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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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Translational Relevance

In the face of developing therapeutic vaccination strategies, it is of vital importance to gain a better understanding of the local tumor environment and the preexisting local anti-tumor response. In this study 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, showing a large polyclonal repertoire of poised non-functional HPV-specific T cells. Addition of immuno-activating compounds could be key in increasing the effector function of these T cells with beneficial effects for therapeutic strategies.

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 V β (TCRV β) 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 TCRV β 's per single peptide-epitope. In most patients only few IFN γ -producing T cells were found and the amount of IFN γ 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, very 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 these tumors are caused by the high-risk human papillomavirus (HPV) which encodes the two clearly 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 these 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 as well as 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 a comprehensive analysis 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 or 1b2) at the department of Gynecology of the Leiden University Medical Centre 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 Committee. The subjects were tested for HPV status using HPV16 and HPV18 specific primers on DNA isolated from surgical 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 scheduled for 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, Zeptomatrix, Buffalo NY, USA) and 5 ng/ml IL-15 (Peprotech, Rocky Hill NJ, USA). On the first day 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 the 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 18×10^6 cells, range $4 - 40 \times 10^6$) 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 for one hour at 37 °C in the presence of collagenase (200 IU/ml, Sigma) and DNase (50 µg/ml, Sigma), then put through a cell strainer (BD, Erebodengem, 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-LCLs) 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 -20°C 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 IFN γ

and IL-10 production was measured by ELISA (29). Antigen-specific cytokine production was defined by a cytokine concentration above the cut-off value (IFN γ 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. TILs 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, IFN γ -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 TCRV β 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 multiparameter 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 TCRV β (Beckman Coulter, Immunotech, France). A TCRV β was considered dominant (>10%), subdominant (3-10%) or minor (<3%) on basis of the percentage of HPV-specific cells using the same TCRV β .

RT-PCR

Expanded bulk 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). V β 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 1ug/ml pool of E6 and E7 peptides, TCGF 10% and IL15. Toll like receptor (TLR) 4 ligand LPS 250 ng/ml (Sigma-Aldrich, USA), TLR 3 ligand Poly(I:C) 12.5 µg/ml (InvivoGen, USA) and TLR 1-2 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 the 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 IFN γ , 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). The TIL all reacted to PHA and proliferation was associated with large amounts of IFN γ and IL-10 (not shown).

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 (Figure 1a), while HPV-specific CD8+ T cells predominantly expressed CD137, yet sometimes co-

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

| Patient | HPV type* | Origin | Days of culture [†] | Proliferation assay (3 d) | | | | T cell | Overnight activation analysis | | |
|---------|-----------|--------|------------------------------|---------------------------|------------------|----------------------------|---------------------|--------|----------------------------------|------|------|
| | | | | Reactivity [§] | SI | IFN γ | IL-10 | | Activated cells (%) [‡] | | |
| | | | | | | | | | 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 | >5,000 | 315 | CD4 | 0.8 | 63 | 0.9 |
| | | | | | | | | CD8 | 0 | 47 | 0.7 |
| 5 | 16 | TIL | 79 | 16E6 | 109 | >5,000 | 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 | >5,000 | <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.

[‡]Percentage of total CD4 or CD8 T-cell population expressing CD154 and/or CD137 after stimulation with the indicated antigen corresponding to the HPV type present in the tumor. A response equal or more than three times the medium control was considered positive.

[§]Reactivity indicates the HPV type and protein to which the T-cell culture specifically reacted.

^{||}SI, stimulation index, average proliferation of test wells divided by the average proliferation of medium control wells. SI > 2 is positive.

^{||}Antigen-specific cytokine production in pg/mL is indicated when test value was above cutoff level and at least more than two times the background production (4).

