations (Figure 1). This indicates that the HPV-specific immune response in cervical cancer patients does comprise IFNγ-producing T cells. The number of these IFNγ-producing HPV-specific T cells is likely to be too low in that they are not detectable in the circulation. However, they can be detected in TIL following a 2-3 week expansion in vitro (Figure 1) and certainly following additional antigen-specific stimulations (Figures 2-4).

Our study shows that in at least 23 of the 54 different HPV16 or HPV18 positive patients, a specific T cell response to E6 and/or E7 can be detected (Table 1). It is possible that vaccination strategies, aiming at the induction of a T-cell response to these antigens, can reinstate an effective anti-tumor response in those patients with a pre-existing immune response. Note, however, that in some cases these pre-existing HPV-specific T cells comprise Th2 cells, non-polarized T cells (5) or even regulatory T cells (40) indicating that vaccination does not necessarily induce HPV-specific T cells capable of attacking the tumor. Importantly, the T-cell epitopes recognized by the T cells in this study constitute physiological targets in the immune response to HPV16 and HPV18 positive tumors. As such they will be valuable for the integrated analysis of the magnitude and

functionality of HPV-specific T-cell subsets at different stages of disease and for the monitoring of immunotherapy trials. The frequent presence of HPV-specific T cells in cervical cancer patients may also constitute a valuable source of tumor-specific T cells that can be used in adoptive T-cell transfer therapies.

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