CHAPTER 3

An unexpectedly large polyclonal repertoire of HPV-specific T cells are poised for action in patients with cervical cancer

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ABSTRACT

The diversity and extent of the local tumor-specific T-cell response in a given individual is largely unknown. We have performed an in-depth study of the local T-cell repertoire in a selected group of cervical cancer patients, by systematic analyses of the proportion, breadth and polarization of HPV E6/E7-specific T cells within the total population of tumor-infiltrating lymphocytes (TIL) and tumor-draining lymph node cells (TDLNC). Isolated T cells were stimulated with sets of overlapping E6 and E7 peptides and analyzed by multiparameter flow cytometry with respect to activation, cytokine production and T-cell receptor Vβ (TCRVβ) usage. HPV-specific CD4+ and CD8+ T-cell responses were detected in TIL and TDLNC and their relative contribution varied between <1% to 66% of all T cells. In general, these HPV-specific responses were surprisingly broad, aimed at multiple E6 and E7 epitopes and involved multiple dominant and subdominant TCRVβ's per single peptide-epitope. In most patients only few IFNγ-producing T cells were found and the amount of IFNγ produced was low suggesting that these are poised T cells, rendered functionally inactive within the tumor environment. Importantly, stimulation of the TIL and TDLNC with cognate antigen in the presence of commonly used Toll like receptor-ligands, significantly enhanced the effector T-cell function. In conclusion, our study suggests that within a given patient with HPV-specific immunity many different tumor-specific CD4+ and CD8+ T cells are locally present and poised for action. This vast existing local T-cell population is awaiting proper stimulation and can be exploited for the immunotherapy of cancer.

INTRODUCTION

Our current knowledge on the diversity and extent of tumor-specific T-cell immunity is largely based on pooled T-cell response data of many different subjects (1-4), the T-cell response to a specific epitope (5;6) and/or studies of tumor-specific T-cell clones (7-9). However, little is known about how extensively one individual's immune system can simultaneously respond to one or more tumor antigens. Cervical carcinoma offers an excellent opportunity to answer this question as they are caused by the high-risk human papillomavirus (HPV) which encodes two defined tumor-specific viral antigens E6 and E7 that are constitutively expressed in each cancer cell (10). Notably, cervical cancer arises more frequently in immunocompromised individuals (11), illustrating the role of T cells in this type of cancer.

Low levels of circulating HPV E6- and E7-specific T cells in patients with cervical cancer or premalignant lesions (4;12-15) indicate that these oncoproteins activate an anti-tumor response. Indeed, cervical tumors are infiltrated by lymphocytes (16) and both CD8+ and CD4+ T cells isolated from such tumors are able to recognize the E6 and E7 tumor antigens (17;18). Furthermore, we showed that 43% of the isolated tumor-infiltrating lymphocyte (TIL) and tumor-draining lymph node cell (TDLNC) cultures from a large cohort of HPV16 or HPV18 positive patients contained T cells specific only for the E6- and/or E7-peptides corresponding to the HPV type present in the tumor (3). When the tumor was negative for HPV16 or 18, the TIL did not react to these HPV16 or HPV18 peptides (3). While these studies clearly indicate that HPV-specific T cells can infiltrate HPV-induced cervical cancer in a substantial number of patients, they do not allow a full comprehension of the contribution and role of HPV-specific TIL and TDLNC to the total tumor-specific immune response. The size of the HPV-specific T-cell pool in the TIL population, the polarization of these T cells and the breadth of this local HPV-specific response within a given individual with an HPV-specific response is unknown. It is important to gain such insights because of several therapeutic strategies under development (19-21). These include vaccines to enhance E6- and/or E7-specific T-cell reactivity (22-27) the results of which could be influenced by the presence of a preexisting HPV-specific local immune response.

Therefore, we performed a more in-depth study of the populations of TIL and TDLNC of patients for which we previously showed that they comprised HPV16- or HPV18-specific T cells (3) by comprehensive analyses of the HPV-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T cells.. Our study revealed that many different HPV-specific T cells are present, but need proper stimulation to become full effector cells.

METHODS

Subjects

Women presenting with histologically proven cervical neoplasia (FIGO 1a2, 1b1/2) at the department of Gynecology of the Leiden University Medical Centre were enrolled in the CIRCLE study, which investigates cellular immunity against cervical lesions after providing informed consent. The study was approved by the Medical Ethical Committee. The subjects were tested for HPV status using HPV16 and HPV18 specific primers on DNA isolated from resection specimens (28).

Antigens

A set of 22-mer peptides overlapping by 12 residues spanning both HPV16 and HPV18 E6 and E7 protein were synthesized and dissolved as described earlier (13;27) and used for T-cell stimulation assays.

Isolation and culture of T cells

Cervical tumor biopsies were obtained from patients with radical hysterectomy as described previously (3). Briefly, fresh cervical tissue was minced 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). At day 1, 5 ng/ml IL-7 (Peprotech) was added to cultures to drive homeostatic expansion of T cells. This does not alter the CD4/ CD8 T-cell composition of TIL(3), but it allows the acquisition of sufficient numbers of T cells for immunological assays. After 2-3 weeks of T-cell expansion (mean $18x10^6$ cells, range 4 - $40x10^6$) the T cells were harvested and stored in liquid nitrogen.

TDLN derived from the pelvic region contained tumor cells, indicative of metastatic cancer. The TDLN were cut into pieces and incubated 1 hour at 37 ºC with collagenase (200 IU/ml, Sigma) and DNAse (50 μg/ml, Sigma), and put through a cell strainer (BD, Erebodemgem, Belgium) to obtain single cells. TDLNC were not expanded but directly stored in liquid nitrogen.

Analysis of T-cell specificity by proliferation assay

T cells (25,000-50,000/well) were stimulated with autologous monocytes or irradiated autologous Epstein-Barr virus transformed B cell lines (B-LCL's) pulsed with HPV16 or 18 E6 and E7 peptides (5 μg/ml) in triplicate wells in a 3-day proliferation assay. After 48 hours supernatant was harvested and stored at -20ºC for cytokine analysis. During the last 16 hours of culture 0.5 μCi/well [3H]thymidine was added to measure proliferation (27) The stimulation index (SI) was calculated as the average of test wells divided by the average of the medium control wells. An SI >2 was considered a positive response. Antigen-specific IFNγ and IL-10 production was measured by ELISA (29). Antigen-specific cytokine production was defined by a cytokine concentration above the cut-off value (IFNγ 100pg/ml; IL-10 20 pg/ml) and >2x the concentration of the medium control (4).

Analysis of T-cell specificity by multiparameter flow cytometry.

T cells were examined directly *ex-vivo* (TDLNC) or after homeostatic expansion (TIL) to quantify the number of HPV-specific T cells. B-LCL were pulsed with 5 ug/ml HPV-16 or 18 E6 and E7 peptide pools. TIL's or TDLNC were thawed, rested in IMDM (BioWhittaker, Verviers, Belgium) containing 10% Fetal Calf Serum (FCS, PAA laboratories, Pasching, Austria) for 5 hours and seeded into a 96-wells round bottom plate at 200,000 cells per well and 40,000 antigen-pulsed B-LCL were added. After one hour Brefeldin A (10ug/ml) was added to the culture and left overnight. Cells were stained with antibodies to CD154-PECy5, CD137-APC, CD3-Pacific Blue, CD4-PECy7, CD8-APCcy7, IFNγ-FITC and IL-2-PE (all from BD Pharmingen, the Netherlands)(30).