**NT, not tested.

expressed CD154 (Figure 1a). The percentage of HPV-specific T cells varied enormously between as little as 0.17% to as much as 66% (Table 1) of the 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 to single peptides in 12 patients (Figure 1b; Supplemental Table 1). The three TIL cultures tested negative before remained negative excluding that the responses detected are primed *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 or more 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

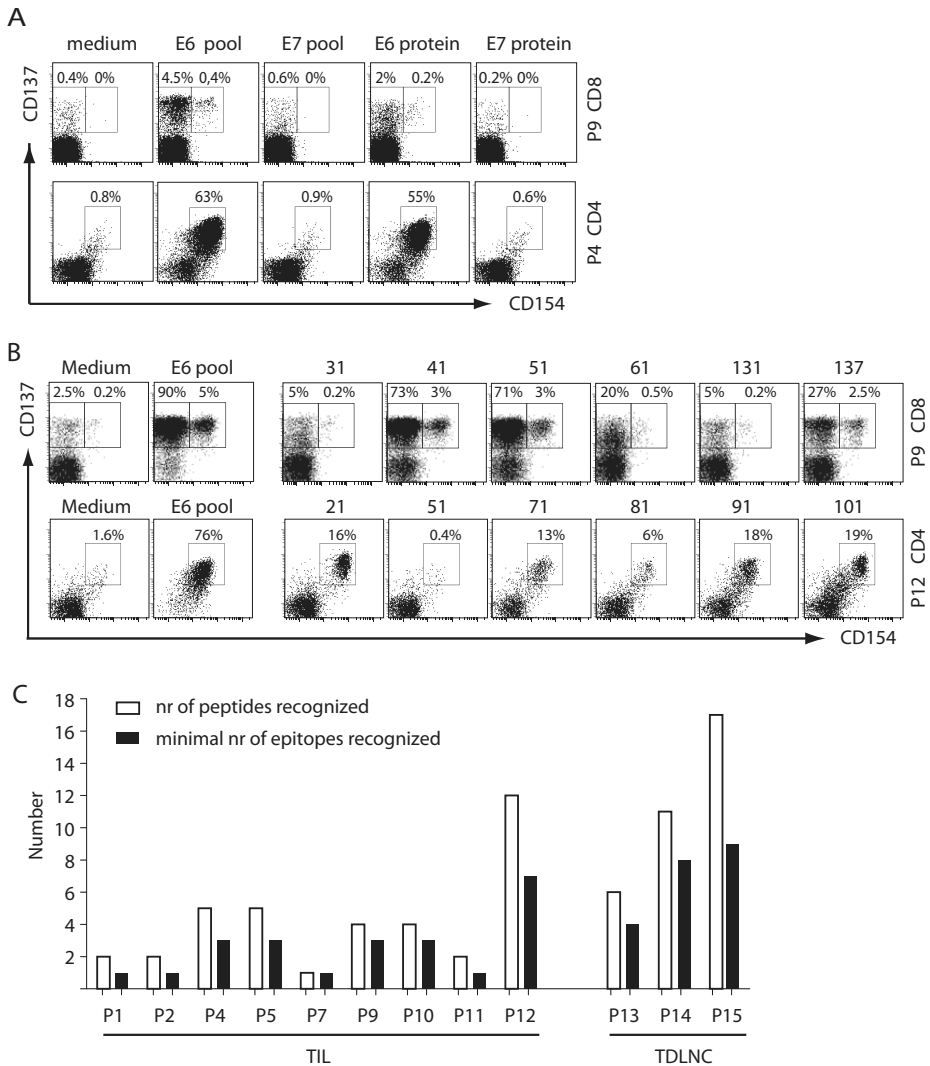


Figure 1. HPV-specific T cells in TIL and TDLNC.

(A) An example of a CD4 and a CD8 HPV-specific response 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 overlapping peptides as one, because the peptides used are 22-mers, overlapping in 12 amino acids.

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 in 12 amino acids. The majority of the patients (8/12) recognized 3 or more 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.

As each individual T-cell epitope can be recognized by different T-cell clones, we studied the number of TCRV β families involved in the recognition of each epitope by using a commercially available TCRV β -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 TCRV β -chain. Figure 2a shows a number of examples on the contribution of several T-cell receptor families reactive to one single peptide. Often one or two dominant TCRV β 's were found (Table 2 and Figure 2b), as well as several sub-dominant and minor TCRV β '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 TCRV β '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 2b). In a number of cases not all TCRV β could be indentified as the available antibodies cover approximately 70-80% of the full TCRV β -repertoire (34). In one case (P7) of whom all the cells in the cultured T-cell population responded exclusively to one peptide - but for which only 22% of the HPV-specific T cells the TCRV β was accounted for by antibodies (Figure 2a, fourth row and Table 2)- a semi-quantitative RT-PCR was applied revealing the presence of TCRV β 24 (Figure 2c). In another case (P15) only 30% of the TCRV β were accounted for by flow cytometry. Here, three additional TCRV β 6C, 6D and 15 (not shown) were detected by RT-PCR. No skewing to a certain TCRV β within this patient group was found, nor was there any skewing of certain TCRV β 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 type 1 cytokines (IFN γ , IL-2) is essential for an effective anti-tumor response and instrumental to functionally characterize antigen-specific T cells (35-40). Therefore, the *ex-vivo* enumerated HPV-specific CD154+ and/or CD137+ T cells were simultaneously analyzed for their production of IFN γ and IL-2. 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 IFN γ 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 IFN γ in

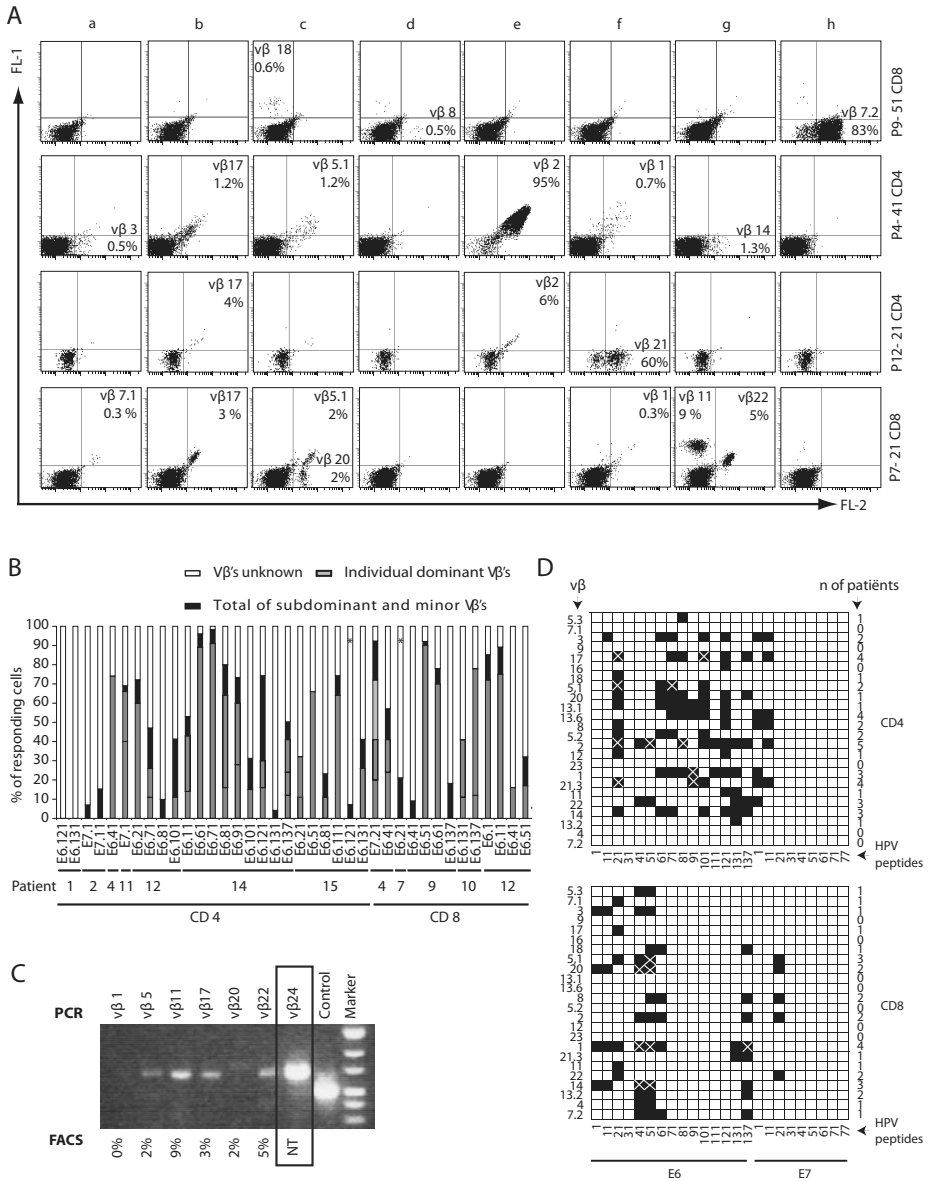


Figure 2. The T-cell response to a single HPV epitope involves the use of multiple dominant and subdominant TCRVβ families.