In addition, TDLNC and TIL were stimulated with a mix of irradiated autologous B-LCL and 5 ug/ml HPV16 or HPV18 E6 or E7 peptide pools and irradiated allogeneic PBMC pool, in order to obtain enough HPV-specific T cells to measure the breadth of the response with respect to single peptide-antigens and for TCRVβ usage analyses (8 sets of antibodies). After a 3-week rest period these cells were tested for their specificity by overnight incubation with each single

peptide. Responses were considered positive when the percentage of HPV stimulated CD154 and/or CD137 positive cells was at least three times the medium control.

Analysis of the breadth of the HPV-specific T-cell response.

In vitro expanded T cells were stimulated with the indicated single peptides of HPV-16 or 18 E6 and E7 (5 ug/ml). Per peptide 500.000 cells were analyzed by flow cytometry as described above. One day later this analysis was repeated for those peptides found positive, but then the antibodies to the cytokines were replaced by antibodies to different TCRVβ (Beckman Coulter, Immunotech, France). A TCRVβ was considered dominant (>10%), subdominant (3-10%) or minor (<3%) on basis of the percentage of HPV-specific cells using the same TCRVβ.

RT-PCR

Expanded cultures were enriched for CD8+ T cells by negative selection using CD4+ isolation dynal beads (Invitrogen, the Netherlands). After RNA was isolated with the RNAeasy mini isolation kit (Qiagen, the Netherlands), cDNA was synthesized using the iScript cDNA Synthese kit (Biorad). Vβ PCR was performed on amplicons as previously described (31). Primers were kindly provided by dr M.H. Heemskerk.

In vitro stimulation with peptides and TLR ligands.

TDLNC were thawed, rested for 5 hours and stimulated with 1μg/ml pool of E6 and E7 peptides, TCGF 10% and IL15. Toll like receptor (TLR) 4 ligand LPS 250 ng/ml (Sigma-Aldrich, USA), TLR3 ligand Poly(I:C) 12.5 μg/ml (InvivoGen, USA) and TLR1-2 ligand Pam3CSK4 20 μg/ml (InvivoGen, USA) were added at the start of culture where indicated. To stimulate TIL, monocytes were pulsed with 1 ug/ml of E6 and E7 peptide pool and the indicated TLR ligands. After 5 hours monocytes were washed and TIL were added. Supernatant was taken every 2 days and analyzed by human Th1/Th2 cytometric bead array (BD Pharmingen, USA). Cells were left to rest for 2-3 weeks before analysis of the percentage of activated and cytokine producing cells by flow cytometry.

RESULTS

Quantification of HPV-specific T cells in tumor and lymph nodes.

TDLNC and homeostatic cytokine-mediated expanded TIL isolated from a selected group of 16 cervical carcinoma patients, comprising 10 patients of whom it was known that their TIL (8 patients) or TDLNC (2 patients) contained T cells that specifically reacted to the peptides of the HPV type present in the tumor and 3 patients in whom we previously failed to detect HPV-specific immunity (3), and 3 patients with unknown reactivity.

The presence of HPV-specific T cells within these homeostatic cytokine-mediated expanded TIL cultures was analyzed by their capacity to proliferate upon stimulation with HPV E6 and E7 peptides (Table 1). As expected proliferating HPV-specific T cells were found in 8/12 tumors tested. All HPV-specific T-cell cultures produced IFNγ, yet the amount of production varied greatly (103 - >5000 pg/ml), irrespective of the proliferative capacity of the cells. In addition, 4/8 positive cultures produced IL-10 (45 – 836 pg/ml). All TIL reacted to PHA and proliferation was associated with large amounts of IFNγ and IL-10 (not shown).

Table 1. Analysis of TIL and TDLNC before antigen specific expansion in vitro

				3 day Proliferation assay					Overnight activation analysis		
									Activated cells (%)§		
Patient	HPV type*	Origin	Days of culture††	Reactivity**	SI†	IFNγ‡	IL10‡	T-cell	Medium	E6	E7
1	16	TIL	9	16E6	2,9	103	<20	CD4	0,16	0,58	0,26
2	18	TIL	13	18E7	5,4	763	45	CD4	0,04	0,03	0,17
3	16	TIL	15	-				-	-	-	-
4	16	TIL	26	16E6	104	>5000	315	CD4	0,8	63	0,9
								CD8	0	47	0,7
5	16	TIL	79	16E6	109	>5000	836	CD4	3,1	66	2,7
6	18	TIL	25	-				-	-	-	-
7	16	TIL	12	-				CD8	2,9	24	3,4
8	16	TIL	29	-				-	-	-	-
9	16	TIL	17	16E6	2,6	110	<20	CD8	0,4	4,5	0,6
10	16	TIL	27	16E6	4,8	>5000	<20	CD4	0,13	0,52	0,14
11	18	TIL	30	18E7	8,9	244	66	NT‖			
12	16	TIL	68	16E6	4	276	<20	NT			
13	18	LN	0	NT				-	-	-	-
14	16	LN	0	NT				CD4	0,12	0,63	0,16
								CD8	0,23	5,92	0,11
15	16	LN	0	NT				CD4	0,51	24	20
16	16	LN	0	NT				-	-	-	-

* HPV type found in the tumor by PCR
†† Days of cytokine mediated homeostatic expansion before immune assay
** Reactivity indicates the HPVtype and protein to which the T-cell culture specifically reacted
† SI, Stimulation Index, average proliferation of test wells divided by the average profileration of medium control wells. SI>2 is positive
‡ Antigen-specific cytokine production in pg/ml is indicated when test value was above cut-off level and at least >2 times background production (4)
§ Percentage of total CD4 or CD8 T cell population expressing CD154 and/or CD137 after stimulation with indicated antigen corresponding to the HPV type present in the tumor. A response equal or more than 3x medium control was considered positive. || NT = Not tested

To type and enumerate HPV-specific T cells within the TIL or TDLNC populations, the percentage of CD4+ and CD8+ T cells specifically expressing the activation markers CD137 and/or CD154 when stimulated with E6 and E7 peptides or proteins was analyzed. CD137 is known as an activation marker for CD8+ T cells and CD154 for CD4+ T cells (32;33). The TIL cultures that were negative in the proliferation assay were taken along as control. In 7/10 TIL cultures tested and 2/4 TDLNC, HPV specific activated T cells were detected and comprised CD4+ or CD8+ T cells or both (Table 1). In two cases not enough TIL were available to perform this analysis. In one TIL culture – in which we previously failed to detect HPV-specific T cells by proliferation - an HPV-specific CD8+ T-cell response was detected. The percentage of HPV-specific T cells generally corresponded with the strength of proliferation. HPV-specific CD4+ T cells expressed both CD154 and CD137, while HPV-specific CD8+ T cells predominantly expressed CD137, yet sometimes co-expressed CD154 (Figure 1a; Supplemental Figure 1). The percentage of HPV-specific T cells varied enormously between as little as 0.17% to as much as 66% (Table 1) of the

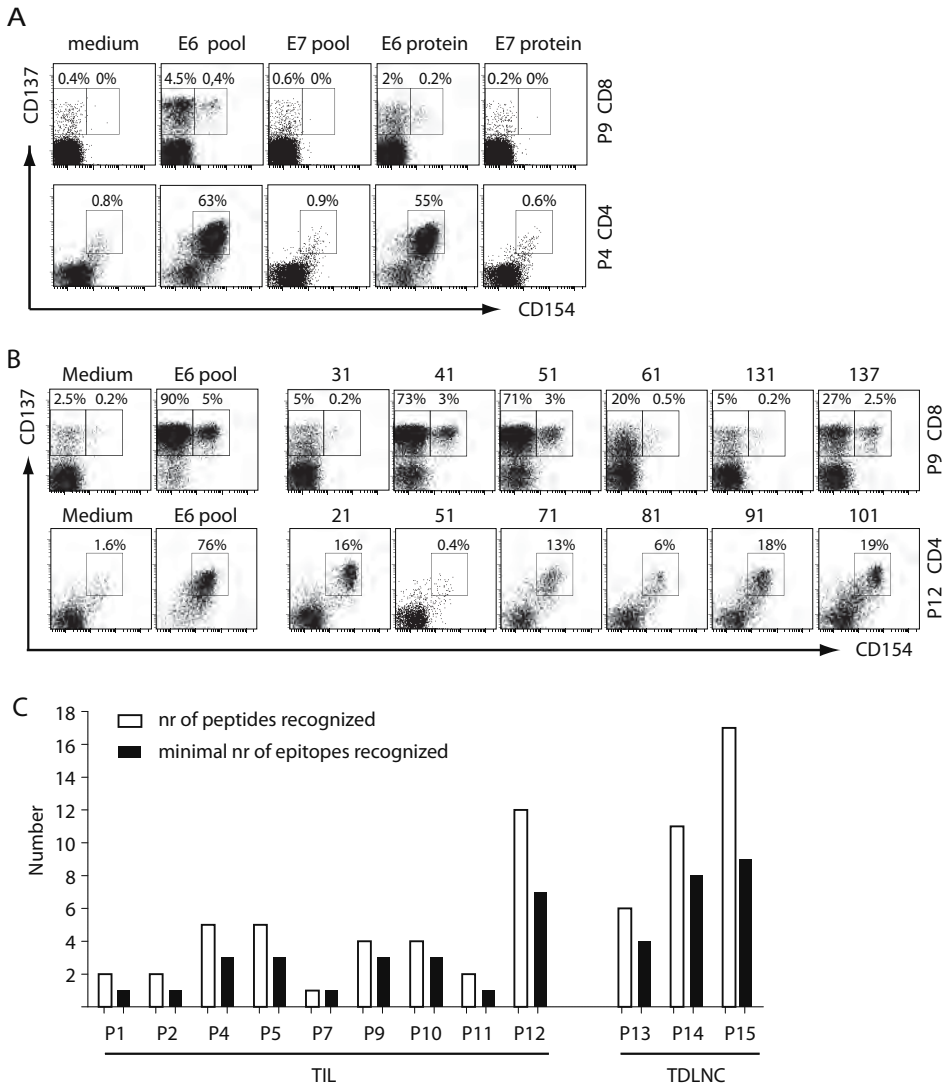


Figure 1. HPV-specific T cells in TIL and TDLNC. (A) An example of CD4 and CD8 HPV-specific responses measured by flow cytometry using the activation markers CD154 and CD137 in two TIL cultures tested before antigen-driven expansion *in vitro* (P4 and P9). (B) The breadth of the response was analyzed after antigen-driven expansion. TIL and TDLNC cultures were stimulated with single peptides and analyzed for the expression of CD154 and CD137. P9 displayed a CD8 response against four different single peptides (notably 41, 51, 61 and 137). P12 displayed a CD4 response against six different single peptides (notably 21, 61, 71, 81, 91 and 101). (C) Summary of the number of different peptides recognized by each single culture (white bars) after antigen-driven expansion. The minimal number of epitopes recognized (black bars) was estimated by counting the response to two 22-mer peptides, which overlap by 12 amino acids, as one.

Table 2. Different TCRVß families found within the HPV-specific T cell population

	Patient	Responding* T cell	Antigen	Peptides**	Number of TCRVß found per peptide** by FACS†			
					Dominant	Sub-dominant	Minor	Total
TIL	2	CD4	E7	1	0	0	4	4
				11	0	2	4	6
	4	CD4	E6	41	1	0	0	1
				51	1	0	0	1
		CD8	E6	41	2	1	3	6
				51	2	1	3	6
		CD8	E7	21	3	2	0	5
	7	CD8	E6	21	0‡	3	4	7‡
	9	CD8	E6	41	0	1	1	2
				51	1	0	3	4
				61	1	1	3	5
				137	0	2	5	7
	10	CD8	E6	131	2	0	0	2
				137	2	0	0	2
		CD4	E7	1	2	0	0	2
	11	CD4	E6	21	1	2	0	3
				71	2	0	1	3
				81	0	1	1	2
				101	1	3	0	4
	12	CD8	E6	1	1	0	3	4
				11	1	0	3	4
				41	1	1	4	6
				51	1	3	2	6
LN	14	CD4	E6	11	2	0	8	10
				61	1	1	5	7
				71	1	1	5	7
				81	2	2	0	4
				91	2	2	0	4
				101	1	3	3	7
				121	2	6	2	10
				131	0	0	2	2
				137	3	2	0	5
	15	CD4	E6	21	2‡	0	0	2‡
				51	1	0	0	1
				81	1	2	1	4
				111	1	1	0	2
				121	0	2	0	2
				131	1	1	3	5

* Depicted are all patients of whom enough T cells were available for TCR Vß analysis after antigen specific expansion by stimulation with E6 or E7 peptide pool

** The number indicates the first aminoacid of the 22-mer peptide of the antigen that the culture specifically responds to by the expression of the activation markers CD154 and/or CD137

† A dominant TCRVß consists of >10% of the activated T cells. A sub-dominant TCR Vß consists of between 3% and 10% of the activated T cells. A minor TCR Vß consists of < 3% of the activated T cells