(A) The TCRVβ families used by T cells responding to a single peptide were analysed in 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 TCRV β 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 TCRV β (s) and the black stacked bars indicate the accumulated percentage of all the sub-dominant and minor TCRV β 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 TCRV β families involved using the TCRV β 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 TCRV β analysis was done by RT-PCR allowing the detection of other TCRV β 's not present in the TCRV β kit. (C) RT-PCR for TCRV β in TIL of patient P7, revealing the presence of TCRV β 24. The percentage of each TCRV β that was detected by flow cytometry is indicated on the left.

(D) A fingerprint overview of all TCRV β 's detected upon response to a single peptide-epitope for the patients investigated. The black squares indicate that the TCRV β (indicated at the left) was involved in response to that particular peptide (indicted at bottom). A white cross means that this TCRV β was found in two independent patients responding to that peptide, the right column indicates in how many patients this particular TCRV β was found.

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 IFN γ 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 IFN γ (Table 1 and Figure 3ab). In mouse models, the local injection of TLR2, TLR3 or TLR 4 ligands can augment the tumor response (41-43). 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 IFN γ 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 IFN γ -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 out 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 which is 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 IFN γ , IL-2 or double- producing T cells after this period (not shown).

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

| | Responding* | | | Peptides [†] | Number of TCRV β found per peptide by FACS [‡] | | | | | | |
|-----|-------------|--------|---------|-----------------------|---|-------------|-------|----------------|---|---|----------------|
| | Patient | T cell | Antigen | | Dominant | Subdominant | 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 | | | |
| | | | | 41 | 2 | 1 | 3 | 6 | | | |
| | | | | 51 | 2 | 1 | 3 | 6 | | | |
| | | | | 21 | 3 | 2 | 0 | 5 | | | |
| | | | | 21 | 0 [§] | 3 | 4 | 7 [§] | | | |
| | 7 | CD8 | E6 | 41 | 0 | 1 | 1 | 2 | | | |
| | | | | 51 | 1 | 0 | 3 | 4 | | | |
| | | | | 61 | 1 | 1 | 3 | 5 | | | |
| | | | | 137 | 0 | 2 | 5 | 7 | | | |
| | | | | 131 | 2 | 0 | 0 | 2 | | | |
| | 10 | CD8 | E6 | 137 | 2 | 0 | 0 | 2 | | | |
| | | | | 137 | 2 | 0 | 0 | 2 | | | |
| | 11 | CD4 | E7 | 1 | 2 | 0 | 0 | 2 | | | |
| | | | | 21 | 1 | 2 | 0 | 3 | | | |
| | | | | 71 | 2 | 0 | 1 | 3 | | | |
| | | | | 81 | 0 | 1 | 1 | 2 | | | |
| | 12 | CD8 | E6 | 101 | 1 | 3 | 0 | 4 | | | |
| 1 | | | | 1 | 0 | 3 | 4 | | | | |
| 41 | | | | 1 | 1 | 4 | 6 | | | | |
| 51 | | | | 1 | 3 | 2 | 6 | | | | |
| 11 | | | | 2 | 0 | 8 | 10 | | | | |
| LN | 14 | CD4 | E6 | 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 TCRV β analysis after antigen-specific expansion by stimulation with E6 or E7 peptide pool.

[†]The number indicates the first amino acid 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 TCRV β consists of >10% of the activated T cells. A subdominant TCRV β consists of between 3% and 10% of the activated T cells. A minor TCRV β consists of <3% of the activated T cells.

[§]Additional TCRV β s were found by PCR analysis for P7 and P15.

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 for immunomonitoring in combination with the activation markers CD154 and CD137,