‡ Additional TCRVβ's were found by PCR analysis for P7 and P15

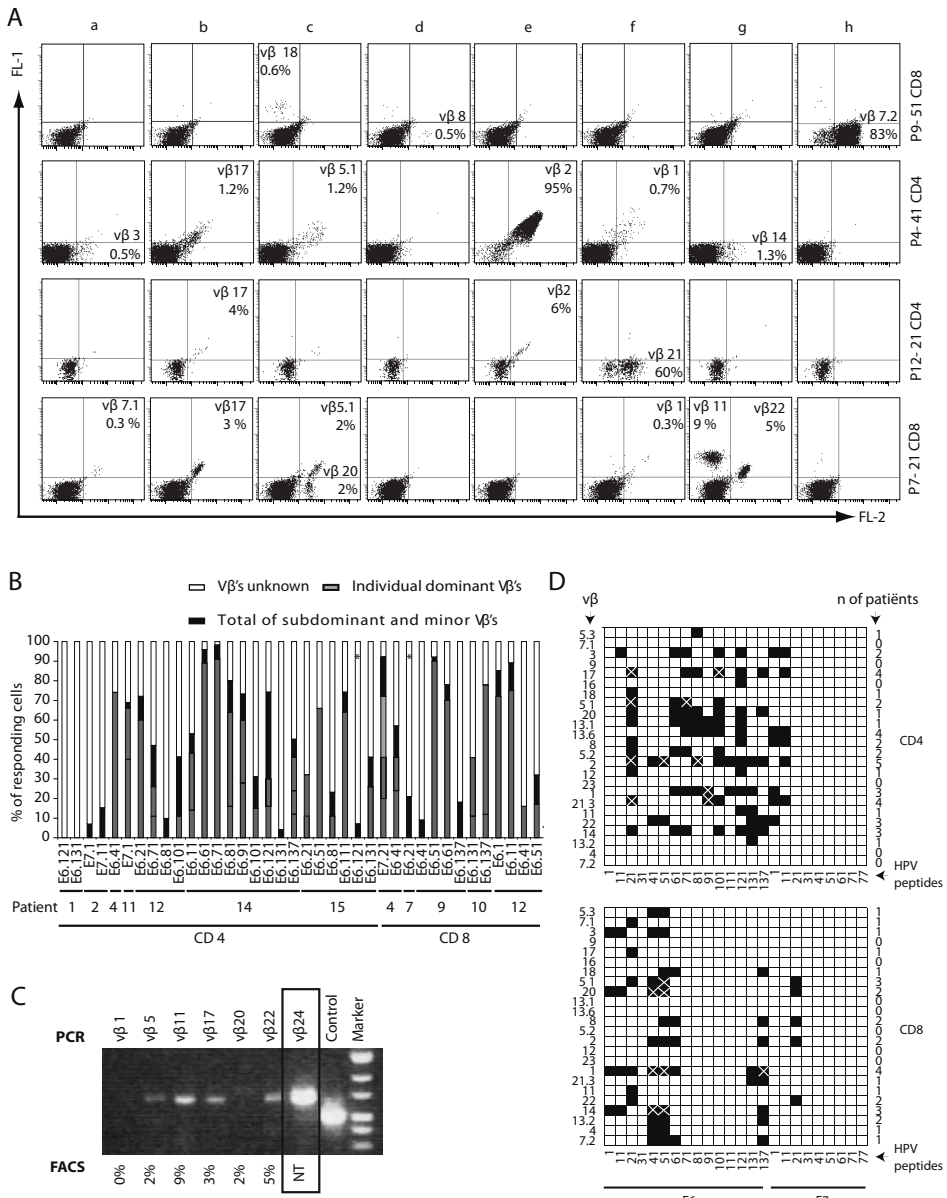
CD4+ or CD8+ T cells present in TIL and from 0.63% - 24% in TDLN. This was independent of the total numbers of T cells isolated, indicating a great variability in the contribution of HPV-specific T cells to the total local anti-tumor response between patients.

The local HPV-specific response consists of a broad T-cell repertoire

To study the breadth of the HPV-specific TIL/TDLNC-repertoire the isolated cells were stimulated with pools of E6 or E7 peptides as otherwise there would not be enough cells to study the response to single peptides. This allowed the analysis of the breadth of the HPV-specific T-cell response – based on antigen-specific expression of CD154 and/or CD137 - to single peptides in 12 patients (Figure 1b; Supplemental Table 1). The three TIL cultures tested negative before remained negative excluding priming *in vitro*. In most of the patients the HPV-specific CD4+ T-cell response was highly diverse as in 5/10 patients CD4+ T cells responded to ≥5 different peptides and in another 4 patients the CD4+ T cells recognized 2 different peptides (Supplemental Table 1). HPV-specific CD8+ T-cell reactivity was detected in 6 of the 12 tested patients, five of whom displayed CD8+ T-cell reactivity to 2-6 different peptides (Figure 1b; Supplemental Table 1).

The minimal number of T-cell epitopes recognized per patient was estimated by counting the response to two overlapping peptides as one because they overlapped by 12 amino acids. The majority of the patients (8/12) recognized ≥3 different T-cell epitopes (Figure 1c). The TDLNC populations reacted against 4-9 different epitopes (Figure 1c). Thus the tumor-induced HPV-specific T-cell repertoire is directed against multiple T-cell epitopes. Based on the patient's HLA-type 4 of the CD4- and 2 of the CD8-responses could involve a reaction against known HLA class I and II T-cell epitopes (not shown) (3;34;35). As each individual T-cell epitope can be recognized by different T-cell clones, we studied the number of TCRVβ families involved in the recognition of each epitope by using a commercially available TCRVβ-specific antibody kit. The different T-cell clones were operationally defined as the cohort of activated HPV single peptide-specific CD4+ or CD8+ T cells expressing the same TCRVβ-chain, within the population of specifically activated CD154+ and/or CD137+ T cells. Figure 2a shows examples of the contribution of several T-cell receptor families reactive to one single peptide. Often one or two dominant TCRVβ's were found (Table 2 and Figure 2b), as well as several sub-dominant and minor TCRVβ's. For example, the HPV-specific CD4+ T-cell response of patient P14 reacted to 9 different peptides and - on the basis of the different TCRVβ's present in the population of activated T cells - this involved the activation of at least 43 different T-cell clones (Table 2 and

Figure 2. The T-cell response to a single HPV epitope involves multiple dominant and subdominant TCRVβ families. (A) The TCRVβ families used by T cells responding to a single peptide were analysed within the T-cell population with high expression of activation marker(s) after peptide stimulation. Depicted is the response against one peptide for four different patients (P4, P7, P9 and P12). The letters (a-h) indicate the eight different antibody pools, each consisting of three differently labeled antibodies (FITC (FL1), PE (FL2) or FITC-PE combined) specific for three different TCRVβ, plotted in separate quadrants. The involved TCRVβ and the percentage of responding cells are indicated in the plots. (B) The relative contribution of dominant and subdominant TCRVβ families to the overall HPV-specific T-cell response is depicted. The grey stacked bars indicate the percentage of HPV-specific T cells comprising a dominant TCRVβ(s) and the black stacked bars indicate the accumulated percentage of all the sub-dominant and minor TCRVβ families (individual percentages for each family not shown) of all T cells responding to one peptide-epitope. The white bars indicate the percentage of HPV-specific T cells for which we could not identify the TCRVβ ▶



▶ families involved using the TCRVβ kit. Patient numbers and 22-mer peptides recognized are indicated by the protein name and the first amino acid in the sequence of this protein. The * identifies the two cultures for which an additional TCRVβ analysis was done by RT-PCR allowing the detection of other TCRVβ's not present in the TCRVβ kit. (C) RT-PCR for TCRVβ in TIL of patient P7, revealing the presence of TCRVβ24. The percentage of each TCRVβ that was detected by flow cytometry is indicated on the left. (D) A fingerprint overview of all TCRVβ's detected upon response to a single peptide-epitope for the patients investigated. The black squares indicate that the TCRVβ (indicated at the left) was involved in response to that particular peptide (indicted at bottom). A white cross means that this TCRVβ was found in two independent patients responding to that peptide, the right column indicates in how many patients this TCRVβ was found.

Figure 2b). In a number of cases not all TCRVβ could be identified as the available antibodies cover approximately 70-80% of the full TCRVβ-repertoire (36). In one case (P7) where all cultured T cells responded exclusively to one peptide - but for which only 22% of the HPV-specific T cells the TCRVβ was accounted for by antibodies (Figure 2a, fourth row and Table 2) - a semi-quantitative RT-PCR was applied revealing the presence of TCRVβ24 (Figure 2c). In another case (P15) only 30% of the TCRVβ were accounted for by antibodies. Here, three additional TCRVβ 6C, 6D and 15 (not shown) were detected by RT-PCR. No skewing to a certain TCRVβ within this patient group was found, nor was there any skewing of certain TCRVβ families to individual peptides observed (Figure 2d). Thus the HPV-specific T-cell repertoire consists of a polyclonal T-cell population able to respond to many different CD4 and CD8 T-cell epitopes.

HPV-specific TIL generally lack type 1 polyfunctional T cells.

The production of IFNγ and IL-2 is essential for an effective anti-tumor response and instrumental to functionally characterize antigen-specific T cells (37-42). Therefore, the *ex-vivo* enumerated HPV-specific CD154+ and/or CD137+ T cells were simultaneously analyzed for their production of these cytokines. When specifically gated on the population of CD4 or CD8 T cells that expressed CD154 and/or CD137 after antigen-specific stimulation, four distinct cytokine profiles were found (Figure 3ab). Profile 1 was found in one patient (P7). Despite the presence of a high percentage of HPV E6-specific CD8+ T cells (24%), only about 4% produced either one of the cytokines. The second profile comprised HPV-specific T cells of which the majority produced both IFNγ and IL-2 (*e.g.* P4 and P5), HPV-specific T cells in the third profile mainly produced IL-2 (*e.g.* P1, P2, P10 and P15), whereas they produced mainly IFNγ in profile four (P14 and P9). These data indicate that while most of the HPV-specific TIL can produce either one of the type 1 cytokines, there are only few patients (2/9) in whom the majority of their HPV-specific TIL simultaneously produce IFNγ and IL-2 (Profile 2; Figure 3ab).

Activation of HPV-specific T cells in the presence of TLR ligands increases the type 1 cytokine effector response.

Our results showed that in many cases the HPV-specific T-cell response is not associated with strong production of IFNγ (Table 1 and Figure 3ab). In mouse models, the local injection of TLR2, TLR3 or TLR4 ligands can augment the tumor response (43-45). To mimic the local delivery of antigen and TLR ligand, homeostatic expanded TIL from 2 patients were stimulated with HPV antigen-pulsed TLR-activated autologous monocytes whereas TDLNC from 2 other patients, which already contained APC, were activated with their cognate HPV-antigens in the absence or presence of TLR-agonist directly *ex-vivo*. Cytokine analyses revealed a faster and higher production of IFNγ during the first 7 days after activation in all four TIL and TDLNC cultures tested when PAM3CSK4 (TLR2) was added and in 3 out of 4 of the cultures with poly(I:C) (TLR3) (Figure 3c). The use of the TLR4 agonist LPS boosted the IFNγ-response in one patient (P14), but with somewhat slower kinetics. Notably, PAM3CSK4 (TLR2) also increased the production of the Th2 cytokine IL-5 in 3 of 4 cultures (not shown).

Analysis of the constitution of the responding cell population after 14 days, allowing the activated T cells to come to rest needed to decrease background staining for the activation markers and cytokines, revealed no overt differences in the number of activated cells or the percentage of IFNγ, IL-2 or double- producing T cells within the CD154- and/or CD137-expressing HPV-specific T-cell population after this period (not shown).

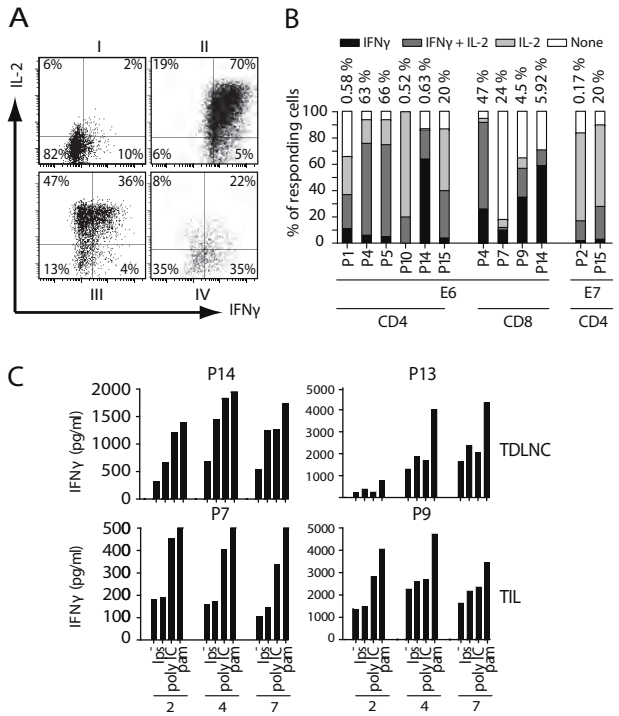


Figure 3. Functionality of HPV-specific TIL and TDLNC . (A) The specific activity to HPV16 or 18 E6 and E7 peptide pools by simultaneous analysis of CD154, CD137, IFNγ and IL-2 in homeostatic cytokine-mediated expanded TIL as well as directly *ex-vivo* in TDLNC. Four different cytokine profiles could be distinguished within the population of HPV antigen-induced CD154- and/or CD137-expressing T cells. Profile I: HPV-specific T-cells with few T-cells producing cytokines (P7). Prolife II: Predominant production of IFNγ and IL-2 (P4). Profile III: predominant production of IL-2 (P15). Profile IV: mainly IFNγ producing HPV-specific T cells (P9). (B) Overview of the cytokine production. The percentage of activated HPV-specific T-cells is indicated on top of the bars. The stacked bars indicate the percentage and type of cytokines (black: IFNγ, grey: IL-2, hatched: IFNγ+IL-2 and white: no IFNγ or IL-2 (none)) produced within the activated HPV-specific T-cell population. (C) The homeostatic expanded TIL of patients 7 & 9 as well as the TDLNC of patients 13& 14 were stimulated with HPV16/18 E6 or E7 peptide pools and TLR-agonist when indicated (medium control indicates peptide stimulation without addition of TLR-agonist). After 2, 4 and 7 days supernatant was harvested and analyzed for cytokine production by cytokine bead array.