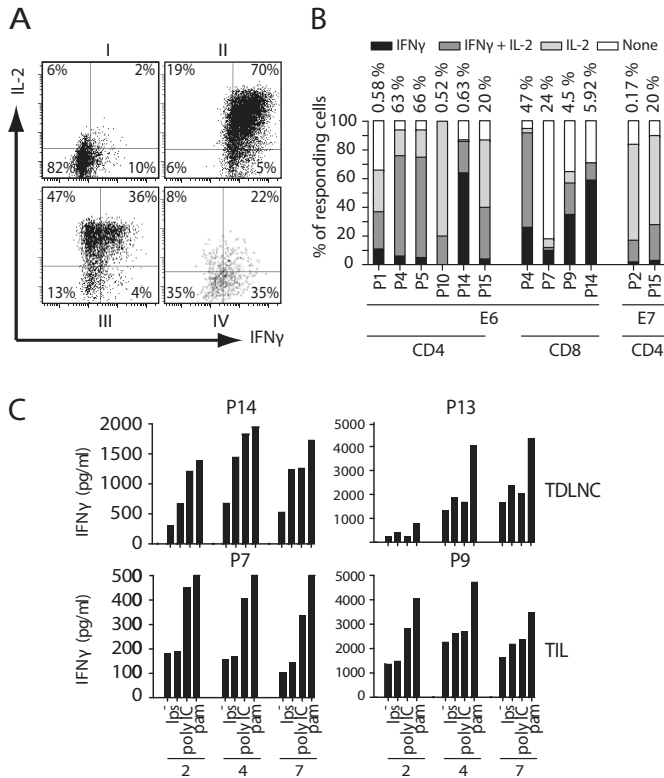


Figure 3. Functionality of HPV-specific TIL and TDLNC .

(A) The specific activity to HPV16 or 18 E6 and E7 peptide pools by simultaneous analysis of CD154, CD137, IFN γ and IL-2 in homeostatic cytokine-mediated expanded TIL as well as directly *ex-vivo* in TDLNC. Four different cytokine profiles could be distinguished. Profile I: HPV-specific T-cells with few T-cells producing cytokines (P7). Proliferative II: Predominant production of IFN γ and IL-2 (P4). Profile III: predominant production of IL-2 (P15). Profile IV: mainly IFN γ 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: IFN γ , grey: IL-2, hatched: IFN γ +IL-2 and white: no IFN γ 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.

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 not exclude that the quantification of HPV-specific T cells among 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 TCRV β families (Table 2, Figure 2). One could argue that our analyzes 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 (34). 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 (44), as well as in the HLA-A*0201-restricted Melan-A/MART-1₂₆₋₃₅-specific CD8 T-cell response (6). The presence of single peptide-specific dominant and subdominant (based on TCRV β -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. It is likely that the different dominant and subdominant HPV-specific T cells 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 (45). 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.

CD4+ and CD8+ T cells as well as the cytokines IFN γ and IL-2 play a key role in the protection against cancer (4;35-38) as well as in the control of chronic viral infections (39;40). In most of the *ex-vivo* tested TIL and TDLNC the population of IFN γ and IL-2 producing T cells or IFN γ -producing T cells among the total population of HPV-specific T cells as well as the amount of IFN γ produced was low (Table 1 & Figure 3b), suggesting that most of the HPV-specific TIL and TDLNC 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 (46). *In vitro* stimulation of these HPV-specific T cells with their cognate antigen resulted in an increase in the total population of HPV-specific T cells (e.g. P9 and P12, compare Figure 1a and Figure 1b) as well as in an increased IFN γ production (P9; compare Table 1 & Figure 3c). We recently reported that the majority of patients vaccinated with a HPV16 E6 and E7 long peptide vaccine, showed a clearly detectable and broad vaccine-induced HPV-specific immune response as detected by IFN γ -ELISPOT (23;24). Most likely this vaccine taps the broad available T-cell repertoire we identified in this study and either primes (in the group of 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), an even more pronounced increase of effector function is observed (Figure 3c). This suggests that local delivery of these innate immune-derived stimulating factors can overcome functional tolerance in human cancers as was also observed in murine tumor models (41-43). Moreover, they are likely to assist therapeutic vaccines in driving T-cell responses with increased function as shown previously for a melanoma peptide vaccine in combination with CpG (47). Such type of responses is highly required as they do not only correlate with clinical efficacy in murine models (41), but also in human trials (48). Interestingly, the widely used TLR4 ligand LPS did not overtly increase IFN γ production of TIL and TDLNC in our study.

In conclusion, the 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.