DISCUSSION

We have comprehensively analyzed the spontaneous tumor-specific immune response in patients with cervical cancer by dissecting local HPV E6- and E7-specific CD4+ and CD8+ T-cell responses down to the level of the percentage, specificity, cytokine polarization and number of different responding T-cells. The expression of the two known tumor antigens E6 and E7 in all cervical cancer cells and the use of overlapping peptide arrays in combination with the activation markers CD154 and CD137, offered the advantage to study the complete cervical cancer-specific local T-cell repertoire - independently of the knowledge of defined T-cell epitopes and not restricted to particular HLA-types – in a quantitative manner. We used a selected panel of

HPV16- and HPV18-typed cervical cancer patients for whom we previously showed that their TIL comprised HPV type-specific T cells, indicating that the current set of data applies to about 40-50% of all patients with an HPV16- or HPV18-positive cervical carcinoma (3). Our data show that while HPV-specific T-cell responses can be detected within the tumors and tumor draining lymph nodes of this group of patients with cervical cancer their relative contribution to the overall local anti-tumor response varied enormously, ranging from <1% to 66% (Table 1). While we can't exclude that the quantification of HPV-specific T cells within the total population of TIL is accurate as it likely to be biased due to the isolation procedure, the results obtained in the *ex-vivo* measurement of HPV-specific T cells among TDLNC still sustains this notion. Strikingly the HPV-specific response of most patients tested was broad as it targeted multiple peptide-epitopes within the E6 and E7 tumor-specific antigens and the T-cell response to each and every peptide-epitope involved multiple dominant and/or subdominant TCRVβ families, or T-cell responses to multiple different epitopes within a single peptide (Table 2, Figure 2). One could argue that our analysis concerning the breadth of the response is biased through the expansion of TIL by either homeostatic cytokines or peptide stimulation, as these rounds of expansion may not equally amplify all possible responding cells and less well proliferating HPV-specific T-cell clones may even become extinct. Yet in view of the broad responses observed already this would only mean that in reality the response is even broader and even now is still underestimated.

The broad and hierarchical responses closely resemble the published pattern of CD4+ and CD8+ T-cell responses to genetically stable viruses, such as CMV (36). This brings forward the question whether the HPV-specific T-cell responses observed in these cancer patients reflect a characteristic antiviral response or an anti-tumor response. As shown previously, patients with HPV-induced pre-malignant disease either fail to mount HPV-specific immunity (4;12) or induce a non-beneficial HPV-specific T-cell response during progression of disease (4;12). Therefore, we deem it more likely that the T-cell responses studied here reflect a typical tumor-specific T-cell response. Indeed, a similar hierarchy of the spontaneous T-cell response was observed in a study of two patients responding to NY-ESO-1 (46), and in the HLA-A*0201-restricted Melan-A/MART-1_{26-35}-specific CD8 T-cell response (6). The presence of single peptide-specific dominant and subdominant (based on TCRVβ-chain expression) T cells within the tumor tissue implies that subdominant TIL participate in the immune surveillance of tumors and not simply act as a reservoir. The different dominant and subdominant HPV-specific T cells may have different functions. This is illustrated by the isolation of both HPV-specific T-helper and T-regulatory cells from the same tumor in a group of cervical patients studied previously (47). Overall, the local HPV-specific immune response consists of a polyclonal T-cell population able to respond to many different CD4 and CD8 T-cell epitopes. We deem it unlikely that this breadth is overestimated due to potential cross-reactivity of the T cells with non-related peptide-MHC complexes as even between the highly homologous sequences of HPV16 and HPV18 this has never been observed (3;13).

CD4+ and CD8+ T cells as well as the cytokines IFNγ and IL-2 play a key role in the protection against cancer (4;37-40) as well as in the control of chronic viral infections (41;42). In most of the *ex-vivo* tested TIL and TDLNC the population of IFNγ and IL-2 producing T cells or IFNγ-producing T cells among the total population of HPV-specific T cells as well as the amount of IFNγ produced was low (Table 1 & Figure 3b), suggesting that most of the HPV-specific TIL and TDLNC with respect to the production of these cytokines are rendered functionally tolerant within the tumor environment and implying that the local tumor-specific immune response in

cervical cancer patients does not differ from others, such as melanoma (48). *In vitro* stimulation of these HPV-specific T cells with their cognate antigen resulted in an increase in the number of HPV-specific T cells (e.g. P9, compare Figure 1a and Figure 1b) as well as in an increased IFNγ production (data not shown). We recently reported that the majority of patients vaccinated with a HPV16 long peptide vaccine, displayed a broad vaccine-induced HPV-specific immune response as detected by IFNγ-ELISPOT (23;24). Most likely this vaccine taps the broad available T-cell repertoire we identified in this study and either primes (in patients without HPV-specific reactivity) or boosts their number. Interestingly, when TIL and lymph node derived T cells are *ex-vivo* stimulated with cognate antigen in the presence of TLR ligands, such as PAM3CSK4 or poly (I:C), a pronounced increase of effector function is observed (Figure 3c). This suggests that local delivery of these innate immune-derived stimulating factors can stimulate a stronger anti-tumor response in human cancers similar to murine tumor models (43-45). Moreover, they may assist therapeutic vaccines in driving T-cell responses with increased function as shown for a melanoma peptide vaccine in combination with CpG (49). Such type of responses is highly required as they correlate with clinical efficacy in murine models (43) and in human trials (50). Interestingly, the widely used TLR4 ligand LPS did not overtly increase IFNγ production of TIL and TDLNC in our study.

In conclusion, TIL or TDNLC isolated from 40-50% of patients with an HPV16 or HPV18-induced cervical tumor contain HPV type-specific T cells (3). Our study of the local T-cell repertoire within this group of patients suggests that within the tumor environment or tumor-draining lymph node of a given patient many different tumor-specific CD4+ and CD8+ T cells are poised for action but are awaiting proper stimulation.

REFERENCES

1. Kawakami Y, Eliyahu S, Jennings C, Sakaguchi K, Kang X, Southwood S, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. J Immunol 1995 Apr 15;154(8):3961-8.
2. Gnjatic S, Atanackovic D, Jager E, Matsuo M, Selvakumar A, Altorki NK, et al. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. Proc Natl Acad Sci U S A 2003 Jul 22;100(15):8862-7.
3. Piersma SJ, Welters MJ, van der Hulst JM, Kloth JN, Kwappenberg KM, Trimbos BJ, et al. 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. Int J Cancer 2008 Feb 1;122(3):486-94.
4. de Jong A, van Poelgeest MI, van der Hulst JM, Drijfhout JW, Fleuren GJ, Melief CJ, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired CD4+ T-cell immunity against early antigens E2 and E6. Cancer Res 2004 Aug 1;64(15):5449-55.
5. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, et al. Use of fluorogenic histocompatibility leukocyte antigen-A*0201/HPV 16 E7 peptide complexes to isolate rare human cytotoxic T-lymphocyte-recognizing endogenous human papillomavirus antigens. Cancer Res 2000 Jan 15;60(2):365-71.
6. Speiser DE, Baumgaertner P, Barbey C, Rubio-Godoy V, Moulin A, Corthesy P, et al. A novel approach to characterize clonality and differentiation of human melanoma-specific T cell responses: spontaneous priming and efficient boosting by vaccination. J Immunol 2006 Jul 15;177(2):1338-48.
7. Mandruzzato S, Rossi E, Bernardi F, Tosello V, Macino B, Basso G, et al. Large and dissimilar repertoire of Melan-A/MART-1-specific CTL in metastatic lesions and blood of a melanoma patient. J Immunol 2002 Oct 1;169(7):4017-24.
8. Sensi M, Traversari C, Radrizzani M, Salvi S, Maccalli C, Mortarini R, et al. Cytotoxic T-lymphocyte clones from different patients display limited T-cell-receptor variable-region gene usage in HLA-A2-restricted recognition of

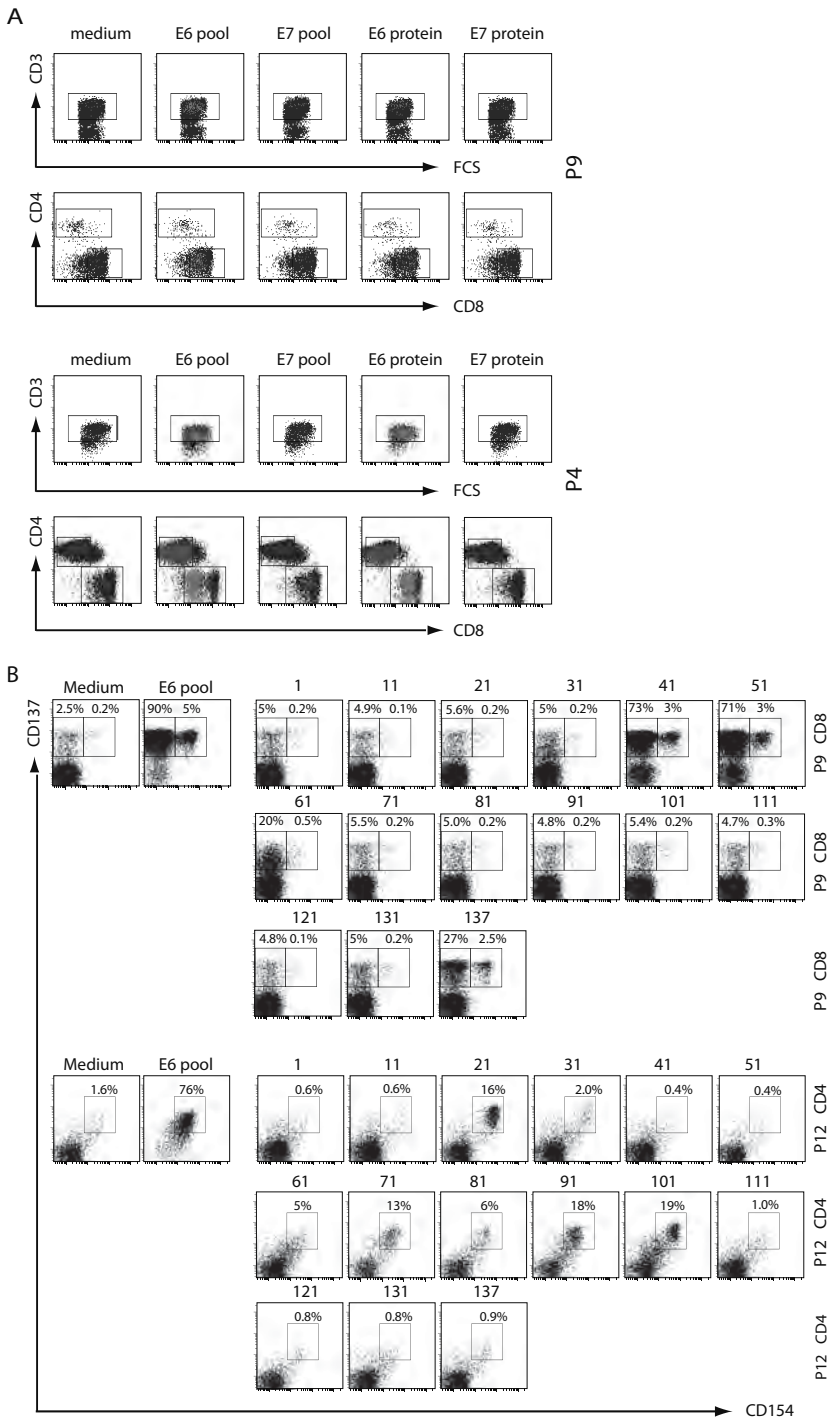
the melanoma antigen Melan-A/MART-1. Proc Natl Acad Sci U S A 1995 Jun 6;92(12):5674-8.

9. Lonchay C, van der BP, Connerotte T, Hanagiri T, Coulie P, Colau D, et al. Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. Proc Natl Acad Sci U S A 2004 Oct 5;101 Suppl 2:14631-8.
10. zur Hausen H. Papillomavirus infections--a major cause of human cancers. Biochim Biophys Acta 1996 Oct 9;1288(2):F55-F78.
11. Bouwes Bavinck JN, Berkhout RJ. HPV infections and immunosuppression. Clin Dermatol 1997 May;15(3):427-37.
12. de Vos van Steenwijk PJ, Piersma SJ, Welters MJ, van der Hulst JM, Fleuren G, Hellebrekers BW, et al. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16-specific T-cell response. Clin Cancer Res 2008 Nov 15;14(22):7188-95.
13. Welters MJ, van der LP, van den Eeden SJ, Kwappenberg KM, Drijfhout JW, Fleuren GJ, et al. Detection of human papillomavirus type 18 E6 and E7-specific CD4+ T-helper 1 immunity in relation to health versus disease. Int J Cancer 2006 Feb 15;118(4):950-6.
14. Bontkes HJ, de Gruijl TD, Bijl A, Verheijen RH, Meijer CJ, Scheper RJ, et al. Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia. J Gen Virol 1999 Sep;80 (Pt 9):2453-9.
15. Welters MJ, de Jong A, van den Eeden SJ, van der Hulst JM, Kwappenberg KM, Hassane S, et al. Frequent display of human papillomavirus type 16 E6-specific memory t-Helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003 Feb 1;63(3):636-41.
16. Piersma SJ, Jordanova ES, van Poelgeest MI, Kwappenberg KM, van der Hulst JM, Drijfhout JW, et al. High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res 2007 Jan 1;67(1):354-61.
17. Evans EM, Man S, Evans AS, Borysiewicz LK. Infiltration of cervical cancer tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res 1997 Jul 15;57(14):2943-50.
18. Hohn H, Pilch H, Gunzel S, Neukirch C, Freitag K, Necker A, et al. Human papillomavirus type 33 E7 peptides presented by HLA-DR*0402 to tumor-infiltrating T cells in cervical cancer. J Virol 2000 Jul;74(14):6632-6.
19. Yuan J, Gnjatic S, Li H, Powel S, Gallardo HF, Ritter E, et al. CTLA-4 blockade enhances polyfunctional NY-ESO-1 specific T cell responses in metastatic melanoma patients with clinical benefit. Proc Natl Acad Sci U S A 2008 Dec 23;105(51):20410-5.
20. Keilholz U. CTLA-4: negative regulator of the immune response and a target for cancer therapy. J Immunother 2008 Jun;31(5):431-9.
21. Blank C, Gajewski TF, Mackensen A. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. Cancer Immunol Immunother 2005 Apr;54(4):307-14.
22. Frazer IH. Prevention of cervical cancer through papillomavirus vaccination. Nat Rev Immunol 2004 Jan;4(1):46-54.
23. Kenter GG, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Phase I immunotherapeutic trial with long peptides spanning the E6 and E7 sequences of high-risk human papillomavirus 16 in end-stage cervical cancer patients shows low toxicity and robust immunogenicity. Clin Cancer Res 2008 Jan 1;14(1):169-77.
24. Welters MJ, Kenter GG, Piersma SJ, Vloon AP, Lowik MJ, Berends-van der Meer DM, et al. Induction of tumor-specific CD4+ and CD8+ T-cell immunity in cervical cancer patients by a human papillomavirus type 16 E6 and E7 long peptides vaccine. Clin Cancer Res 2008 Jan 1;14(1):178-87.
25. Ferrara A, Nonn M, Sehr P, Schreckenberger C, Pawlita M, Durst M, et al. Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. J Cancer Res Clin Oncol 2003 Sep;129(9):521-30.
26. Santin AD, Bellone S, Palmieri M, Zanolini A, Ravaggi A, Siegel ER, et al. Human papillomavirus type 16 and 18 E7-pulsed dendritic cell vaccination of stage IB or IIA cervical cancer patients: a phase I escalating-dose trial. J Virol 2008 Feb;82(4):1968-79.
27. van der Burg SH, Ressing ME, Kwappenberg KM, de Jong A, Straathof K, de Jong J, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 2001 Mar 1;91(5):612-8.
28. Claas EC, Melchers WJ, van der Linden HC, Lindeman J, Quint WG. Human papillomavirus detection in paraffin-embedded cervical carcinomas and metastases of the carcinomas by the polymerase chain reaction. Am J Pathol 1989 Oct;135(4):703-9.
29. van der Burg SH, Kwappenberg KM, Geluk A, van der KM, Pontesilli O, Hovenkamp E, et al. Identification of a conserved universal Th epitope in HIV-1 reverse transcriptase that