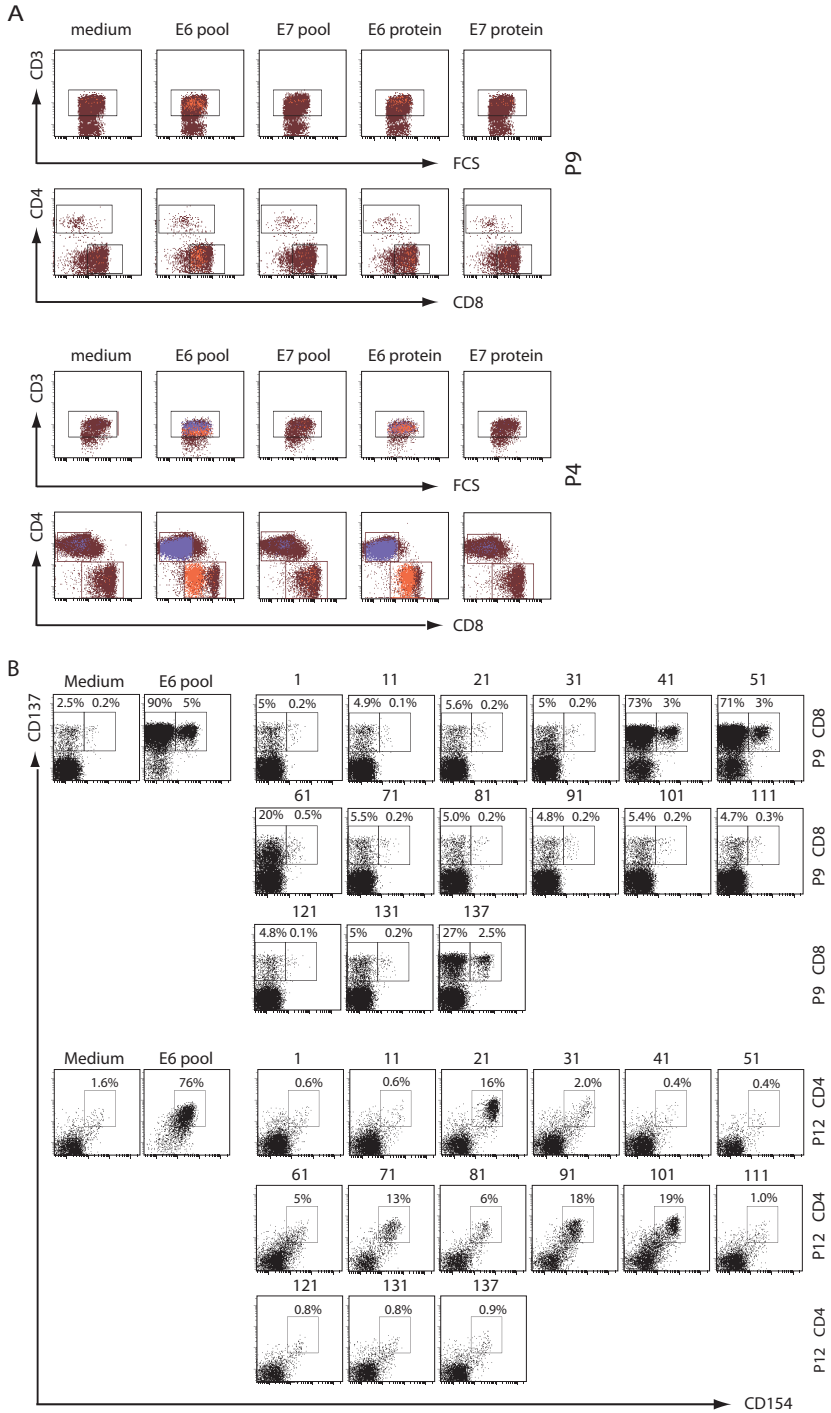
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Supplemental figure 1.

(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 protein-activated 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).

Supplemental table 1. Breadth of the HPV specific response detected in TIL and TDLNC

| Responding* | | | | | Activated cells (%)† | Responding | | | | | Activated cells (%)† | |
|-------------|--------|---------|------------|--------|----------------------|------------|--------|---------|------------|--------|----------------------|----|
| Patient | T-cell | Antigen | Peptides** | | | Patient | T-cell | Antigen | Peptides** | | | |
| TIL | 1 | CD4 | E6 | medium | 2 | LN | 13 | CD4 | E6 | medium | 0.1 | |
| | | | E6 | 121 | 22 | | | | E6 | 1 | 1 | |
| | | | E6 | 131 | 23 | | | | E6 | 41 | 1 | |
| | 2 | CD4 | E7 | medium | 2 | | E6 | 51 | 1 | | | |
| | | | E7 | 1 | 10 | | E6 | 71 | 1 | | | |
| | | | E7 | 11 | 10 | | E6 | 121 | 1 | | | |
| | 4 | CD4 | E6 | medium | 4 | | E6 | 131 | 1 | | | |
| | | | E6 | 41 | 89 | | 14 | CD4 | E6 | medium | 0.1 | |
| | | | E6 | 51 | 65 | | | | E6 | 11 | 15 | |
| | | | CD8 | E6 | medium | | | | 0.6 | E6 | 61 | 78 |
| | | | | E6 | 41 | | | | 73 | E6 | 71 | 78 |
| | | | | E6 | 51 | | | | 60 | E6 | 81 | 2 |
| | | CD8 | E7 | medium | 2 | | | | E6 | 91 | 3 | |
| | | | E7 | 21 | 15 | | E6 | 101 | 3 | | | |
| | | | 5 | CD4 | E6 | | medium | 13 | E6 | 121 | 9 | |
| | | E6 | | | 31 | | 51 | E6 | 131 | 6 | | |
| | | E6 | | | 51 | | 60 | E6 | 137 | 2 | | |
| | | E6 | | | 61 | | 64 | CD8 | E6 | medium | 4 | |
| | E6 | 71 | | | 56 | | E6 | | 21 | 29 | | |
| | E6 | 81 | | | 60 | | E6 | | 137 | 58 | | |
| | 7 | CD8 | E6 | medium | 12 | | 15 | | CD4 | E6 | medium | 1 |
| | | | E6 | 21 | 47 | | | | | E6 | 1 | 14 |
| | | | 9 | CD8 | E6 | | | | | medium | 3 | E6 |
| | E6 | 41 | | | 73 | | E6 | 41 | 6 | | | |
| | E6 | 51 | | | 71 | | E6 | 51 | 6 | | | |
| | E6 | 61 | | | 20 | | E6 | 71 | 2 | | | |
| | E6 | 137 | | | 27 | | E6 | 81 | 4 | | | |
| | 10 | CD4 | | | E6 | | medium | 9 | E6 | 111 | 5 | |
| | | | E6 | 1 | 33 | | E6 | 121 | 56 | | | |
| | | | E6 | 137 | 14 | | E6 | 131 | 38 | | | |
| | | CD8 | E6 | medium | 0.4 | | E6 | 137 | 20 | | | |
| | | | E6 | 1 | 18 | | CD4 | E7 | medium | 1 | | |
| | | | E6 | 131 | 13 | | | E7 | 1 | 39 | | |
| | E6 | 137 | 14 | E7 | 11 | | | 37 | | | | |
| | 11 | CD4 | E7 | medium | 5 | | | E7 | 41 | 4 | | |
| | | | E7 | 1 | 92 | | | E7 | 51 | 5 | | |
| | | | E7 | 11 | 67 | | | E7 | 61 | 3 | | |
| | 12 | CD4 | E6 | medium | 2 | | E7 | 71 | 14 | | | |
| | | | E6 | 21 | 16 | | E7 | 77 | 4 | | | |
| | | | E6 | 61 | 5 | | | | | | | |
| | | | E6 | 71 | 14 | | | | | | | |
| | | | E6 | 81 | 6 | | | | | | | |
| E6 | | | 91 | 18 | | | | | | | | |
| CD8 | | E6 | 101 | 19 | | | | | | | | |
| | | E6 | medium | 1 | | | | | | | | |
| | | E6 | 1 | 47 | | | | | | | | |
| | | E6 | 11 | 42 | | | | | | | | |
| | | E6 | 41 | 37 | | | | | | | | |
| | | E6 | 51 | 27 | | | | | | | | |
| | | E6 | 61 | 2 | | | | | | | | |
| | | E6 | 71 | 3 | | | | | | | | |

* Depicted are all patients of whom T cells were tested after antigen specific expansion with E6 or E7 peptide pools
 ** HPV 16 or 18 (corresponding to the HPV type present in the tumor) E6 and E7 22-amino acid long overlapping peptides were used to test specificity
 Medium is activity present in cultures when no E6 or E7 22-mer peptide is added
 The number indicates the first aminoacid of the peptide the culture responded to or no peptide (medium)
 † The percentage of HPV-specific T cells that responded by upregulation of activation markers CD154 and/or CD137 to the peptide depicted in the column on the left. A response is considered positive when the percentage is more than 3 times medium control