is processed and presented to HIV-specific CD4+ T cells by at least four unrelated HLA-DR molecules. J Immunol 1999 Jan 1;162(1):152-60.

30. Speetjens FM, Kuppen PJ, Welters MJ, Essahsah F, Voet van den Brink AM, Lantrua MG, et al. Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer. Clin Cancer Res 2009 Feb 1;15(3):1086-95.
31. Heemskerk MH, Hoogeboom M, de Paus RA, Kester MG, van der Hoorn MA, Goulmy E, et al. Redirection of antileukemic reactivity of peripheral T lymphocytes using gene transfer of minor histocompatibility antigen HA-2-specific T-cell receptor complexes expressing a conserved alpha joining region. Blood 2003 Nov 15;102(10):3530-40.
32. Wolfl M, Kuball J, Eyrich M, Schlegel PG, Greenberg PD. Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. Cytometry A 2008 Nov;73(11):1043-9.
33. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med 2005 Oct;11(10):1113-7.
34. Gallagher KM, Man S. Identification of HLA-DR1- and HLA-DR15-restricted human papillomavirus type 16 (HPV16) and HPV18 E6 epitopes recognized by CD4+ T cells from healthy young women. J Gen Virol 2007 May;88(Pt 5):1470-8.
35. Wang X, Moscicki AB, Tsang L, Brockman A, Nakagawa M. Memory T cells specific for novel human papillomavirus type 16 (HPV16) E6 epitopes in women whose HPV16 infection has become undetectable. Clin Vaccine Immunol 2008 Jun;15(6):937-45.
36. Bitmansour AD, Waldrop SL, Pitcher CJ, Khatamzas E, Kern F, Maino VC, et al. Clonotypic structure of the human CD4+ memory T cell response to cytomegalovirus. J Immunol 2001 Aug 1;167(3):1151-63.
37. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001 Apr 26;410(6832):1107-11.
38. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell 1993 Apr 9;73(1):5-8.
39. Melief CJ, Toes RE, Medema JP, van der Burg SH, Ossendorp F, Offringa R. Strategies for immunotherapy of cancer. Adv Immunol 2000;75:235-82.
40. Ostrand-Rosenberg S. CD4+ T lymphocytes: a critical component of antitumor immunity. Cancer Invest 2005;23(5):413-9.
41. Ciuffreda D, Comte D, Cavassini M, Giostra E, Buhler L, Perruchoud M, et al. Polyfunctional HCV-specific T-cell responses are associated with effective control of HCV replication. Eur J Immunol 2008 Oct;38(10):2665-77.
42. Harari A, Vallelian F, Meylan PR, Pantaleo G. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. J Immunol 2005 Jan 15;174(2):1037-45.
43. van Mierlo GJ, Boonman ZF, Dumortier HM, den Boer AT, Fransen MF, Nouta J, et al. Activation of dendritic cells that cross-present tumor-derived antigen licenses CD8+ CTL to cause tumor eradication. J Immunol 2004 Dec 1;173(11):6753-9.
44. Currie AJ, van der Most RG, Broomfield SA, Prosser AC, Tovey MG, Robinson BW. Targeting the effector site with IFN-alphabeta-inducing TLR ligands reactivates tumor-resident CD8 T cell responses to eradicate established solid tumors. J Immunol 2008 Feb 1;180(3):1535-44.
45. Asprodites N, Zheng L, Geng D, Velasco-Gonzalez C, Sanchez-Perez L, Davila E. Engagement of Toll-like receptor-2 on cytotoxic T-lymphocytes occurs in vivo and augments antitumor activity. FASEB J 2008 Oct;22(10):3628-37.
46. Jackson H, Dimopoulos N, Mifsud NA, Tai TY, Chen Q, Svobodova S, et al. Striking immunodominance hierarchy of naturally occurring CD8+ and CD4+ T cell responses to tumor antigen NY-ESO-1. J Immunol 2006 May 15;176(10):5908-17.
47. van der Burg SH, Piersma SJ, de JA, van der Hulst JM, Kwappenberg KM, van den HM, et al. Association of cervical cancer with the presence of CD4+ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A 2007 Jul 17;104(29):12087-92.
48. Mantovani A, Romero P, Palucka AK, Marincola FM. Tumour immunity: effector response to tumour and role of the microenvironment. Lancet 2008 Mar 1;371(9614):771-83.
49. Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 2005 Mar;115(3):739-46.
50. Kenter G, Welters MJ, Valentijn AR, Lowik MJ, Berends-van der Meer DM, Vloon AP, et al. Effective Treatment of Vulvar Intraepithelial Neoplasia by Vaccination against the Oncoproteins E6 and E7 of Human Papillomavirus Type 16. 2009.

SUPPLEMENTAL FIGURES



Supplemental figure 1. (A) Activated cells (CD154+ and/or CD137+) within CD3+ T cell population and within the CD3+ T-cell gated population expressing either CD4 or CD8. In these plots the homeostatic expanded TIL from two patients (P9 and P4) after stimulation with medium only, E6 or E7 peptide pools and E6 or E7 protein is shown. The peptide or proteinactivated CD154+ and/or CD137+ T cells as shown in Figure 1 where back-gated and indicated by a red color (activated CD8 T cells) or a blue color (activated CD4 T cells). Down regulation of CD8 is seen in the CD8+ T-cell population stimulated with the peptide pool and to a lesser extent in CD8 T cells stimulated with protein and this is not observed for CD4 on activated CD4 T cells. (B) An overview of all the single peptides used for analysis of the breadth of response after antigen-driven expansion for P9 and P12 (as partly shown in figure 1). Shown here are all the positive and negative responses as measured by the expression of CD154 and CD137. P9 displayed a CD8 response against four different single peptides (notably 41, 51, 61 and 137). P12 displayed a CD4 response against six different single peptides (notably 21, 61, 71, 81, 91 and 101).